MOLECULAR BIOLOGY

A Project Approach

by Susan J. Karcher

Academic Press

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A Project Approach

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To the students.

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SUGGESTED SCHEDULE OF LABORATORY PROTOCOLS

Week	Day 1	Day 2	Day 3	Day 4
1	Review: Use of micropipettors; sterile technique; streaking for single colonies Protocol 1.2 Part A	Protocol 1.2 Part B	Protocol 1.2 Part B, starting at step 10; Protocol 1.1	
2	Protocol 1.3	Protocol 1.3	Protocol 1.4	
3	Protocol 1.5; Protocol 2.1a		Protocol 1.6[a]; Protocol 2.1b; Protocol 2.2	
4	Protocol 2.3[b]		Protocol 2.3, continued	
5	Protocol 2.4[c]	Protocol 2.4; ligation	Protocol 2.4; transformation[d]	Protocol 2.4 completion
6	Protocol 2.6a or b		Protocol 2.6a or b	
7	Protocol 3.1a	Protocol 3.1a	Protocol 3.2[e]; Protocol 3.3a, b, or c	
8	Protocol 3.4a or b[f]; hybridization	Protocol 3.4a or b; posthybridization washes	Protocol 3.4a or b; detection of probe	
9	Protocol 4.1		Protocol 4.1	
10	Protocol 4.2		Protocol 4.2	
11	Hybridization of blot from Protocol 4.2; using Protocol 3.4a or b	Posthybridization washes; using Protocol 3.4a or b	Detection of probe; using Protocol 3.4a or b	
12	Protocol 5.1		Protocol 5.1 and Protocol 5.2	

(continues)

(continued)

Week	Day 1	Day 2	Day 3	Day 4
13	Protocol 5.3; Protocol 5.4		Protocol 5.6[g]	
14	Hybridization of blot from Protocol 5.4; using Protocol 3.4a or b	Posthybridization washes; using Protocol 3.4a or b	Detection of probe; using Protocol 3.4a or b	
15	Protocol 6.1		Analysis of results of Protocol 6.1 by gel electrophoresis; using Protocol 2.2	

[a] Protocol 1.6 is continued by students outside the scheduled lab.
[b] For a course of less than 15 weeks, the instructor may wish to demonstrate Protocol 2.3 to the students.
[c] The timetable for Protocol 2.4 can be varied, depending on whether a one-hour or an overnight ligation is used.
[d] The competent cells needed in Protocol 2.5 are prepared using Protocol 2.5a or 2.5b. The instructor may wish to prepare frozen competent cells for the students. Alternatively, if the students will prepare their own competent cells, this may be done in Week 4 (for frozen cells, Protocol 2.5a) or in Week 5, Day 2 and 3 (for fresh cells, Protocol 2.5b).
[e] For a course of less than 15 weeks, the instructor may wish to omit Protocol 3.2.
[f] Alternatively, Protocol 3.5 may be used.
[g] Alternatively, Protocol 5.5 may be used.

PREFACE

I hear, and I forget,
I see, and I remember,
I do, and I understand.
Ancient Chinese Proverb

This manual of experiments is intended for beginning students who have a basic understanding of genetics and molecular biology, but who may not have had any laboratory experience in these areas. Included is an extensive introduction and a large amount of background material so that this text might "stand alone" without the need of a second textbook for the laboratory course. I have also included an extensive bibliography so students may learn more about areas that have piqued their interest.

This manual is based on the laboratory class in molecular biology and molecular genetics I have taught to undergraduate juniors and seniors and masters students at Purdue University in the Department of Biological Sciences since 1982. The approach I have used in teaching the molecular biology laboratory at Purdue has been to have students perform multipart projects, with parts that build on each other, rather than to have the students perform a new exercise each laboratory period. It is that project-oriented approach that I bring to this manual.

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West Lafayette, Indiana

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I greatly appreciated the book by W. B. Wood, J. H. Wilson, R. M. Benbow, and L. E. Hood, "Biochemistry: A Problems Approach" (The Benjamin/Cummings Publishing Co., Menlo Park, CA, first and second Eds.), the source of the ancient Chinese proverb in the preface.

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NOTE TO USERS

Although an effort has been made to indicate the precautions necessary when handling certain materials used in the procedures of this manual, the individual using this manual must assume all responsibility for the correct use of materials.

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1

TRANSPOSON MUTAGENESIS OF *Escherichia coli*

Introduction to Transposons

A transposon is a genetic entity that promotes its own movement or transposition; it can insert as a discrete segment of DNA into many different sites in the genome.

In the 1940s, Barbara McClintock's pioneering studies on the genetics of pigment variation in maize kernels first indicated the existence of transposable elements. Such elements were understood on a molecular level when bacterial insertion sequences (IS elements) were isolated and characterized in the late 1960s. The discovery of insertion sequences showed that rearrangements can occur within the bacterial genome and sometimes result in alterations in gene expression. At the time of this discovery, the genome was thought to be a very stable entity. IS elements were first found in studies of gene expression in *Escherichia coli* as highly polar, rather unstable mutations in the galactose and lactose operons and in bacteriophage λ early genes (Galas and Chandler, 1989). Analysis of hybrid DNA molecules (heteroduplexes) in the electron microscope showed that these mutations resulted from the insertion of the same few segments of DNA into different places of the genome. These elements, subsequently called insertion sequences, were shown to be present in the wild-type *E. coli* genome as well; the elements move to new locations and generate mutations as they insert into active genes. IS elements vary in copy number from a few to a few hundred in different organisms, are common on naturally occurring plasmids, and are often found associated with antibiotic resistance genes (Tables 1.1 and 1.2).

Table 1.1
Insertion Sequences

Name of element	Size (bp)	Inverted repeats (bp)	Target duplications (bp)	Source
IS1	768	20/23	9 (8-11)	*E. coli*
IS3	1258	29/40	3	*E. coli*
IS10	1329	17/22	9	R100 (Tn10)
IS50	1534	8/9	9	Tn5
IS66	2548	18/20	8	*A. tumefaciens*

Source: D. J. Galas and M. Chandler (1989).

Table 1.2
Transposons

Name	Antibiotic resistance	Size (kb)	Structure	Insertion specificity mode of transposition
Tn3	Ampicillin	5	No IS elements; 38-bp inverted repeats	Prefers to insert in AT-rich region; transposes at high frequency into plasmids, at low frequency into chromosome; replicative transposition; 5-bp target duplication
Tn5	Kanamycin Bleomycin Streptomycin	5.8	1.5-kb IS50 elements as inverted repeats, 19-bp inverted repeats important for transpostion	Random insertions; occasional hot spots; high-frequency transposition nonreplicative transposition; 9-bp target duplication
Tn9	Chloramphenicol	2.6	0.8-kb IS1 as direct repeats	Inserts in many sites, insertional hot spots; 9-bp (or 8-bp) target duplication
Tn10	Tetracycline	9.3	1.3-kb IS10 elements as inverted repeats; 23-bp inverted repeats important for transposition	Inserts in many sites, insertional hot spots; 9-bp target duplication
Mu	Immunity to Mu	37.5	2-bp inverted repeats important for transposition	Random insertion with some hot spots; bacteriophage, transposes as part of phage replication; 5-bp target duplication
Tn916	Tetracycline	16.4	No IS elements, 26-bp inverted repeats important for transposition	Conjugative transposon; transfer 10^{-8} to 10^{-5} per donor; no target site duplication
Tn554	Erythromycin Spectinomycin	6.7	No IS elements No inverted repeats	Transposes by site-specific recombination to att554 site; no target site duplication

Source: Berg *et al.* (1989).

Kleckner (1981) divided prokaryotic transposons into three classes based on the structure of the elements; later, this classification was modified somewhat as more diverse types of transposons were discovered (Kleckner, 1990). Class Ia consists of IS-like elements that have terminal inverted repeated DNA sequences at the ends of the elements and encode only the genes necessary for the transposition of the elements. The IS-like transposons are small, ranging from about 800 to 2500 bp in length with terminal inverted repeats of about 9–30 bp. See Table 1.1 for a list of some IS elements. When the IS element inserts into the host DNA, a portion of the host DNA is replicated, generating a short (about 10) base pair direct repeat of target DNA sequences flanking the IS element. The members of Class Ib are composite IS elements, containing IS elements at the ends of the transposons (generally present as inverted repeats) and additional genes (usually encoding antibiotic resistance). Figure 1.1 shows the structures of some composite Class I transposons. Class II prokaryotic transposons are noncomposite drug resistance transposons. A major group of such Class II transposons is the Tn*3* family of transposons. Members of this group are large (greater than 5 kb), contain antibiotic resistance genes, and have inverted repeated sequences at the ends of the elements. The repeated element, however, is not an IS sequence. This group of transposons encodes a transposase protein that is involved in the formation of a replicative cointegrate (a covalently joined plasmid molecule containing the original transposon and the new plasmid where a transposon inserted) that is subsequently resolved by a site-specific recombinase, the resolvase.

Another major group of this second class of transposons is the conjugative transposons. Conjugative transposons are completely different from the classical transposons described above. For conjugative transposons the donor and target genomes are in different bacterial cells. Contact between two bacterial cells triggers the excision of the transposon, which forms a covalently closed circle that is transferred to the recipient cell. In the recipient cell, the transposon may integrate into many sites. More than one transposon per recipient genome is sometimes observed (Scott, 1992). Conjugative transposons do not generate a duplication of the target site into which they insert. They have great medical importance because they are highly promiscuous and are likely responsible for the dispersal of antibiotic resistance genes among many different bacteria. This type of transposon is often found in clinical isolates of streptococci and staphylococci. All known conjugative transposons encode tetracycline resistance (*tetM* gene), but other antibiotic resistance genes have also been observed. Two examples of conjugative transposons are Tn*916*, which is 16.4 kb long, and Tn*1545*.

A third group of Class II transposons is the Tn*7* family, which contains five different transposition functions and can transpose by different

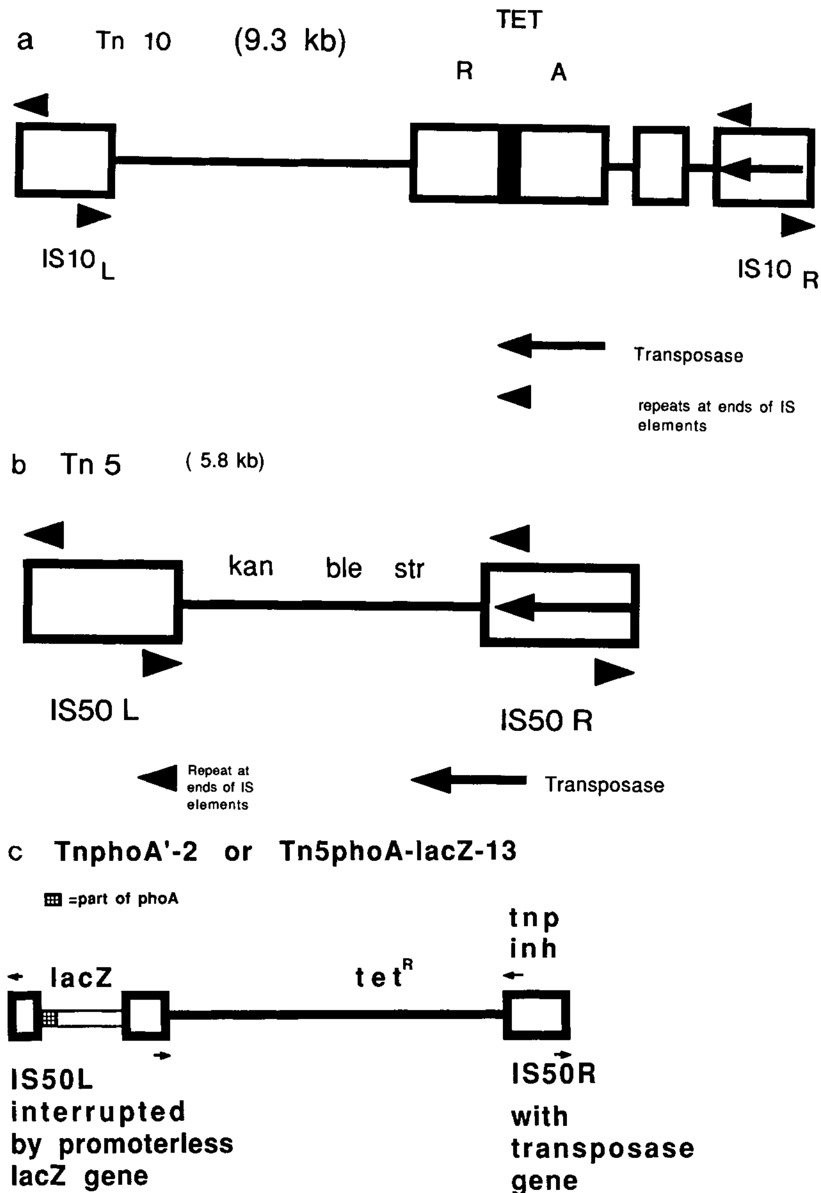


Figure 1.1 (a) A sketch of the transposon Tn*10*. (b) A sketch of the transposon Tn5. (c) A sketch of the transposon Tn*phoA*, transposon with a promoterless reporter gene, *LacZ* (β-galactosidase). IS, insertion sequence. L and R refer to left and right. TET is the tetracycline resistance determinant, *tetR*, which encodes a repressor that regulates the transcription of itself and the *tetA*, which encodes the structural protein for tetracycline resistance. *tnp* is the gene for transposase. *inh* codes for an inhibitor protein that regulates transposition of Tn5. *Kan*, kanamycin resistance; *ble*, bleomycin resistance; *str*, streptomycin resistance, which is not expressed in *E. coli*.

mechanisms. One mode of Tn7 transposition is the specific insertion of Tn7 into a unique chromosomal attachment site. The other mode of transposition causes the transposition of the element into many different sites.

The third class of transposons, Class III, includes bacteriophage Mu and related phages. These phage DNAs do not have repeated sequences at their ends, but transpose as part of their normal mode of replication. When Mu (for mutator) phage infects *E. coli,* its linear DNA enters the host cell, where it becomes a noncovalently closed circle. Using the MuA transposase protein (and with greater efficiency if MuB protein is present), Mu DNA is integrated into the host DNA by a nonreplicative (conservative) transposition. In the lytic cycle, by replicative transposition (using MuA and MuB proteins and some host DNA replication proteins), copies of the Mu DNA insert approximately at random in the *E. coli* genome. There may be as many as 100 copies of the Mu DNA in the host genome by the time the Mu prophage DNA is packaged into mature phage particles. Mu is packaged by a "headful" mechanism, and chromosomal DNA flanking the inserted Mu DNA can also be excised and packaged into phage particles.

The transposon Tn5, discovered in 1975, is 5.8 kb long. It is a composite IS element (Class Ib), with IS50 elements present as terminal inverted repeats. The IS50 element is 1533 bp long with 8-bp terminal inverted repeats and generates a 9-bp duplication of the target site on insertion. About 19 bp at each end of Tn5 are important for transposition. Tn5, like Tn*10*, is thought to transpose by a conservative, cut and paste mechanism (Berg, 1989). IS50R (right) encodes two proteins, Tnp and Inh. Tnp is a 476-amino-acid transposase that is primarily *cis*-acting; that is, the transposase functions best to promote the transposition of IS50 ends near its gene. When Tnp is overexpressed, however, it is possible to observe *trans*-complementation of the gene. Why the ends of the element must be close to the tnp^+ gene is not clear, but it is possible that an unstable transposase protein is rapidly degraded or that the transposase, once it binds DNA, can move only inefficiently to the ends of the transposon element. Alternatively, more than one molecule of transposase may be needed. The gene *inh,* which is coded for by the same open reading frame as Tnp but 55 codons smaller, codes for a 421-amino-acid *trans*-acting inhibitor protein. The inhibitor protein acts to regulate the transposition of Tn5. Although the mode of action of the inhibitor protein is not clear, it is known that the Inh protein does not inhibit the expression of the *tnp* gene. Perhaps the Inh protein interacts with the transposase protein or with a transposition complex at the ends of the element. The presence of a resident Tn5 decreases the ability of an incoming Tn5 to transpose because of the action of the inhibitor protein.

Tn5 inserts into DNA more nearly randomly than other transposons. Bacteriophage Mu also inserts randomly. The target sequences of Tn5

insertion sites show no consensus sequence, unlike the situation with Tn*10*. There are occasional hot spots in certain genes where Tn5 inserts with higher frequency. The target site of Tn5 insertion at these hot spots usually contains GC pairs at each end of the 9-bp target duplication.

Tn*10* is another transposon that has been studied in detail by Kleckner and is well characterized. See the references for Tn*10* under Suggested Reading.

Advantages of Transposon Mutagenesis

There are numerous advantages to using transposons to generate mutations. First, transposon mutagenesis is safe, simple to do, and relatively inexpensive. Other advantages include:

1. The transposon contains a genetic marker, the antibiotic resistance gene, for which there is a strong positive selection. It is easy to select for cells that contain transposons by simply selecting for the appropriate antibiotic resistance marker.
2. The transposon also provides a physical marker. The insertion of the transposon results in an increase in the length of DNA within the mutagenized gene that can be used as a physical marker. This physical marker can be used to map the location of the insertion by electron microscopic examination of heteroduplexes formed between plasmids containing the transposon and the unmutagenized plasmid. The mutagenized and unmutagenized plasmids are completely homologous except for the insertion site of the transposon. An electron micrograph of such heteroduplexes shows a loop of single-stranded DNA the size of the transposon. If the plasmids can be linearized at a unique restriction endonuclease site, the distance of this loop from that restriction site can be measured to map the location of the insertion. Transposons can also provide known restriction endonuclease sites. To map the insert, DNA can be cut with restriction endonucleases at these sites, and the sizes of the fragments generated can be determined by gel electrophoresis. For example, in a case where the restriction enzyme used does not cut within the transposon, one restriction fragment would disappear in the mutagenized sample and would be replaced by a restriction fragment that would be the size of the lost fragment plus the transposon. Transposons such as Tn5 and Tn*10* are well characterized, with restriction endonuclease site maps or even DNA sequences available. See Fig. 1.1. In addition, transposons can be used as hybridization probes to purify sequences in genes of interest flanking the inserted DNA.

3. Transposon insertion is almost random; that is, transposons can insert into many sites. A consensus sequence of the preferred site of insertion has been determined for Tn*10*; however, that sequence occurs on average about once every kilobase of DNA. Tn5 has occasional hot spots of insertion, but no consensus sequence for insertion has been found. Tn5 and bacteriophage Mu insert the most randomly of the transposons. When mutagenesis of a particular DNA sequence fails because one transposon does not seem to insert into that sequence, using a different transposon will often result in insertions into that sequence.
4. Insertion mutations can be recovered at high frequency because the transposon moves at high frequency.
5. Insertion of a transposon results in a single mutation, that is, one transposon insertion per cell. If a phage system introduces the transposon into the cells to be mutagenized, the multiplicity of infection (ratio of phage to bacterial cells) can be controlled to make it very likely that there will be only one transposition event per cell. With Tn5, the presence of the Inh inhibitor protein makes the insertion of a second Tn5 unlikely once there is one resident Tn5.
6. Transposon-generated mutations are fairly stable because once the transposon has inserted, the frequency of further movement of the element is fairly low. In addition, there are ways of stabilizing the transposon once it has transposed into the gene of interest. For example, in the Tn3-HoHo1 mutagenesis system (Stachel *et al.*, 1985), the transposon used for insertional mutagenesis is a Tn3-based element that lacks a functional transposase. The transposase is supplied *in trans;* the gene for the transposase is on a different plasmid, which can be eliminated. In this way, once insertions are generated and the transposase–gene containing plasmid is eliminated, the insertions will not undergo further transposition. Another system is the modified Tn5 series of transposons of Wilmes-Riesenberg and Wanner (1992). Insertions are generated with a modified Tn5 that has a functional transposase. The insertion can be made stable by exchanging the first Tn5 for a Tn5 element with a defective transposase. The exchange occurs by homologous recombination between the two elements. The two elements contain different antibiotic resistance markers to select for the exchange of the elements. (Wilmes-Riesenberg also used this selection method to exchange different elements and thereby generate transcriptional or translational fusions.)
7. When a transposon inserts into a gene, there is generally a complete loss of function in the interrupted gene; that is, transposon insertions usually result in nonleaky mutations.

8. Transposon insertions into operons are usually polar. Transcription begun at the promoter of an operon terminates within the transposon so that downstream genes of the operon are not transcribed. This can be very useful for elucidating the order of genes in an operon. Occasionally, certain transposon insertions give rise to transcription of downstream genes because the sequence of the transposon and adjacent sequences are fortuitously brought together to make a promoter.
9. Insertion mutations revert by precise excision of the transposon and one of the duplicated target site sequences. It is relatively easy to show that the transposon insertion caused the mutant phenotype observed. Revertants of the mutant phenotype can be selected and then examined to show that they have lost the antibiotic resistance marker of the transposon. Alternatively, cells that have lost the antibiotic resistance marker can be examined to show that they have now reverted to the wild-type phenotype.
10. Transposon insertions are "well-behaved" genetic markers; they behave much like point mutations in fine-structure mapping such as three-point crosses. The antibiotic resistance marker can be used as a genetic marker in such crosses.
11. Transposon insertions can be used to generate duplications or deletions of regions of the chromosomes; they can serve as regions of homology for recombinational events.
12. Transposons can be used to map "silent regions" of a genome, using an antibiotic resistance marker to follow the manipulation of a region for which there is no other phenotype.
13. Transposons can be used for localized mutagenesis, such as the mutagenesis of a cloned gene.
14. Transposons can be used to insert a portable "reporter gene," a gene with a readily assayable phenotype, behind promoters of interest, creating a fusion of the reporter gene with the foreign promoter.

Kleckner (1977) and Berg and Berg (1983) discuss these many advantages in detail.

Eukaryotic Transposable Elements

Eukaryotic transposable elements have been classified by structure (Finnegan, 1985) as:

1. Elements with long terminal direct repeats
2. Elements with long inverted repeats

3. Elements with short inverted repeats
4. Elements without repeats.

Readers interested in eukaryotic transposable elements should refer to the Suggested Reading at the end of this chapter.

Transposons and Gene Fusions

Gene fusions from transposable elements linked to promoterless reporter genes are valuable tools for studying gene expression. For example, *lacZ* fusions have been used to study regions of genes involved in transcriptional and translational control. *phoA* gene fusions are used to study cell envelope proteins and sequences that target proteins to the cytoplasmic membrane (Gutierrez *et al.*, 1987; Hoffman and Wright, 1985; Manoil and Beckwith, 1986). The Tn*phoA* fusions encode bacterial alkaline phosphatase. The fusion protein is active only when the protein is localized to the cell surface. Wilmes-Riesenberg and Wanner (1992) have created a series of useful transposons that can be used to generate *lacZ* fusions. Their transposons have a promoterless *lacZ* gene that can be used to generate transcriptional or translational fusions. In addition, the sequences at the ends of these transposons are homologous. This sequence homology allows homologous recombination to occur and one element to be exchanged for another, for example, after one of the transposons is used in a mutagenesis experiment. Once an insertion of the transposon in a gene of interest has been identified, the transposon insertion can be stabilized by exchanging a transposon element with a defective transposase by recombination. An element without a functional transposase cannot transpose. Because different elements in their series have different antibiotic resistance markers, it is easy to select for the exchange of different elements. One of their elements contains a promoter sequence that is oriented outward from one of the repeated ends of the element. This element can be exchanged for another element to remove any possible polar effects of the transposon insertion at that site.

Preparing for Laboratory Exercises

Common Laboratory Rules

1. Always clean up at the end of the lab session.
2. Make sure that all materials are well-labeled, including the date.
3. Do not return a solution to the stock shelves once it has been opened.

4. Do not mouth pipet.
5. Do not bring food or drink into the laboratory.
6. Wash hands before leaving lab.

Guidelines for Laboratory Notebooks

Many different styles are acceptable in laboratory notebooks because each notebook is an individual creation. However, the following guidelines apply to all notebooks:

1. Use a bound or a spiral notebook, not loose leaf paper.
2. Make notes in this manual as needed, but do not use this manual or handouts as a lab notebook! Calculations and observations should be recorded directly in the lab notebook.
3. Include the following in the lab notebook:
   a. The title of each experiment.
   b. The objectives of each experiment.
   c. An overview of how the experiment is to be accomplished.
   d. Daily entries of objectives for that day's lab.
   e. Daily entries of how the day's lab was performed—this does not mean that the entire procedure from this manual should be copied into your notebook!
   f. Notes on modifications in procedures that were made as the experiment was performed.
   g. All data and observations.
   h. Interpretation and analysis of the data. All calculations and manipulation of data should be shown in the notebook.
   i. A brief conclusion and summary of experimental results at the end of each subsection of the experiments and at the end of a complete experiment, a brief analysis of how the experiment could be improved, and a brief indication of further experiments suggested by this experiment.

Write in the notebook daily in lab. Use ink. Crossing out errors is perfectly acceptable. Strive to make the lab notebook as neat and well organized as possible. Read Price (1990) for an assessment of the importance of laboratory notebooks.

Guidelines for Laboratory Reports

The following format should be followed for laboratory reports. Look at the format used in journal articles. Examine the instructions to authors in several journals (generally found in either the first or the last issue of the year) for suggestions.

Your report *must* follow the format given.

ABSTRACT: In a brief paragraph summarize the whole experiment and results. An abstract should be able to stand alone—it must contain a summary of all the key points of the entire paper.

INTRODUCTION: Include any background information and summarize the objectives and goals of the experiment.

MATERIALS AND METHODS: Briefly and concisely summarize the procedures used. Include information about sources of material used, identify strains and plasmids used, etc. Be sure to include key features, such as drug resistance markers and other selectable markers, of each.

RESULTS: Report the results of your experiment.

DISCUSSION AND CONCLUSIONS: Analyze the results. Explain the observations made. Summarize the results of the experiment. Discuss the significance of the findings.

REFERENCES: List the references cited.

Using Micropipettors

1. Read the manufacturer's information sheet about the micropipettor you will use.
2. Learn to adjust the volume of solution the pipettors will pick up by dialing the adjustable knob and reading the digital scale (the three vertical numbers). Note the volume that is measured for a reading on the digital scale for each type of pipettor.
3. Note the first stop (the first resistance met) and second stop (push harder) as the plunger of a pipettor is depressed. Practice pipeting various amounts of water onto a piece of waxed paper or plastic wrap. Observe how high the liquid level ascends into the pipettor tip whendifferent volumes of water are measured. Develop the habit of examining the level of liquid in the pipettor tip when measuring solutions.

4. If an analytical balance is available, measure the weight of a particular volume of water delivered onto the balance using a pipettor. Repeat the pipeting and weighing several times with the same volume of water. How do the weights compare?

Review of Sterile Technique

This laboratory exercise reviews sterile technique and procedures for handling bacteria. Students must master these techniques before beginning the experiments.

Single-Colony Isolation

It is important to begin with a bacterial strain that is pure. Do this by streaking for single colonies and then using a single colony to inoculate further cultures. Also, streaking the bacteria on a plate allows the colony morphology and color to be closely examined. This is useful for checking that a bacterial culture is correct and does not contain a contaminating microorganism.

Materials

- L agar plates
- *E. coli* strain on an agar plate or in liquid culture
- Bunsen burner and matches
- inoculation loop
- 37°C incubator

Procedure

1. Hold the wire of an inoculation loop in the flame of a Bunsen burner until the wire is bright red. Remove the loop from the flame and allow the loop to cool. To be sure that the loop is cool, touch it to a clean sterile surface, such as the agar in an agar plate or the inside lid of a sterile petri plate. See Figure 1.2.
2. Using the flamed loop, pick up a small amount of bacteria—either from bacteria growing on a plate or from bacteria in liquid culture.
3. Spread the bacteria on a new agar plate. Be careful not to gouge the agar surface with the loop. Spread the bacteria in a streak across the plate; move the loop back and forth across the surface of the agar three or four times.
4. Flame the loop again. When the loop is cool, move the loop once through the streak of bacteria just made on the plate. Again, streak the loop back and forth across a new area of the plate.

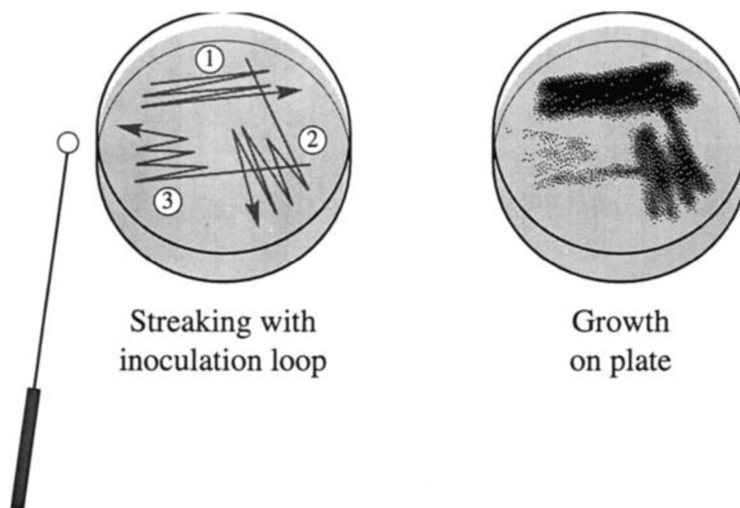


Figure 1.2 The use of an inoculation loop to streak for single colonies. Numbered lines show the order and direction of streaking with the inoculation loop.

5. Repeat the flaming and streaking three or four times, each time going through the previous streak only once to pick up bacteria and streaking onto a new part of the plate.
6. Incubate the plate inverted—with the agar-containing part of the plate up—at 37°C overnight.

The next morning, individual colonies should be visible in the last streaks on the plate. If there are no individual colonies, repeat the procedure until single colonies are readily obtained. With a bit of practice, this method will consistently give individual colonies.

Once this streaking procedure has been learned, it is possible to streak several colonies for individual colonies on the same agar plate. This reduces the number of agar plates needed. It is helpful to divide the plate into sections by marking the plate with a permanent marker. The experienced microbiologist can readily streak 8 to 12 different colonies for single colonies on the same plate.

Agar plates are incubated upside down, with the agar-containing part of the plate on top, so that any condensation that forms as the plate warms does not drop onto the agar surface. Droplets of condensation on the agar surface would smear the bacterial streaks. In general, plates that have been stored in the cold should be warmed to room temperature before use. This will minimize condensation. Plates can be warmed rapidly in a 37°C incubator.

Checking Sterile Technique

This exercise is performed to check that solutions can be handled without risk of contamination.

Materials

- *E. coli* strain on agar plate or liquid culture
- inoculation loop
- rich medium, such as L broth or nutrient broth
- Bunsen burner and matches or lighter
- several sterile capped test tubes in a rack
- sterile 10-ml pipets in canister
- pipet aid
- 37°C incubator or shaking water bath

Procedure

Under sterile conditions, transfer 2 ml of medium to each of three sterile test tubes. To do this:

1. Loosen the lid on a bottle of medium.
2. Briefly pass the lid of a canister of sterile 10-ml pipets through the flame of a Bunsen burner. Note that in this step the outside of the canister does not become hot enough to be sterilized. The flame heats the canister only enough to set up air currents that should remove bacteria adhering to the outside of the canister.
3. With the canister of pipets laying horizontally on the lab bench, slide the canister lid off.
4. Select a pipet. Touch the pipet only at the end. Slide the pipet out of the canister by lifting the pipet so that the pipet tip does not touch the ends of the other pipets in the canister as it is removed.
5. Insert the end of the pipet into the pipet aid.
6. While holding the pipet in the pipet aid in one hand, use the other hand to pass the lid and neck of the bottle of medium through the Bunsen burner flame.
7. Remove the lid of the bottle and hold it between the little finger and the palm of the hand that is holding the pipet.
8. Use a flamed sterile pipet to remove 6 ml of medium from the bottle.
9. Flame the opening of the bottle and replace the lid.
10. Flame the lid of a metal-capped sterile test tube. Remove the lid of the test tube with the hand that is holding the pipet.
11. Dispense 2 ml of medium into the test tube.

12. Flame the opening of the test tube and replace the metal cap.
13. Dispense 2 ml of medium into two other test tubes in the same way.
14. Inoculate one test tube with bacteria but do not inoculate the other two test tubes.
15. Incubate all three test tubes and the bottle of medium used at 37°C overnight.
16. Check the containers for bacterial growth. Only the test tube inoculated with bacteria should show bacterial growth.

If growth occurred in one of the containers that was not intentionally inoculated, repeat the whole procedure until sterile technique is achieved.

Tn5 Mutagenesis of *Escherichia coli* and Analysis of Auxotrophs: Overview

In this mutagenesis experiment using a modified Tn5, created by Wilmes-Riesenberg and Wanner (1992), a series of random insertions of the Tn (tet-resistant cells) is generated in *E. coli* (strain 10738).

Following mutagenesis, the target tetracycline-resistant cells are examined. Some transposon insertions will generate auxotrophs. Auxotrophs are isolated by failure to grow on a minimal medium and identified by growth on "pool plates." Pool plates are minimal plates supplemented with certain components. Many other transposon insertions will still be prototrophs. (How can the location of the modified Tn5 in such *E. coli* cells be determined?)

After the experimental part of this project, the students will select one auxotroph to examine in detail. In the literature search part of this project, the students will design experiments to localize the transposon.

Strain List

E. coli strains	Genotype
BW11397	*lac-169* DE10(*phoA8 phoA-E15*) *creB510 supF58 supE44 hsdR514 galK2 galT22 trpR55 metB1 tonA*
BW10748	*lac-169 phn*(EcoB) *creB510 hsdR514*
λ phage vehicle λ 4253	λ c_I857 *rex*::Tn*phoA'-2* O29(am) P80(am) b221
Tn*phoA'-2* = Tn*phoA-lacZ-132*	This transposon is a derivative of Tn5, which has a promoterless *lacZ* gene and a tetracycline resistance gene (in place of the kanamycin resistance gene)

Comments: The λ phage vehicle used to introduce the transposon into *E. coli* contains the following mutations:

b221: deletion of the attachment site (att^-) for λ integration. This mutation prevents integration of λ at its preferred site in the *E. coli* chromosome. The prophage insertion and excision genes are also deleted (int^- and xis^-). This deletion has removed approximately 10 kb (22%) of λ DNA and also allows room in the λ genome to carry the transposon. λ DNA is packaged by a headful mechanism that allows λ DNA of only a certain size range to be packaged into the phage head.

c_I857: temperature-sensitive allele of the λ repressor protein. At 30°C, the c_I product is active and will allow lysogeny, but at higher temperatures, such as 39°C, the c_I product is inactive; hence, lysogeny would not be permitted.

rex: the location into which the transposon Tn*phoA'-2* has been inserted. The *rex* gene has a role in blocking multiplication of rII mutants of phage T4 in λ lysogens.

O and P genes: involved in DNA replication of the λ phage and required for lytic growth of the phage.

O29(am) and P80(am): amber mutations. An amber mutation is a base change resulting in the sequence ATC in the sense strand and therefore UAG in the mRNA. The UAG codon signals polypeptide chain termination, so if a base change results in a UAG codon in the middle of a gene, the polypeptide chain will be prematurely truncated.

supF and *supE:* suppressors of amber mutations. Such suppressor mutations result when a change occurs in the DNA sequence of a tRNA species at its anticodon. The tRNA will then recognize the termination codon UAG as sense and insert an amino acid allowing completion of the protein chain. Often such proteins are active despite having a new amino acid. *supE* inserts a glutamine residue at the stop codon UAG. *supF* inserts a tyrosine residue at UAG.

Escherichia coli strain BW11397 contains two amber suppressors, *supF58* and *supE44*. Thus, the phage 4253 can replicate in this strain, and stocks of this phage can be prepared in this strain of *E. coli*.

Escherichia coli strain BW10748 does not contain a suppressor mutation—it is sup^+—and the λ phage cannot replicate and form a plaque in that strain.

For the mutagenesis experiment, infect *E. coli* strain BW10748 with phage 4253. Select tetracycline-resistant derivatives of BW10748. Alternatively, other *E. coli* strains that are tetracycline sensitive and sup^+ can be mutagenized with this transposon. Because the phage cannot lysogenize (and thus replicate along with the bacterial chromosome) or replicate

autonomously (to be maintained as a plasmid), any tetracycline-resistant bacterial colonies must result from a transposition of Tn*phoA'-2* (Fig. 1.1c) from the rex gene of the λ phage to some place on the bacterial chromosome (or resident plasmid, if present in the bacterium).

Medium Recipes

Unless otherwise specified, autoclave all media 20 min, slow exhaust, and store at room temperature.

L Broth

L broth is also called Luria or Luria–Bertani broth (L-B).

1. For each liter, combine
   10 g tryptone
   5 g yeast extract
   5 g sodium chloride (NaCl)
2. Add water to almost 1 liter. Adjust to pH 7 with sodium hydroxide (NaOH). Bring volume to 1 liter with water (H_2O).
3. For plates, add 15 g Bacto agar per liter.
4. For top agar, add 7 g Bacto agar per liter.

λ Broth

For each liter, use

10 g tryptone
2.5 g NaCl

λ Ym Broth

Use λ broth (above) *plus*

0.2% (0.2 g/100 ml) maltose
0.01% (0.01 g/100 ml) yeast extract

SM Buffer

0.02 *M* Tris, pH 7.5
0.1 *M* NaCl
0.01 *M* $MgSO_4$

Tris Phage Buffer

1. For each liter, combine
   1.2 g Trizma base
   2.45 g magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$)
   2.9 g sodium chloride

2. Adjust pH to 7.5 with HCl.
3. Add 10 ml 1% (1 g/100 ml) gelatin solution; mix well. Autoclave.

λ Top Agar or λ Soft Agar

For each liter, use
10 g tryptone
2.5 g NaCl
6.5 g Bacto agar

λ Agar

For each liter, combine
10 g tryptone
2.5 g NaCl
10 g Bacto agar

Minimal Medium

Minimal medium is also called VBC, Vogel–Bonner minimal medium, or E medium. See Davis *et al.* (1980, pp. 202–203).

50× VBC minus $MgSO_4$ Stock

For 1 liter, add to about 670 ml H_2O
100 g citric acid·$1H_2O$
500 g potassium phosphate, dibasic K_2HPO_4 (anhydrous)
175 g sodium ammonium phosphate, tetrahydrate ($NaNH_4HPO_4 \cdot 4H_2O$).

Dissolve salts one at a time in order listed with stirring at 45°C. Do not add the next salt until the previous salt has dissolved completely. Bring volume to 1000 ml with H_2O. Filter sterilize or store over chloroform and do not autoclave until diluted. Add appropriate amount of $MgSO_4$ stock solution after diluted and autoclaved. Store at room temperature.

0.5 M $MgSO_4$ Stock

Dissolve 60 g $MgSO_4$ (if anhydrous) per liter H_2O. Autoclave.

Minimal 1× VBC Bottom Agar

Autoclave the following separately:

2× VBC

20 ml 50× VBC
470 ml H_2O

Autoclave 20 min. Store at room temperature.

2× Agar (3%)

15 g Bacto agar
500 ml H_2O

Autoclave 20 min. Store at room temperature.

20% Glucose

20 g glucose

1. Add H_2O to bring volume to 100 ml.
2. Autoclave 20 min. Store at room temperature.

To make **Minimal 1× VBC bottom agar:** Melt 500 ml 2× **agar;** add to 490 ml **prewarmed 2× VBC;** also add 1.6 ml **0.5 *M* $MgSO_4$;** add 8 ml **20% glucose.** (Glucose will caramelize and cause the medium to darken if warmed to too high a temperature.) Pour plates. These plates are 0.2% glucose. Minimal 1× VBC contains 98 mg $MgSO_4$ per liter.

2× EM Medium (Enriched Minimal Medium)

To minimal 1× VBC medium, add 20 ml nutrient broth per liter.
For plates, use 15 g bacto agar per liter.

Nutrient Broth

For each liter, combine
8 g nutrient broth (Difco)
5 g NaCl

PROTOCOL 1.1: Phage λ Titer

This procedure is used to determine the titer—number of plaque forming units per milliliter—of a phage solution.

Materials

- *E. coli* strain that will support lytic replication of the λ phage used
- shaking water bath at 37°C
- λ Ym medium
- λ broth
- small sterile test tubes (Wassermann test tubes) in rack
- λ top (or soft) agar, melted and placed in water bath (45–50°C)
- rich agar plates, such as L agar or λ agar plates
- water bath or heating block at 45–50°C

- Bunsen burner and matches
- 0.1-, 1-, 5-, and 10-ml sterile pipets in canisters
- pipet aids
- sterile glass capillary pipets and appropriate pipet aid

Procedure

1. Grow recipient *E. coli* strain overnight in λ Ym medium. Inoculate several culture tubes, each containing 2 ml λ Ym medium, with bacteria from an agar plate. Incubate at 37°C with shaking.
2. Melt λ top or soft agar in a boiling H_2O bath or a microwave. Place the melted top agar in a heating block or water bath at 45–50°C to cool.
3. Make serial dilutions of phage using λ broth. (Suggested dilutions are 0.1 ml of a dilution plus 0.9 ml of broth for a 10-fold dilution, but 100-fold dilutions—0.01 ml of a dilution plus 0.99 ml of broth—could be made also.) Use a separate pipet for each dilution.
4. Transfer 0.1 ml of each phage dilution to be plated to appropriately labeled sterile test tubes. Add 0.1 ml of overnight bacterial culture to each tube.
5. Mix and incubate the aliquots of the phage dilutions with bacteria at room temperature for about 20 min. This allows time for the phage to adsorb to the bacteria. The time is not crucial; 10 min may be adequate.
6. Carefully label the L agar plates on which the phage dilutions will be plated. Remember that only 0.1 ml of a phage dilution will be plated. This is a 10-fold dilution of the contents of the dilution tube and is sometimes called the "plating factor."
7. Add 2.5 ml of melted λ top agar from the heating block or water bath to a test tube of phage and bacteria to be plated. Mix without introducing bubbles.
8. Quickly pour the phage, bacteria, and top agar mixture before it hardens onto an L agar plate (or other rich media). Quickly spread the top agar evenly onto the top of the L agar plate by moving and tipping the agar plate. Moving the plate rapidly in a figure 8 pattern is an easy way to spread the top agar.

CAUTION: Top agar will set up too fast (before it can be spread) if the agar is too cool.

Once the top agar has been evenly distributed on the plate, do not tip the plate until the top agar has completely solidified.

9. Plate all the phage and bacteria mixtures in the same way.
10. Incubate the agar plates inverted at 37°C overnight.

11. The next day, count the number of plaques per plate and calculate the phage titer (number of plaque forming units per milliliter) of the original phage solution. Plaques are small clear circles where the phage have lysed the bacteria on a cloudy lawn of bacterial growth.

NOTES

1. When determining a phage titer, prepare duplicate plates of each dilution to be plated. Also prepare a no-phage, bacteria-only control.
2. When making dilutions, do not inadvertently bring extra solution from the *outside* of the pipet. Such extra droplets clinging to the outside of the pipet could result in dilution errors if they are added to the test tubes. One way to avoid such errors is to wipe the tip of the pipet carefully with a sterile tissue before delivery. Another way is to take care *not* to put the pipet too far into the liquid.
3. Warm the plates to be used to room temperature. If the plates used are too cold, the top agar may solidify before being completely spread on the surface of the plate. If plates had been stored in the cold, the plates can be rapidly warmed by incubation at 37°C.
4. Take care that the top agar is not too cool when mixed with phage and bacteria and poured on top of the agar plate. If the top agar sets up too quickly before the bacteria with adsorbed phage have been mixed into the top agar, after overnight incubation at 37°C, the plate will show small irregular-shaped clumps of top agar that are clear, that is, do not show any bacterial growth. Such irregular clear zones can interfere with the observation of plaques.
5. If some plates contain too many plaques to count easily, make an estimate of the number of plaques on the plate by counting the number of plaques on a quarter of the plate and multiplying the number counted by 4.

PROTOCOL 1.2: Making a Phage Stock—Growing λ-Tn5′

In this procedure, a few individual plaques are isolated and grown to make a stock of the λ-Tn5′ vector.

Part A: Making Fresh Plaques

Materials

- λ::*Tn5′*—λ phage Tn*phoAlacZ-132* (4253, also called Tn*phoA′-2*)

- *E. coli* strain that contains a nonsense suppressor and can support the lytic growth of λ::Tn5′—BW11397
- λ Ym medium
- sterile test tubes
- spectrophotometer to monitor bacterial cell density, such as Spectronic 20, or Klett meter
- λ agar plates poured thick, within the last 5 days
- water bath or heating block at 45–50°C
- Bunsen burner and matches
- 0.1-, 1-, 5-, and 10-ml sterile pipets in canisters
- pipet aids
- sterile glass capillary pipets and appropriate pipet aid

Procedure

1. Inoculate an *E. coli* nonsense suppressor strain (BW11397) into 5 ml of λ Ym broth; incubate culture overnight with shaking at 37°C.
2. The next day, start a culture of the *E. coli* nonsense suppressor strain (BW11397) in λ Ym broth using an inoculum from the overnight culture. Grow the *E. coli* culture to mid-log phase. This is an optical density of approximately 0.6 at 660 nm in a spectrophotometer or approximately 100–120 Klett units in a Klett meter. For example, 0.1 ml of an overnight culture diluted into 2.5 ml of λ Ym broth will grow to mid-log phase in about 3 hr with a vigorous shaking at 37°C.
3. Melt λ top or soft agar in a boiling H_2O bath or a microwave. Place the melted top agar in a heating block or water bath at 45–50°C to cool.
4. Make serial dilutions of the λ phage Tn*phoAlacZ-132* (4253, also called Tn*phoA′-2*) in λ Ym broth. Prepare to plate phage dilutions that will give 50 to 100 plaques per plate.
5. Transfer 0.1 ml of each phage dilution to be plated to appropriately labeled sterile test tubes. Add 0.1 ml of the mid-log phase bacterial culture to each tube.
6. Mix and incubate the aliquots of the phage dilutions with bacteria at room temperature for 20 min to allow time for phage to adsorb to bacteria.
7. To each tube, add 2.5 ml of melted λ top agar, cooled to 45–50°C. Quickly overlay the mixture onto fresh λ agar plates. These plates should have been poured within the last 5 days and should be very thick (i.e., ~2× usual volume of medium per plate). (Phage plaques will grow larger on very fresh plates with a high moisture content.) Let the overlay solidify completely before moving the plates.

8. Incubate the plates inverted at 37°C overnight.
9. The next day do Part B.

Part B: Making Phage Lysate Stocks

The plates prepared in Part A the day before are used to amplify individual plaques to make phage lysate stocks.

Materials

- plates with individual plaques of phage prepared in Part A the day before
- overnight culture of *E. coli* strain that contains a nonsense suppressor and can support the lytic growth of λ::Tn5′—BW11397 grown in λ Ym broth
- Tris phage buffer
- sterile Pasteur pipets
- pipet bulbs or pipet aids
- sterile test tubes
- λ top agar
- λ agar plates poured within the last 24 hr
- water bath or heating block at 45–50°C
- Bunsen burner and matches
- 0.1-, 1-, 5-, 10-ml sterile pipets in canisters
- pipet aids
- sterile glass capillary pipets and appropriate pipet aid
- clinical centrifuge or preparative centrifuge
- sterile centrifuge tubes for the above centrifuge
- chloroform

Procedure

Begin with phage plates prepared in Part A the day before. Several different phage lysates, five for example, are prepared.

For each phage lysate:

1. For each lysate to be made, add 0.1 ml of Tris phage buffer to each test tube. Use a sterile Pasteur pipet and a pipet bulb to "core out" or pick up a plaque. Place a well-isolated individual plaque or several individual plaques from the overnight plates in Part A in the tube. Select λ plaques that are "average looking," *not* extreme in size or morphology. The λ plaques can be "cored out" and picked up using a sterile Pasteur pipet. The use of very fresh plates helps accentuate the differences in plaque morphologies.

2. Allow the phage plaque to incubate in the Tris phage buffer for 30 min at room temperature.
3. Melt λ top agar in a boiling H_2O bath or a microwave. Place the melted top agar in a heating block or water bath at 45–50°C to cool.

Note: For this step, use top agar that has been melted *only once*. For phage titers, top agar melted several times may be used.

4. Add 0.1 ml of an overnight culture of bacteria (BW11397) in λ Ym medium to the test tube. Mix. Adsorb phage to bacteria for 20 min at room temperature.
5. Add 3 ml of *freshly* melted top agar, cooled to 45–50°C, to the test tube.
6. Pour onto a *very fresh* λ agar plate. Use plates poured within the last 24 hr or less (poured with ~25 ml agar per plate).
7. Be sure to make a control plate (bacteria alone, no phage).
8. Incubate at 37°C for 6 to 8 hr until there is evidence of lysis on the plate. The plates should look "lacy" or almost clear because there are many plaques very close together. The no-phage control plate, which should have a solid lawn of bacteria, is very helpful for comparison to determine when the phage-containing plates have lysed or cleared.
9. After the plates have cleared, flood each agar plate with 5 ml of Tris phage buffer. Store the plates in the refrigerator for 12 to 24 hr. Be careful not to tip flooded plates.
10. After 12 to 24 hr, tilt each plate and remove the buffer containing the phage lysate using a sterile Pasteur pipet or other glass pipet. Place the phage suspension in a screw-capped centrifuge tube.
11. Add a few drops of chloroform to the tube and cap securely. Mix by inversion.

CAUTION: Wear gloves and protective goggles when handling chloroform. DO NOT mouth pipet chloroform. Keep chloroform away from heat and open flames.

12. Spin the sample for 10 min in a clinical centrifuge at 3000 rpm or in a preparative centrifuge for 5 min at 5000 rpm.
13. Carefully remove the supernatant solution with a pipet and transfer the supernatant fluid to a new screw-capped centrifuge tube. Do not remove the debris or chloroform.
14. Add a few drops of chloroform to the supernatant, mix, and centrifuge again. Carefully remove the supernatant solution to a new tube. Add a few drops of chloroform to the tube. *Label* the tube clearly. This is a λ::Tn*phoA'*-2 lysate. Store at 4°C.
15. Determine the titer of the phage lysate using Protocol 1.1.

NOTES

1. The phage lysates may be stored at 4°C indefinitely. Phage titers of 10^9 to 10^{10} are typically obtained. In step 8, if it is inconvenient to check plates for lysis after 6 to 8 hr, the plates can be prepared in the afternoon and incubated overnight. This may reduce the phage titer somewhat, but still gives satisfactory results.
2. If there is not enough time to allow the phage to diffuse from the top agar into the buffer, phage can also be removed from top agar by scraping the top agar off a plate and into a centrifuge tube. Add 5 ml of Tris phage buffer and 0.1 ml of chloroform. Vortex to mix thoroughly. Centrifuge 5 min at 10,000 rpm. The supernatant solution is the phage lysate.

PROTOCOL 1.3: Transposon Mutagenesis Using λ::Tn*phoA'*-2

A transposon can be introduced into *E. coli* cells to be mutagenized by the infection process of bacteriophage λ. The *E. coli* strain to be mutagenized must be sup^+ so the phage containing amber mutations in the O and P genes needed for lytic DNA replication of λ cannot grow lytically in the strain. The host strain must be tetracycline sensitive so the *E. coli* cells with a transposon that renders them tetracycline resistant can be selected.

Materials

- *E. coli* strain to be mutagenized, freshly grown on an L agar plate the day before this procedure (BW10748 that is nonpermissive for λ phage replication)
- Phage lysate stock of λ phage Tn*pho*AlacZ-132
- L broth + 0.4% maltose
- MC buffer (10 m*M* $MgCl_2$, 10 m*M* $CaCl_2$)
- sterile test tubes
- shaking water bath at 37°C
- L_{tet15} agar plates (15 μg/ml tetracycline)
- bent glass rod and beaker with alcohol to spread bacterial cells on agar plates
- clinical or preparative centrifuge
- sterile centrifuge tubes

Procedure

1. The day before use, streak the bacterial strain (BW10748) to be mutagenized on an L agar plate and incubate overnight at 37°C.

2. The day of this procedure, inoculate a single colony of BW10748 into 5 ml of L broth + 0.4% maltose.
3. Grow the culture 4 to 6 hr with shaking at 37°C. The bacterial culture will be in mid- to late log phase growth at that time.
4. Harvest the bacterial cells by centrifugation for 5 min at 3000 rpm in a clinical centrifuge or for 5 min at 5000 rpm in a preparative centrifuge.
5. Decant the medium and resuspend the cell pellet in 1 ml of MC buffer.
6. Distribute 0.1 ml of resuspended cells to each of several small sterile test tubes.
7. Add to the tube a series of different phage concentrations. Make dilutions in MC buffer. Prepare a range of tubes similar to those suggested.

   To tube 1 add 10 μl of the phage lysate.

   To tube 2 add 10 μl of a 1:10 dilution of the phage lysate.

   To tube 3 add 10 ml of a 1:100 dilution of the phage lysate.

   To tube 4 add nothing.
8. Incubate 20 min at room temperature.
9. After adsorption, spread the cells and phage or dilutions of the cells and phage on L_{tet15} agar plates (15 μg/ml tetracycline). To spread the cells on the agar plate, sterilize a bent glass rod by dipping it in a beaker of ethanol. Drain excess ethanol off the glass rod. Briefly pass the rod through a Bunsen burner flame. When all the ethanol has burned off, touch the glass rod to the inside of the petri plate top to cool it. Use the glass rod to spread the aliquot of cells evenly on the agar plate.
10. Incubate at 40°C overnight.

NOTES

1. The *E. coli* strain to be mutagenized is grown in medium containing maltose because growth in maltose in the absence of glucose induces the formation of more λ phage receptors on the surface of the *E. coli* cells. The receptor for bacteriophage λ binding and uptake into the *E. coli* cell, encoded by the *lamB* gene, is also the channel for maltose uptake into the *E. coli* cell. Growth of *E. coli* in the presence of maltose induces the formation of more receptors for maltose uptake on the surface of the *E. coli* cell. Maltose is broken down to glucose in the *E. coli* cell. Growth of *E. coli* in the presence of glucose prevents the induction of more maltose receptors on the cell because of catabolite repression.
2. An alternative way to prepare the *E. coli* cells to be mutagenized is to

grow an overnight culture of bacteria in L broth + 0.4% maltose. A few hours before it is needed, dilute the overnight culture in L broth + 0.4% maltose and grow the cells until mid- to late log phase. For example, 0.2 ml of an overnight culture diluted into 5 ml of medium will grow to mid-log phase in about 3 hr with vigorous shaking at 37°C.

3. The 40°C incubation is recommended for phage vehicles containing the temperature-sensitive C_I repressor mutation (Miller, 1992, p. 355). The repressor protein is not active at this higher temperature. Growth at 40°C reduces the background of cells that begin to grow briefly and then lose the transposon.

Introduction to Auxotrophs

The laboratories of Joshua Lederberg and Bernard Davis pioneered work in the field of biochemical genetics. Their work to isolate mutants was essential for the determination of biochemical pathways. It was Bernard Davis who coined the term "auxotroph" (Davis, 1993). Both Davis and Lederberg working independently developed the auxotroph screening method using enriched minimal plates. In an enriched minimal medium, an auxotroph is limited in an essential component and grows only into a small colony, whereas wild-type colonies, prototrophs, produce large colonies. Again working independently, Lederberg and Davis developed a selection procedure for auxotrophs using penicillin (Davis, 1948; Lederberg and Zinder, 1948). The antibiotic penicillin causes the death and lysis of sensitive bacterial cells. However, to be killed by penicillin, a bacterium must be growing and actively metabolizing. To select for auxotrophs, a mutagenized culture of penicillin-sensitive bacteria is grown in a minimal medium containing penicillin. A prototrophic bacterium will grow in minimal medium and be killed by the penicillin present. However, because an auxotrophic bacterium lacks a component essential for growth, it will not grow in the minimal medium and will not be killed by penicillin. The cells are washed to remove the penicillin and then planted on a rich plate that will allow the growth of auxotrophs.

PROTOCOL 1.4: Isolation of Auxotrophs—Replica Plating, Toothpicking, or Screening on 2 EM Plates

All the cells that have grown on the L_{tet} plates after the mutagenesis in Protocol 1.3 should contain a transposon. There are several ways to search for auxotrophs among the mutagenized cells.

Materials

- Mutagenized *E. coli* cells prepared in Protocol 1.3 that are tetracycline-resistant colonies on L_{tet15} agar plates
- L_{tet15} agar plates
- $minimal_{tet10}$ agar plates
- 2 EM_{tet10} agar plates
- sterile toothpicks (To sterilize toothpicks, autoclave for 20 min at a fast and dry setting.)
- sterile velvets: Square pieces of cloth about $\frac{1}{2}$ in. larger than the petri plates with a thick nap, such as velvet or velveteen. (As an alternative to velvets, sterile circular pieces of filter paper with a diameter about 2 mm less than the inside diameter of a petri dish bottom may be used.)
- platform to put velvets on for replica plating
- sterile test tubes and rack
- 1× VBC broth, a minimal broth

Procedures

Replica Plating

1. Select L_{tet15} plates grown in Protocol 1.3 that have between 50 and 125 well-spaced colonies.
2. Carefully replica plate the colonies onto a minimal plate and another L_{tet} plate.
   a. Place a sterile velvet on the replica plating platform.
   b. Press an L_{tet} agar plate with colonies onto the platform. Apply gentle, uniform pressure to the bottom of the petri plate to ensure that all colonies on the agar surface touch the velvet. Remove the agar plate.
   c. Carefully press a $minimal_{tet10}$ agar plate to the velvet. Again, using gentle pressure, make sure all areas of the agar surface make contact with the velvet. Mark the top of the plate with a permanent marker. Remove the agar plate.
   d. Carefully press a new L_{tet15} agar plate to the velvet. Mark the top of the plate with a permanent marker. Remove the agar plate.
3. Incubate the plates at 40°C overnight.
4. The next day, compare the growth of bacteria on both plates. An auxotroph will not grow on the minimal plate, but will grow on the L_{tet} plate.
5. When an auxotroph is found, locate the corresponding colony on the L_{tet} plate. With a sterile toothpick or an inoculation loop, transfer some of the bacterial colony to a new L_{tet} plate. Streak for single-colony isolation. Incubate the plates at 40°C overnight.

6. Retest that the colony isolated is an auxotroph by streaking single colonies on a $minimal_{tet10}$ agar plate.

Note: When replica plating, use agar plates that are thoroughly dry. If plates are too wet, the colonies can smear together. Do not apply too much pressure when transferring the colonies or colonies may run together.

Toothpicking

1. Choose L_{tet15} plates grown in Protocol 1.3 that have too many colonies to use for replica plating, but still have separate, distinct colonies.
2. Using a sterile toothpick, touch a colony.
3. With the same toothpick, make a $\frac{1}{4}$-in. streak on the surface of a $minimal_{tet10}$ agar plate.
4. Make a second streak with the toothpick on the corresponding place on an L_{tet15} agar plate.
5. With a new sterile toothpick, touch a new colony.
6. Repeat the process.
7. Incubate the plates at 40°C overnight.
8. Identify streaks that fail to grow on minimal agar plates. Find the corresponding streak on the L_{tet15} agar plate.
9. Streak some of that colony for single colonies on a new L_{tet15} agar plate. Once it has grown, retest by streaking on a $minimal_{tet10}$ agar plate.

NOTES

1. Be sure to make the streaks in the corresponding places on the pair of plates. At least 40 to 50 different colonies can be streaked on an agar plate. A template can be used if desired. Examples are given of a template in Appendix 1. Do not try to pick colonies so close together that it is extremely difficult to pick only one colony with the toothpick.
2. Used toothpicks can be collected, resterilized, and reused.
3. Once an auxotrophic strain has been isolated, be sure to make a permanent stock of that strain (See Appendix 2). Label the strain clearly.

Screening for Auxotrophs on 2 EM Agar (Minimal Medium Supplemented with a Small Amount of Nutrient Broth) Plates. Crowded plates containing from 500 to 1000 bacterial colonies are used. The basis of this screen is that in the limiting amount of nutrients on 2 EM plates containing a large number of colonies, the growth of auxotrophs will be slowed or stopped as the crowded plate becomes depleted of the nutrients the auxotrophs require for growth. Colonies that are tiny compared to the

majority of the colonies may be auxotrophs; such colonies will be picked and retested.

1. Remove bacteria from an L_{tet} plate containing more than 100 colonies from the mutagenesis protocol by flooding the plate with 5 ml of 1× VBC minimal broth.
2. With a sterile 5-ml pipet, scrape the bacteria off the agar surface. Remove the bacteria with a pipet and place bacteria in a centrifuge tube.
3. Centrifuge the bacteria for 5 min at 5000 rpm. Decant the buffer from the bacterial cell pellet.
4. Resuspend the cells in 1 ml of minimal medium. This stock of mutagenized bacteria can now be tested on 2 EM plates. The 2 EM plates should be crowded to enhance selection for potential auxotrophs. A crowded plate should have about 500–1000 bacterial colonies on it.
5. Make serial dilutions of the mutagenized bacteria in 1× VBC minimal medium.
6. Using an ethanol-flamed bent glass rod, spread 0.1 ml of that dilution estimated to give 500–1000 colonies per plate on a 2 EM plate (minimal medium supplemented with 20 ml of nutrient broth per liter). Make a series of such plates. Also spread 0.1 ml of bracketing dilutions, such as 10 times higher and 10 times lower concentrations, on 2 EM plates.
7. Incubate plates overnight at 40°C.
8. Examine the plates the next day. Growth will be slower on this minimal plate. Examine the plates closely (a dissecting microscope may be helpful here) for the presence of colonies that are much smaller than the majority of colonies on the plate. These tiny colonies are *potential* auxotrophs. The tiny colonies may also be less opaque than the average colony because the tiny auxotrophic colonies are not as thick. The plates may need to be incubated longer before the tiny colonies are readily visible.
9. On the outside of the petri plate, mark the location of the tiny colonies with a marking pen.
10. Using sterile toothpicks or an inoculation wire, streak the tiny colonies onto L_{tet} plates.
11. Incubate plates overnight at 37°C.
12. Replica plate or toothpick the colonies onto minimal plates.

Colonies that grow on L_{tet} but not on minimal medium are auxotrophs. These auxotrophs will be tested to characterize their biochemical defects.

PROTOCOL 1.5: Identification of Auxotrophs on Pool Plates

Materials

- auxotrophs isolated in Protocol 1.4 and retested to be auxotrophs, streaked for single colonies on L_{tet15} plates
- pool plates, one of each of 11 different pools
- sterile toothpicks or inoculation loop

Procedure

1. Using sterile toothpicks, transfer a small number of bacteria of each auxotroph onto each of the 11 pool plates. Many different auxotrophs can be tested on the same pool plates. Be sure to place each auxotroph in the same location on each of the pool plates.
2. Also streak a prototroph on each of the pool plates as a positive control. The prototroph will grow on all of the pool plates.
3. Incubate at 37°C overnight.
4. Observe growth on pool plates the next day. Score the amount of growth for each auxotroph on each pool plate.
5. Return the plates to 37°C and allow further growth.
6. Score growth again after the second day.
7. By examining the components in the pool plates, identify the type of auxotroph.

Making Pool Plates

The following components should be filter-sterilized because they may be degraded at the high temperatures used in autoclaving. All other components may be autoclaved.

Adenine
Asparagine
Potassium aspartate
Cysteine
Sodium glutamate
Tryptophan
Tyrosine

Additional comments:

Solutions containing tryptophan should be stored in the dark.

The salts of glutamic acid and aspartic acid rather than the free acids should be used.

The following components dissolve more readily in acidic solutions. Use the acid strength indicated to dissolve the component.

Adenine, 0.1 *N* HCl

Adenosine, 0.1 *N* HCl

Guanosine, 0.3 *N* HCl

Phenylalanine, 0.01 *N* HCl

Stock solutions of each of the individual components can be made and combined into the pool groups.

Stocks of each amino acid can be made at a concentration of 10 mg/ml of the L form or 20 mg/ml of the DL form. Adenosine, guanosine, thymine, and uracil stocks can be made at 5 mg/ml. Stocks of vitamins and other components can be made at the specific concentrations indicated: thiamine (1 μg/ml), diaminopimelic acid (DAP, 300 μg/ml), pyridoxine (50 μg/ml), nicotinic acid (50 μg/ml), biotin (1 μg/ml), pantothenate (50 μg/ml). Note that vitamins are trace growth factors; that is, vitamins are required at very low concentrations. Sterilize the components as indicated.

To make the stocks for pool plates, using aseptic or sterile technique, combine the stocks of individual components: Mix equal volumes of the stocks of individual components indicated for each pool.

Pool stocks may be stored indefinitely at room temperature or at 4°C. Tightly close caps or wrap caps with Parafilm. Wrap pool stocks containing tryptophan with aluminum foil to keep out light.

Components of Pool Plates[a]

Pool	1	2	3	4	5
6	Adenosine	Guanosine	Cysteine	Methionine	Thiamine
7	Histidine	Leucine	Isoleucine	Lysine	Valine
8	Phenylalanine	Tyrosine	Tryptophan	Threonine	Proline
9	Glutamine	Asparagine	Uracil	Aspartic acid	Arginine
10	Thymine	Serine	Glutamic acid	Diaminopimelic acid	Glycine
11	Pyridoxine	Nicotinic acid	Biotin	Pantothenate	Alanine

[a] The components of pools 1 to 5 are listed vertically; the components of pools 6 to 10 are listed horizontally. *How to use the pool plates:* If a mutant grows on two pool plates, the auxotrophic requirement is for the component in common to both pools. For example, a mutant which grows only on pools 3 and 9 is an auxotroph for uracil. If a mutant grows only on one pool plate of plates 1 to 10, the mutant must require more than one component from that pool. If a mutant grows only on pool 11, each component of pool 11 must be tested individually for growth to identify the auxotrophy.

To make pool plates, add 10 ml of a pool stock to 1 liter of minimal medium or minimal agar.

If only a small number of pool plates are needed: To make individual pool plates, using sterile technique, place 0.2 ml of the appropriate pool stock in a sterile petri plate. Pour approximately 20 ml of minimal agar into the petri plate. The pool components will diffuse throughout the plate as the plate solidifies and is stored.

Additional Information about the Growth of Auxotrophs on Specific Pool Plates

1. Purine mutants: Some purine mutants will grow on pools 1, 2, and 6 because the mutants can grow on adenosine or guanosine. Some other purine mutants will grow only on pool 6 because they need both adenosine and thiamine.
2. Pyrimidine mutants: *pyrA* mutations require uracil and arginine and will grow on pool 9.
3. Aromatic amino acid biosynthetic pathway mutants: Mutants that are blocked early in the aromatic pathway will grow only on pool 8.
4. Isoleucine and valine mutants: Mutants that require isoleucine and valine will grow only on pool 7. Growth of *E. coli* K12 strains is inhibited by the presence of valine in the absence of isoleucine. When *E. coli* K12 strains are used, isoleucine must be added to pool 5 to avoid this inhibition.
5. Many, but not all, auxotrophs isolated can be identified by these pool plates. Some mutations may cause pleiotrophic effects. Such mutations may fail to grow on any of the pool plates.

The pools presented here are based on those of Davis *et al.* (1980, p. 209). Other researchers have devised slightly different pools to identify auxotrophs. For example, see Clowes and Hayes (1968, p. 228) or Eisenstadt *et al.* (1994, p. 308).

Concentrations of components in pools can be obtained from Lederberg (1950), Davis *et al.* (1980, p. 209), or Clowes and Hayes (1968, p. 228).

PROTOCOL 1.6: Analysis of Auxotrophs Using a Literature Search

Select an auxotroph to analyze in further detail. Through a literature search and an understanding of the biochemical pathway for that auxotroph, devise a method to locate the modified Tn5 insertion. Design the

experimental plans, but proceed with the experiments only to the extent that time and materials are available.

Suggestions for getting started on an auxotroph literature study:

1. See references for the Auxotroph Library Report, pages 42–44.
2. Check the loci in *E. coli* known to be involved in the auxotroph. See Bachmann (1983, 1990) and Bachmann and Low (1980) for information on the *E. coli* linkage map. Note the loci on the *E. coli* map.
3. Check a biochemistry textbook for information about the biochemical pathway involved.
4. Use any of the following methods to localize the Tn5:
   a. Nutritional data, such as the feeding of intermediates in a pathway
   b. Biochemical assays for enzyme activities
   c. Genetic mapping strategies (for a review of methods, see Low (1973) and Wollmen *et al.* (1956), as well as genetic texts)
   d. Physical mapping, using the Tn5 as a probe
   e. Inverse PCR from the transposon ends; use as probe in Kohara-ordered λ clones of *E. coli* (Kohara, 1987).

Be sure to include a reasonable amount of detail of how each type of experiment would be done. For example, in a particular enzyme assay, do not report the exact millimolar salt concentrations needed in the enzyme assay, but indicate the substrate for the assay, the wavelength for monitoring the substrate if it is a colorimetric assay, etc. Likewise, in genetic mapping experiments, indicate specific genetic markers to be used. In physical mapping, indicate (and reference) appropriate restriction enzymes and plasmids to be used.

Genetic Mapping Strategies

Use of Transduction to Localize the Transposon

Consider several genes involved in the production of cysteine. For the purposes of this example, assume that the transposon is in either the *cysB* or the *cysG* gene. The modified Tn5′ is tet^R.

Gene	Map location	Nearby selectable marker
cysB	28 min	*trp* 27.7 min
cysG	74 min	*argD* 73.8 min

For *cysB*, the donor strain is trp$^+$cys::Tn5′. The recipient strain is trp$^-$cys$^+$.

1. Make a P1 phage lysate on donor strain, infect recipient strain.
2. Select for recipient strain transduced to trp^+ by plating on min plus cys; score how many trp^+ cells are also cys^- (cannot grow on a min plate) or tet^R (can grow on a min_{tet5} plus cys plate or an L_{tet15} plate).

For *cysG*, the donor strain is *argD*$^+$*cys*::Tn5′. The recipient strain is *argD*$^-$*cys*$^+$.

1. Make a P1 phage lysate on donor strain, infect recipient strain.
2. Select for recipient strain transduced to arg^+ by plating on min plus cys; score how many arg^+ cells are also cys^- (cannot grow on a min plate) or tet^R (can grow on a min_{tet5} plus cys plate or an L_{tet15} plate).

The results of the transduction experiments will be that the co-transduction of tet^R or *cys*$^-$ with *arg* will be high and that with *trp* will be zero, indicating the transposon is closely linked to *argD*—that is, the transposon inserted into *cysG*, or the co-transduction of tet^R or *cys*$^-$ with *trp* will be high and with *arg* will be zero, indicating the transposon inserted into *cysB*.

NOTE

Auxotrophic markers can be used readily in such co-transduction experiments. Vinopal (1987) lists many (10.5 pages worth!) selectable markers. See Miller (1992, pp. 263–274) for details of using bacteriophage P1 for transduction.

References

Bachman, B. J. (1983). Linkage map of *Escherichia coli* K-12, ed. 7. *Microbiol. Rev.* **47,** 180–230.

Bachman, B. J. (1990). Linkage map of *Escherichia coli* K-12, ed. 8. *Microbiol. Rev.* **54,** 130–197.

Bachman, B. J., and Low, K. B. (1980). Linkage map of *Escherichia coli* K-12, ed. 6. *Microbiol. Rev.* **44,** 1–56.

Berg, C. M., Berg, D. E., and Groisman, E. A. (1989). Transposable elements and the genetic engineering of bacteria. *In* "Mobile DNA" (D. E. Berg and M. M. Howe, eds.), pp. 881–888. American Society for Microbiology, Washington, DC.

Berg, D. E. (1989). Transposon Tn5. *In* "Mobile DNA" (D. E. Berg and M. M. Howe, eds.), pp. 185–210. American Society for Microbiology, Washington, DC.

Berg, D. E., and Berg, C. M. (1983). The prokaryotic transposable element Tn5—A review article. *Biotechnology* **1,** 417–435.

Clowes, R. C., and Hayes, W., eds. (1968). "Experiments in Microbial Genetics." Wiley, New York.

Davis, B. D. (1948). Isolation of biochemically deficient mutants by penicillin. *J. Am. Chem. Soc.* **70,** 4267.

Davis, B. D. (1993). The penicillin method of mutant selection. *BioEssays* **15,** 837–839.
Davis, R. W., Botstein, D., and Roth, J. R. (1980). "A Manual for Genetic Engineering: Advanced Bacterial Genetics." Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Eisenstadt, E., Carlton, B. C., and Brown, B. J. (1994). Gene mutation. *In* "Methods for General and Molecular Bacteriology" (P. Gerhardt, R. G. E. Murray, W. A. Wood, and N. R. Krieg, eds.), pp. 297–316, American Society for Microbiology, Washington, DC.
Finnegan, D. J. (1985). Transposable elements in eukaryotes. *Int. Rev. Cytol.* **93,** 281–326.
Galas, D. J., and Chandler, M. (1989). Bacterial insertion sequences. *In* "Mobile DNA" (D. E. Berg and M. M. Howe, eds.), pp. 112–113. American Society for Microbiology, Washington, DC.
Gutierrez, C., Barondess, J., Mannoil, C., and Beckwith, J. (1987). The use of transposon Tn*phoA* to detect genes for cell envelope proteins subject to a common regulatory stimulus. *J. Mol. Biol.* **195,** 289–297.
Hoffman, C. S., and Wright, A. (1985). Fusions of secreted proteins to alkaline phophatase: An approach for studying protein secretion. *Proc. Natl. Acad. Sci. U.S.A.* **82,** 5107–5111.
Kleckner, N. (1977). Genetic engineering *in vivo* using translocatable drug-resistance elements. *J. Mol. Biol.* **116,** 125–159.
Kleckner, N. (1981). Transposable elements in prokaryotes. *Annu. Rev. Genet.* **15,** 341–404.
Kleckner, N. (1990). Regulation of transposition in bacteria. *Annu. Rev. Cell Biol.* **6,** 297–327.
Lederberg, J. (1950). *In* Isolation and characterization of biochemical mutants of bacteria. "Methods in Medical Research," **3,** (R. W. Gerard, ed.), pp. 5–22. The Year Book Publishers, Inc.
Lederberg, J., and Zinder, N. (1948). Concentration of biochemical mutants of bacteria with penicillin. *J. Am. Chem. Soc.* **70,** 4267–4268.
Low, K. B. (1973). Rapid mapping of conditional and auxotrophic mutations in *E. coli* K-12. *J. Bacteriol.* **113,** 798–812.
Manoil, C., and Beckwith, J. (1986). A genetic approach to analyzing membrane protein topology. *Science* **233,** 1403–1408.
Price, C. A. (1990). Laboratory notebooks: Sacred works. *Plant Mol. Biol. Report.* **8,** 220–221.
Scott, J. R. (1992). Sex and the single circle: Conjugative transposition. *J. Bacteriol.* **174,** 6005–6010.
Stachel, S., An, G., Flores, C., and Nester, E. W. (1985). A Tn*3 lacZ* transposon for the random generation of β-galactosidase gene fusions: Application to the analysis of gene expression in *Agrobacterium*. *EMBO J.* **4,** 891–898.
Vinopal, R. T. (1987). Selectable phenotypes. *In* "*Escherichia coli* and *Salmonella typhimurium* Cellular and Molecular Biology." (F. C. Neidhardt, ed.), pp. 990–1015. American Society for Microbiology, Washington, D.C.
Wilmes-Riesenberg, M. R., and Wanner, B. L. (1992). Tn*phoA* and Tn*phoA'* elements for making and switching fusions for study of transcription, translation, and cell surface localization. *J. Bacteriol.* **174,** 4558–4575.
Wollman, E. L., Jacob, F., and Hayes, W. (1956). Conjugation and genetic recombination in *E. coli* K-12. *Cold Spring Harbor Symp. Quant. Biol.* **21,** 141–162.

Suggested Reading

Phage λ

Friedman, D. I., Olson, E. R., Georgopoulos, C., Tilly, K., Herskowitz, I., and Banuett, F. (1984). Interactions of bacteriophage and host macromolecules in the growth of bacteriophage λ. *Microbiol. Rev.* **48,** 299–325.

Hendrix, R. W., ed. (1983). "Lambda II." Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Lewin, B. (1994). Phage strategies: Lytic cascades and lysogenic repression. *In* "Genes V," Chap. 17, pp. 491–523. Oxford University Press, New York.

Phage λ Receptor

Hall, M. N., Gabay, J., and Schwartz, M. (1983). Evidence for a coupling of synthesis and export of an outer membrane protein in *E. coli*. *EMBO J.* **2,** 15–19.

Ludwig, R. A. (1987). Gene tandem-mediated selection of coliphage λ-receptive *Agrobacterium*, *Pseudomonas*, and *Rhizobium* strains. *Proc. Natl. Acad. Sci. U.S.A.* **84,** 3334–3338.

Roessner, C. A., and Ihler, G. M. (1984). Proteinase sensitivity of bacteriophage lambda tail proteins gpJ and pH in complexes with the lambda receptor. *J. Bacteriol.* **157,** 165–170.

Schwartz, M. (1983). Phage λ receptor (*LamB* protein) in *E. coli*. *In* "Methods in Enzymology" (S. Fleischer and B. Fleischer, eds.), Vol. 97, pp. 100–112. Academic Press, San Diego.

Prokaryotic Transposons

Adzuma, K., and Mizuuchi, K. (1988). Target immunity of Mu transposition reflects a differential distribution of MuB protein. *Cell (Cambridge, Mass.)* **53,** 257–266.

Calos, M. P., and Miller, J. H. (1980). Transposable elements. *Cell* **20,** 579–595.

Grindley, N. D. F. (1983). Transposition of Tn3 and related transposons. *Cell* **32,** 3–5.

Kleckner, N. (1977). Genetic engineering *in vivo* using translocatable drug-resistance elements. *J. Mol. Biol.* **116,** 125–159.

Kleckner, N. (1981). Transposable elements in prokaryotes. *Annu. Rev. Genet.* **15,** 341–404.

Kleckner, N. (1990). Regulation of transcription in bacteria. *Annu. Rev. Cell Biol.* **6,** 297–327.

Lewin, B. (1994). Transposons that mobilize via DNA. *In* "Genes V," Chap. 34, pp. 999–1031. Oxford University Press, New York.

Reyes, O., Beyon, A., Mignotte-Vieux, C., and Richaud, F. (1987). Mini-Mu transduction: Cis-inhibition of the insertion of Mud transposon. *Plasmid* **18,** 183–192.

Details of Tn5

Berg, D. E. (1989). Transposon Tn5. *In* "Mobile DNA" (D. E. Berg and M. M. Howe, eds.), pp. 185–210. American Society for Microbiology, Washington, DC.

Berg, D. E., and Berg, C. M. (1983). The prokaryotic transposable element Tn5—a review article. *Biotechnology* **1,** 417–435.

Reznikoff, W. S. (1993). The Tn5 transposon. *Annu. Rev. Microbiol.* **47,** 945–963.

How Tn5 Works

Biek, D., and Roth, J. R. (1980). Regulation of Tn5 transposition in *Salmonella typhimurium*. *Proc. Natl. Acad. Sci. U.S.A.* **77,** 6047–6051.

de la Cruz, N. B., Weinreich, M. D., and Wiegand, R. W. (1993). Characterization of the Tn5 transposase and inhibitor proteins: A model for the inhibition of transposition. *J. Bacteriol.* **175,** 6932–6938.

Jorgensen, R. A., Rothstein, S. J., and Reznikoff, W. S. (1979). A restriction enzyme cleavage map of Tn5 and location of a region encoding neomycin resistance. *Mol. Gen. Genet.* **177,** 65–72.

Kendrick, K. E., and Reznikoff, W. S. (1988). Transposition of IS50 L activates downstream genes. *J. Bacteriol.* **170,** 1965–1968.

Kuan, C.-T., and Tessman, I. (1991). LexA protein of *Escherichia coli* represses expression of the Tn5 transposase gene. *J. Bacteriol.* **173,** 6406–6410.

Kuan, C.-T., and Tessman, I. (1992). Further evidence that transposition of Tn5 in *Escherichia coli* is strongly enhanced by constitutively activated RecA proteins. *J. Bacteriol.* **174,** 6872–6877.

Kuan, C.-T., Liu, S.-K., and Tessman, I. (1991). Excision and transposition of Tn5 as an SOS activity in *Escherichia coli. Genetics* **128,** 45–57.

Lodge, J. K., and Berg, D. E. (1988). Transposon Tn5 target specificity: Preference for insertion at G/C pairs. *Genetics* **120,** 645–650.

Lodge, J. K., and Berg, D. E. (1990). Mutations that affect Tn5 insertion into pBR322: Importance of local DNA supercoiling. *J. Bacteriol.* **172,** 5956–5960.

Makris, J. C., Nordmann, P. L., and Reznikoff, W. S. (1988). Mutational analysis of insertion sequence 50 (IS50) and transposon 5 (Tn5) ends. *Proc. Natl. Acad. Sci. U.S.A.* **85,** 2224–2228.

Makris, J. C., Nordmann, P. L., and Reznikoff, W. S. (1990). Integration host factor plays a role in IS50 and Tn5 transposition. *J. Bacteriol.* **172,** 1368–1373.

Merrick, M., Filser, M., Kennedy, C., and Dixon, R. (1978). Polarity of mutations induced by insertion of transposons Tn5, Tn7 and Tn10 into the *nif* gene cluster of *Klebsiella pneumoniae. Mol. Gen. Genet.* **165,** 103–111.

Rothstein, S. J., and Reznikoff, W. S. (1981). Functional differences in the inverted repeats of Tn5 are caused by a single base pair nonhomology. *Cell* **23,** 191–199.

Rothstein, S. J., Jorgensen, R. A., Postle, K., and Reznikoff, W. S. (1980). The inverted repeats of Tn5 are functionally different. *Cell (Cambridge, Mass.)* **19,** 795–805.

Weinreich, M. D., and Reznikoff, W. S. (1992). Fis plays a role in Tn5 and IS50 transposition. *J. Bacteriol.* **174,** 4530–4537.

Weinreich, M. D., Makris, J. C., and Reznikoff, W. S. (1991). Induction of the SOS response in *Escherichia coli* inhibits Tn5 and IS50 transpostion. *J. Bacteriol.* **173,** 6910–6918.

Wiegand, T. W., and Reznikoff, W. S. (1992). Characteristion of two hypertransposing Tn5 mutants. *J. Bacteriol.* **174,** 1229–1239.

Yin, J. C. P., and Reznikoff, W. S. (1987). *dnaA*, an essential host gene, and Tn5 transposition. *J. Bacteriol.* **169,** 4637–4645.

Yin, J. C. P., and Reznikoff, W. S. (1988). P2 and inhibition of Tn5 transposition. *J. Bacteriol.* **170,** 3008–3015.

Applications of Tn5

de Bruijn, F. J., and Lupski, J. R. (1984). The use of transposon Tn5 mutagenesis in the rapid generation of correlated physical and genetic maps of DNA segments cloned into multicopy plasmids—a review. *Gene* **27,** 131–149.

Manoil, C., and Beckwith, J. (1985). Tn*phoA*: A transposon probe for protein export signals. *Proc. Natl. Acad. Sci. U.S.A.* **82,** 8129–8133.

Nag, D. K., Huand, H. V., and Berg, D. E. (1988). Bidirectional chain-termination nucleotide sequencing: Transposon Tn5seq1 as a mobile source of primer sites. *Gene* **64,** 135–145.

Phadnis, S. H., Huang, H. V., and Berg, D. E. (1989). Tn5supF, a 264-base-pair transposon derived from Tn5 for insertion mutagenesis and sequencing DNAs cloned in phage λ. *Proc. Natl. Acad. Sci. U.S.A.* **86,** 5908–5912.

Saskawa, C., and Yoshikawa, M. (1987). A series of Tn5 variants with various drug-resistance markers and suicide vector for transposon mutagenesis. *Gene* **56,** 283–288.

Sharma, S. B., and Signer, E. R. (1990). Temporal and spatial regulation of the symbiotic genes of *Rhizobium meliloti in planta* revealed by transposon Tn5-gusA. *Genes Dev.* **4,** 344–356.

Wang, G., Blakesley, R. W., Berg, D. E., and Berg, C. M. (1993). pDUAL: A transposon-based cosmid cloning vector for generating nested deletion and DNA sequencing templates *in vivo*. *Proc. Natl. Acad. Sci. U.S.A.* **90,** 7874–7878.

Details of Tn*10*

Bender, J., and Kleckner, N. (1986). Genetic evidence that Tn*10* transposes by a nonreplicative mechanism. *Cell* **45,** 801–815.

Bender, J., and Kleckner, N. (1992). IS*10* transposase mutations that specifically alter target site recognition. *EMBO J.* **11,** 741–750.

Bender, J., Kuo, J., and Kleckner, N. (1991). Genetic evidence against intramolecular rejoining of the donor DNA molecule following IS*10* transposition. *Genetics* **128,** 687–694.

Benjamin, H. W., and Kleckner, N. (1989). Intramolecular transposition by Tn*10*. *Cell* **59,** 373–383.

Benjamin, H. W., and Kleckner, N. (1992). Excision of Tn*10* from the donor site during transposition occurs by flush double-strand cleavages at the transposon termini. *Proc. Natl. Acad. Sci. U.S.A.* **89,** 4648–4652.

Bender, J., and Kleckner, N. (1992). Tn*10* insertion specificity is strongly dependent upon sequences immediately adjacent to the target-site consensus sequence. *Proc. Natl. Acad. Sci. U.S.A.* **89,** 7996–8000.

Ciampi, M. S., Schmid, M. B., and Roth, J. R. (1982). Transposon Tn*10* provides a promoter for transcription of adjacent sequences. *Proc. Natl. Acad. Sci. U.S.A.* **79,** 5016–5020.

Davis, M. A., Simons, R. W., and Kleckner, N. (1985). Tn*10* protects itself at two levels from fortuitous activation by external promoter. *Cell* **43,** 379–387.

Halling, S. M., and Kleckner, N. (1982). Symmetrical six-base-pair target site sequence determines Tn*10* insertion specificity. *Cell* **28,** 155–163.

Halling, S. M., Simons, R. W., Way, J. C., Walsh, R. B., and Kleckner, N. (1982). DNA sequence organization of IS10-right of Tn*10* and comparison with IS10-left. *Proc. Natl. Acad. Sci. U.S.A.* **79,** 2608–2612.

Haniford, D. B., Chelouche, A. R., and Kleckner, N. (1989). A specific class of IS*10* transposase mutants are blocked for target site interactions and promote formation of an excised transposon fragment. *Cell (Cambridge, Mass.)* **59,** 385–394.

Haniford, D. B., Benjamin, H. W., and Kleckner, N. (1991). Kinetic and structural analysis of a cleaved donor intermediate and a strand transfer intermediate in Tn*10* transposition. *Cell (Cambridge, Mass.)* **64,** 171–179.

Harayama, S., Oguchi, T., and Iino, T. (1984). Does Tn*10* transpose via the cointegrate molecule? *Mol. Gen. Genet.* **194,** 444–450.

Jorgensen, B. A., Berg, D. E., Allet, B., and Reznikoff, W. S. (1979). Restriction enzyme cleavage map of Tn*10*, a transposon which encodes tetracycline resistance. *J. Bacteriol.* **137,** 681–685.

Kleckner, N. (1979). DNA sequence analysis of Tn*10* insertions: Origin and role of 9 bp flanking repetitions during Tn*10* translation. *Cell* **16,** 711–720.

Kleckner, N. (1989). Transposon Tn*10*. *In* "Mobile DNA" (D. E. Berg and M. M. Howe, eds.), Chap. 8, pp. 227–268. Americal Society for Microbiology, Washington, DC.

Kleckner, N. (1990). Regulating Tn*10* and IS*10* transposition. *Genetics* **124,** 449–454.

Kleckner, N., Bender, J., and Gottesman, S. (1991). Uses of transposons with emphasis on Tn*10*. *In* "Methods in Enzymology" (J. H. Miller, ed.), Vol. 204, pp. 139–180. Academic Press, San Diego, CA.

Morisato, D., and Kleckner, N. (1984). Transposase promotes double strand breaks and single strand joints at Tn*10* termini *in vivo*. *Cell* **39,** 181–190.

Morisato, D., Way, J. C., Kim, H-J., and Kleckner, N. (1983). Tn*10* transposase acts preferentially on nearby transposon ends *in vivo*. *Cell* **32,** 799–807.

Roberts, D., Hooper, B. C., McClure, W. R., and Kleckner, N. (1985). IS*10* transposition is regulated by DNA adenine methylation. *Cell* **43,** 117–130.
Roberts, D. E., Ascherman, D., and Kleckner, N. (1991). IS*10* promotes adjacent deletions at low frequency. *Genetics* **128,** 37–43.
Ross, D. G., Swan, J., and Kleckner, N. (1979). Physical structures of Tn*10*—promoted deletions and inversions: Role of 1400 bp inverted repetitions. *Cell* **16,** 721–731.
Shen, M. M., Raleigh, E. A., and Kleckner, N. (1987). Physical analysis of Tn*10*- and IS10-promoted transpositions and rearrangements. *Genetics* **116,** 359–369.
Simons, R. W., and Kleckner, N. (1988). Biological regulation by antisense RNA in prokaryotes. *Annu. Rev. Genet.* **22,** 567–600 (especially p. 571, 589, 590 about IS10).
Way, J. C., and Kleckner, N. (1984). Essential sites at transposon Tn*10* termini. *Proc. Natl. Acad. Sci. U.S.A.* **81,** 3452–3456.

Tn*10* and Anti-Sense RNA in Prokaryotes

Case, C. C., Roels, S. M., Gonzalez, J. E., Simons, E. L., and Simons, R. W. (1988). Analysis of the promoters and transcripts involved in IS*10* anti-sense RNA control. *Gene* **72,** 219–236.
Simons, R. W. (1988). Naturally occurring antisense RNA control—a brief review. *Gene* **72,** 35–44.
Simons, R. W., and Kleckner, N. (1988). Biological regulation by antisense RNA in prokaryotes. *Annu. Rev. Genet.* **22,** 567–600. See pp. 571, 589, 590 about IS*10*.

Tet^R Gene of Tn*10*

Bertrand, K. P., Postle, K., Wray, L. V., Jr., and Reznikoff, W. S. (1983). Overlapping divergent promoters control expression of Tn*10* tetracycline resistance. *Gene* **23,** 149–156.
Bertrand, K. P., Postle, K., Wray, L. V., Jr., and Reznikoff, W. S. (1984). Construction of a single-copy promoter vector and its use in analysis of regulation of the transposon Tn10 tetracycline resistance determinant. *J. Bacteriol.* **158,** 910–919.
Braus, G., Argast, M., and Beck, C. F. (1984). Identification of additional genes on transposon Tn*10*:*tetC* and *tetD*. *J. Bacteriol.* **160,** 504–509.
Hillen, W., Gatz, C., Altschmied, L., Schollmeir, K., and Meier, I. (1983). Control of expression of Tn*10*-encoded tetracycline resistance genes. *J. Mol. Biol.* **169,** 707–721.
Hillen, W., Schollmeier, K., and Gatz, C. (1984). Control of expression of the Tn*10*-encoded tetracycline resistance operon II interaction of RNA polymerase and TET repressor with the tet operon regulatory region. *J. Mol. Biol.* **172,** 185–201.
Isackson, P. J., and Bertrand, K. P. (1985). Dominant negative mutations in the Tn*10* tet repressor: Evidence for use of the conserved helix-turn-helix motif in DNA binding. *Proc. Natl. Acad. Sci. U.S.A.* **82,** 6226–6230.
Moyed, H. S., Nguyen, T. T., and Bertrand, K. P. (1983). Multicopy Tn*10* tet plasmids confer sensitivity to induction of tet gene expression. *J. Bacteriol.* **155,** 549–556.
Tovar, K., and Hillen, W. (1989). Tet repressor binding induced curvature of *tet* operator DNA. *Nucleic Acids Res.* **17,** 6515–6522.

Models of Mechanism of Transposition in Prokaryotes

Bennett, P. M., de la Cruz, F., and Grinsted, J. (1983). Cointegrates are not obligatory intermediates in transposition of Tn3 and Tn*21*. *Nature (London)* **305,** 743–744.
Chaconas, G., and Surette, M. G. (1988). Mechanism of Mu DNA transposition. *BioEssays* **9,** 205–208.
Galas, D. J., and Chandler, M. (1981). On the molecular mechanisms of transposition. *Proc. Natl. Acad. Sci. U.S.A.* **78,** 4858–4862.

Harshey, R. M., and Bukhari, A. I. (1981). A mechanism of DNA transposition. *Proc. Natl. Acad. Sci. U.S.A.* **78,** 1090–1094.
Shapiro, J. A. (1979). Molecular model for the transposition and replication of bacteriophage Mu and other transposable elements. *Proc. Natl. Acad. Sci. U.S.A.* **76,** 1933–1937.

Other Transposon Mutagenesis Systems

Olson, E. R., Sadowsky, M. J., and Verma, D. P. S. (1985). Identification of genes involved in *Rhizobium*-legume symbiosis by Mu-DI (Kan, lac)-generated transcription fusion. *Biotechnology,* **3,** 143–148.
Stachel, S. E., An, G., Flores, C., and Nester, E. W. (1985). A Tn*3* *lacZ* transposon for random generation of β-galactosidase gene fusions: Application to the analysis of gene expression in *Agrobacterium. EMBO J.* **4,** 891–898.
Way, J. C., Davis, M. A., Morisato, D., Roberts, D., and Kleckner, N. (1984. New Tn*10* derivatives for transposon mutagenesis and for construction of *lacZ* operon fusions by transposition. *Gene* **32,** 369–379.

Eukaryotic Transposable Elements

Berg, D. E., and Howe, M. M., (eds.) (1989) "Mobile DNA." American Society of Microbiology, Washington, DC.
Boehe, J. D., Garfinkel, D. J., Styles, C. A., and, Fink, G. R. (1985). Ty elements transpose through an RNA intermediate. *Cell* **40,** 491–500.
Dujon, B. (1989). Group I introns as mobile genetic elements: Facts and mechanistic speculations—a review. *Gene* **82,** 91–114.
Döring, H.-P., and Starlinger, P. (1984). Barbara McClintock's controlling elements: Now at the DNA level. *Cell* **39,** 253–259.
Döring, H.-P., and Starlinger, P. (1986). Molecular genetics of transposable elements in plants. *Annu. Rev. Genet.* **20,** 175–200.
Eichinger, D. J., and Boehe, J. D. (1988). The DNA intermediate in yeast Tyl element transposition copurifies with virus-like particles: Cell-free Ty*l* transposition. *Cell* **54,** 955–966.
Fedoroff, N., Masson, P., and Banks, J. A. (1989). Mutations, epimutations, and the developmental programming of the maize suppressor-mutator transposable element. *BioEssays* **10,** 139–144.
Fink, G. R., Boeke, J. D., and Garfinkel, D. J. (1986). The mechanism and consequences of retrotransposition. *Trends Genet.* **2,** 118–123.
Finnegan, D. J. (1985). Transposable elements in eukaryotes. *Int. Rev. Cytol.* **93,** 281–326.
Finnegan, D. J. (1989). Eukaryotic transposable elements and genome evolution. *Trends Genet.* **5,** 103–107.
Finnegan, D. J., and Fawcett, D. H. (1986). Transposable elements in *Drosophila melanogaster. In* "Oxford Surveys on Eukaryotic Genes" (N. Maclean, ed.), Vol. 3, pp. 1–62. Oxford University Press, Oxford.
Georgiev, G. P. (1984). Mobile genetic elements in animal cells and their biological significance. *Eur. J. Biochem.* **145,** 203–220.
Hastie, N. (1985). Middle repetitive DNA: Amplication, homogeneity, and mobility. *Trends Genet.* **1,** 37.
Lewin, B. (1994). Retroviruses and retroposons. *In* "Genes V," Chap. 35, pp. 1053–1056. Oxford University Press, New York.
Rubin, G. M., and Spradling, A. C. (1982). Genetic transformation of *Drosophila* with transposable element vectors. *Science* **218,** 348–353.
Shapiro, J. A. (ed.). (1983). "Mobile Genetic Elements." Academic Press, New York.

Spradling, A. C., and Rubin, G. M. (1982). Transposition of cloned P elements into *Drosophila* germ line chromosomes. *Science* **218**, 314–347.

Wessler, S. R., (1989). The splicing of maize transposable elements from pre-mRNA—a minireview. *Gene* **82**, 127–133.

Mammalian Mobile DNA

Deininger, P. L., and Daniels, G. R. (1986). Recent evolution of mammalian repetitive DNA elements. *Trends Genet.* **2**, 76–80.

Paulson, K. E., Deka, N., Schmid, C., Mistra, R., Schindler, C., Rush, M., Kadyk, L., and Leinwand, L. (1985). A transposon-like element in human DNA. *Nature (London)* **316**, 359–363.

Rogers, J. (1983). Retroposons defined. *Nature (London)* **301**, 460.

Rogers, J. (1985). The origin and evolution of retroposons. *Int. Rev. Cytol.* **93**, 187–279.

Sakaki, Y. M., Hattori, M., Fujita, A., Yoshioka, K., Kuhara, S., and Takenaka, O. (1986). The LINE-1 family of primates may encode a reverse transcriptase-like protein. *Cold Spring Harbor Symp. Quant. Biol.* **51**, 456–469.

Singer, M. (1982). Sine's and line's: Highly repeated short and long interspersed sequences in mammalian genomes. *Cell (Cambridge, Mass.)* **28**, 433–434.

Spence, S. E., Young, R. M., Garner, K. J., and Lingrel, J. B. (1985). Localization and characterization of members of a family of repetitive sequences in the goat β-globin locus. *Nucleic Acids Res.* **13**, 2171–2186.

Trabuchet, G., Chebloune, Y., Savatier, P., Lachuer, J., Faure, C., Verdier, G., and Nigon, V. M. (1987). Recent insertion of an *Alu* sequence in the β-globin gene cluster of the gorilla. *J. Mol. Evol.* **25**, 288–291.

Weiner, A. M., Deininger, P. L., and Efstratiadis, A. (1986). Nonviral retroposons: Genes, pseudogenes, and transposable elements generated by reverse flow of genetic information. *Annu. Rev. Biochem.* **55**, 631–661.

The Auxotroph Library Report

General Reading

Herrmann, K. M., and Somerville, R. L. (1983). Amino Acids: Biosynthesis and Genetic Regulation." Addison–Wesley, Reading, MA.

Mandelstam, J., McQuillen, K., and Dawes, I. W. (1982). "Biochemistry of Bacterial Growth" 3rd ed. Wiley, New York.

Miller, J. H. (1992). "A Short Course in Bacterial Genetics: A Laboratory Manual and Handbook for *Escherichia coli* and Related Bacteria." Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. (Especially see the section on P1 transduction, pp. 263–274, and the section on physical mapping, pp. 2.3–2.43.)

Neidhardt, F. C., Ingraham, J. L., Low, K. B., Magasanik, B., Schaechter, M., and Umbarger, H. E., eds. (1987). "*Escherichia coli* and *Salmonella typhimurium*: Cellular and Molecular Biology." American Society for Microbiology, Washington, DC.

Umbarger, H. E. (1978). Amino acid biosynthesis and its regulation. *Annu. Rev. Biochem.* **47**, 533–606.

Escherichia coli Linkage Map

Bachman, B. J. (1990). Linkage map of *Escherichia coli* K-12, 8th ed. *Microbiol. Rev.* **54**, 130–197.

Bachman, B. J. (1983). Linkage map of *E. coli* K-12, ed. 7. *Microbiol. Rev.* **47**, 180–230.

Bachman, B. J., and Low, K. B. (1980). Linkage map of *E. coli* K-12. ed. 6. *Microbiol. Rev.* **44,** 1–56.
Bachman, B. J., Low, K. B., and Taylor, A. L. (1976). Recalibrated linkage map of *E. coli* K-12. *Bacteriol. Rev.* **40,** 116–167.

Other Escherichia coli Information

Neidhardt, F. C., Vaughn, V., Phillips, T. A., and Bloch, P. L. (1983). Gene-protein index of *E. coli* K-12. *Microbiol. Rev.* **48,** 231–284.
Riley, M. (1993). Functions of the gene products of *Escherichia coli*. *Microbiol. Rev.* **57,** 862–952.

Hfr Mapping and Bacterial Conjugation

Berg, C. M., and Berg, D. E. (1986). Uses of transposable elements and maps of known insertions. *In* "*Escherichia coli* and *Salmonella typhimurium:* Cellular and molecular biology" (F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger, eds.), pp. 1071–1109. American Society for Microbiology, Washington DC.
Chumley, F. G., Menzel, R., and Roth, J. R. (1978). Hfr formation directed by Tn*10*. *Genetics* **91,** 639–655.
Low, K. B. (1972). *E. coli* K-12 F-prime factors old and new. *Bacteriol. Rev.* **36,** 587–607.
Low, K. B. (1973). Rapid mapping of conditional and auxotrophic mutations in *E. coli* K-12. *J. Bacteriol.* **113,** 798–812.
Low, K. B. (1987). Mapping techniques and determination of chromosome size. *In E. coli* and *Salmonella typhimurium:* Cellular and Molecular Biology" (F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger, eds.), pp. 1184–1189. American Society for Microbiology, Washington DC.
Wollman, E. L., Jacob, F., and Hayes, W. (1956). Conjugation and genetic recombination in *E. coli* K-12. *Cold Spring Harbor Symp. Quant. Biol.* **21,** 141–162.

Physical Mapping

Braus, G., Argast, M., and Beck, C. F. (1984). Identification of additional genes on transposon Tn*10: tetC* and *tetD. J. Bacteriol.* **160,** 504–509.
Clarke, L., and Carbon, J. (1976). A colony bank containing synthetic ColE1 hybrid plasmids representative of the entire *E. coli* genome. *Cell* **9,** 91–99.
Hillen, W., and Schollmeier, K. (1983). Nucleotide sequence of the Tn*10* encoded tetracycline resistance gene. *Nucleic Acids Res.* **11,** 525–539.
Jorgensen, R. A., Berg, D. E., Allet, B., and Reznikoff, W. S. (1979). Restriction enzyme cleavage map of Tn*10*, a transposon which encodes tetracycline resistance. *J. Bacteriol.* **137,** 681–685.
Kohara, Y. K., Adiyama, and Isono, K. (1987). The physical map of the whole *E. coli* chromosome: Application of a new strategy for rapid analysis and sorting of a large genomic library. *Cell* **50,** 495–508.
Médigue, C., Viari, A., Hénaut, A., and Danchin, A. (1993). Colibri: A functional database for the *E. coli* genome. *Microbiol. Rev.* **57,** 623–654.
Médigue, C., Viari, A., Hénaut, A., and Danchin, A. (1991). *Escherichia coli* molecular genetic map (1500 kbp): Update II. *Mol. Microbiol.* **5,** 2629–2640.
Noda, A., Courtright, J. B., Denor, P. F., Webb, G., Kohara, Y., and Ishihama, A. (1991). Rapid identification of specific genes in *E. coli* by hybridization to membranes containing the ordered set of phage clones. *Biotechniques* **10,** 474–477.

Postle, K., Nguyan, T. T., and Bertrand, K. P. (1984). Nucleotide sequence of the repressor gene of the Tn*10* tetracycline resistance determinant. *Nucleic Acids Res.* **12,** 4849–4863.

Transduction

Wu, T. T. (1966). A model for three point analysis of random general transduction. *Genetics* **54,** 405–410.
Young, K. K. Y., and Edlin, G. (1983). Physical and genetic analysis of bacteriophage T4 generalized transduction. *Mol. Gen. Genet.* **192,** 241–246.

Selection of tet Revertants

Bochner, B. R., Huang, H.-C., G. L. Schieven, and B. N. Ames (1980). Positive selection for loss of tetracycline resistance. *J. Bacteriol.* **143,** 926–933.
Foster, T. J. (1975). Tetracycline-sensitive mutants of the F-like R factors R100 and R100-1. *Mol. Gen. Genet.* **137,** 85–88.
Maloy, S. R., and Nunn, W. D. (1981). Selection for loss of tetracycline resistance by *Escherichia coli*. *J. Bacteriol.* **145,** 1110–1112.

Mapping Strategy

Kukral, A. M., Strauch, K. L., Maurer, R. A., and Miller, C. G. (1987). Genetic analysis in *Salmonella typhimurium* with a small collection of randomly spaced insertions of transposon Tn10v16v17. *J. Bacteriol.* **169,** 1787–1793.
Singer, M., Baker, T. A., Schnitzler, G., Deischel, S. M., Goel, M., Dove, W., Jaacks, K. J., Grossman, A. D., Erickson, J. E., and Gross, C. A. (1989). A collection of strains containing genetically linked alternating antibiotic resistance elements for genetic mapping of *E. coli*. *Microbiol. Rev.* **53,** 1–24.

2

RECOMBINANT DNA CLONING

Introduction to Recombinant DNA Technology

In this experiment on recombinant DNA technology, the student will learn a method of cloning DNA with restriction endonucleases, learn how to perform a number of different types of cloning, and come to understand the advantages of recombinant DNA cloning. In addition, the clones generated in this experiment may be used as hybridization probes in a Southern blot experiment (see Chapter 3).

This chapter presents the background of recombinant DNA cloning and then presents the procedures for cloning. To clone something means to obtain identical copies of that item. In tissue culture, one speaks of clonal lines that are derived from an individual cell. In recombinant DNA cloning, one obtains an individual fragment of DNA that is amplified readily to yield as much of that DNA as desired.

Why clone DNA? DNA is cloned to obtain a specific fragment of DNA that can be used for analysis because it is free from other DNA fragments. Cloning a DNA fragment also allows the researcher to obtain large enough amounts of the specific DNA sequence to analyze without high background interference from other DNA sequences. It is interesting to note that before the advent of recombinant DNA cloning, the only specific DNA sequences that could be studied readily were highly repeated sequences because this was the only class of DNA that could be obtained sufficiently pure and in large enough quantities (Brown, 1994). Because many repeated sequences have buoyant densities different from that of the bulk of an organism's DNA in a cesium chloride density gradient, they can be isolated from the majority of the DNA and studied. Such DNA, known as satellite DNA, bands in a gradient at a different position than the majority of the DNA,

the main band DNA. Once recombinant DNA cloning was developed, it became possible to obtain large amounts of virtually any specific DNA sequence. To illustrate what an increase in sensitivity of detection DNA cloning gives, consider a gene of 1 kb. If this gene were cloned from the *Escherichia coli* chromosome, which is 4000 kb in size, this is more than a 1000-fold enrichment. If the gene were cloned from *Drosophila*, with its haploid genome size of 1.75×10^5 kb, or from a higher eukaryote, such as humans, with a haploid genome size of 2.75×10^6 kb, the enrichment is even more striking.

Coupled with the other extremely powerful tools of molecular biology, recombinant DNA cloning has made possible numerous types of detailed analyses of DNA. Most notably, combined with the Southern blotting method, hybridization information about specific DNA sequences is now available.

The types of analyses made possible by recombinant DNA cloning include nucleotide sequence determination and experiments that characterize genes, such as determination of the promoter for gene transcription, the poly(A) addition site, and enhancer sequences. The cloned gene can also be used in hybridization studies to examine temporal and spatial patterns of gene expression. Messenger RNA isolated from different stages can be examined by Northern blotting to determine whether the gene of interest hybridizes to mRNA isolated at each stage. In addition, the clone of a specific gene can be used to make a specific gene product to raise an antibody to that protein. These antibodies can be used to localize the protein. The clone can also be used to make the specific protein product. Striking examples of the results of recombinant DNA methods include the production of proteins that have pharmaceutical value, such as human insulin, interferon, and TPA (tissue plasminogen activator, used to treat heart attacks) (Van Brunt, 1990).

The development of recombinant DNA technology depended on a number of individual discoveries that were put together in the 1970s. The first important discovery that contributed to the development of recombinant DNA manipulations was the finding that DNA ligases repair nicks in DNA and join DNA molecules together if the molecules are annealed by complementary single-stranded ends (Gefter *et al.*, 1967; Olivera and Lehman, 1967; Weiss and Richardson, 1967; Zimmerman *et al.*, 1967). A second advancement that occurred during this time was the discovery of a terminal transferase, calf thymus terminal deoxynucleotidyl transferase (Kato *et al.*, 1967), which adds nucleotides to the 3′ end of DNA molecules.

Lobban and Kaiser (1973) and Jackson *et al.* (1972) used these two advances to join DNA molecules. They used terminal transferase to add a poly(dA) tail to one DNA and a poly(dT) tail to another DNA. When these two DNAs were mixed, their complementary single-stranded tails

annealed, and DNA ligase was used to join the DNA molecules. Also in 1972, Sgaramella showed that T4 DNA ligase could join blunt-ended DNA molecules, that is, DNA molecules without cohesive ends.

Work on restriction endonucleases and their sequence-specific cleavage of DNA developed at the same time. In 1970, Smith and Wilcox published their work on the site specificity of a restriction enzyme, *Hin*dII isolated from *Haemophilus influenzae*. Mertz and Davis (1972) characterized a restriction enzyme, *Eco*RI from *E. coli*, that made sequence-specific staggered cuts in the two strands of the DNA helix, producing single-stranded ends that could reanneal. They showed that the single-stranded "sticky ends" or "cohesive ends" made by *Eco*RI digestion were identical for all DNAs cut. Any fragment of DNA with a cohesive end generated by *Eco*RI digestion could anneal with any other fragment of DNA with a cohesive end that was also generated by *Eco*RI digestion. This work led to the use of restriction enzymes to generate the specific DNA fragments to be combined in recombinant DNA experiments.

The improvement in methods for introducing DNA into bacteria was another needed advancement. The treatment of *E. coli* with calcium ions improved bacterial transformation (the ability of bacteria to take up DNA) (Mandel and Higa, 1970; Cohen *et al.*, 1972).

The development of plasmids to serve as vectors for recombinant DNA cloning was also essential. Cohen and Chang developed the first plasmid for use as a cloning vector, pSC101 (Cohen *et al.*, 1973; Cohen and Chang, 1977).

Denniston and Enquist (1981) compiled a collection of reprints of many of the scientific papers that represented significant advancements. Their book *Recombinant DNA* also includes comments about the contributions these papers made.

Other important developments included the use of agarose gel electrophoresis to separate DNA fragments on the basis of size and the use of ethidium bromide staining of agarose gels to visualize the DNA fragments (Helling *et al.*, 1974; Sharp *et al.*, 1973).

Beginning in 1971 and 1972, as the methodology for combining DNA from different organisms through recombinant DNA cloning was developing, scientists became concerned about the potential hazards of some combinations of DNA from different sources. At the June 1973 Gordon Conference on Nucleic Acids, as progress in recombinant DNA cloning was discussed, many scientists questioned the safety of some proposed experiments. This growing concern was reflected in a letter sent to *Science* (Singer and Soll, 1973). A short time later, a second letter to *Science* (Berg *et al.*, 1974) expressed concern about the potential hazards of (1) creating new bacterial plasmids with new drug resistances and (2) joining DNA from oncogenic viruses or other animal viruses to plasmid vectors and

asked that scientists contemplating such experiments "voluntarily defer" until the hazards of such experiments could be evaluated. In February 1975, 150 scientists involved with molecular biology met for 3 days at a conference center in Asilomar, California, to discuss the need to regulate this kind of recombinant DNA research. Although the risks of such recombinant DNA research were unknown, the consensus of the Asilomar conference was that "restraint and caution" were needed. The conference attendees agreed on a moderate approach, calling for the use of biological and physical containment procedures and stating that certain experiments not be done under the present containment conditions (Berg *et al.*, 1975). It is especially noteworthy that scientists were proposing self-regulation of their own research. The Director of the National Institutes of Health (NIH) formed a Recombinant DNA Molecule Program Advisory Committee, which established a detailed set of guidelines for recombinant DNA work.

Four levels of physical containment were proposed: P1 (minimal), P2 (low), P3 (moderate), and P4 (high). The P1 level of containment applies to experiments of no or minimal biohazard. The laboratory is that used for standard microbiological work done on open lab benches. There is public access to the laboratory. P2 containment applies to work evaluated as low biohazard. The P2 lab has all the features of the P1 lab plus access to an autoclave in the building. The access to the laboratory is limited during low-level biohazard experiments. The P3 laboratory for work involving moderate biohazard risks is specially designed to be separate from other laboratories. Access to the laboratory is controlled. The P3 facility is separated from the rest of the building by double-doors, air locks, controlled access corridors, or locker rooms. The P4 laboratory is used for work with microorganisms that are extremely hazardous or can cause serious epidemic disease. The facility is completely separated and access to the facility is under strict control (Watson and Tooze, 1982, p. 153.)

In addition to the levels of physical containment, levels of biological containment were designated (Grobstein, 1977). Biological containment level EK1 designates work of minimal biohazard, such as the use of the normal K-12 laboratory strains of *E. coli*. Biological containment level EK2, for work of greater biohazard risk, involves the use of "crippled" strains of *E. coli* K-12 that contain genetic mutations to prevent the bacteria from surviving outside the laboratory conditions (χ1776). Work at the EK3 level involves EK2 level host–vector systems that have undergone additional field tests.

The public concern over the safety of recombinant DNA research grew from 1975 and became very great in 1977. One example of the strong public response was the reaction in Cambridge, Massachusetts, to a proposal that an existing laboratory building on the Harvard campus be converted to a P3 containment facility. The public outcry led to a 6-month

moratorium. In 1977, after the formation of a safety committee that included members from the public, the P3 project was allowed to proceed.

By 1978, the great public concern about recombinant DNA research began to subside. This was probably due in part to the implementation of the NIH safety guidelines and in part to the reassessment of the potential hazards of recombinant DNA work. Watson and Tooze (1982) have collected a variety of documents on the beginnings of recombinant DNA into a book. Numerous other books have been written about these events, including those by Grobstein (1979), Jackson and Stich (1979), Zilinskas and Zimmerman (1986), and Krimsky (1982).

More information was gathered about recombinant DNA as experiments were performed. This new information led to the modification and relaxation of the NIH recombinant DNA guidelines. Guidelines issued in earlier years were changed to a large extent. Current guidelines can be obtained from the National Institutes of Health.

Although the public is no longer alarmed by the prospects of recombinant DNA research, there is still the need for public input and evaluation of developments stemming from this research. Current issues include the release of modified organisms into the environment and the safety of plant and animal products from organisms that contain modified genes produced by recombinant DNA technology (Dixon, 1993; Flavell *et al.*, 1992; McEnvoy *et al.*, 1992; H. I. Miller, 1992; Van Brunt, 1990; Vandele, 1992; Wrubel *et al.*, 1992).

The tremendous significance of this area of research was recognized by the awarding of Nobel Prizes for work related to recombinant DNA. In 1978, Daniel Nathans, Hamilton O. Smith, and Werner Arber received the Nobel Prize in medicine for the discovery and use of restriction enzymes. In 1980, the Nobel Prize in chemistry was awarded jointly to Paul Berg for cloning the first recombinant DNA molecules and to Walter Gilbert and Frederick Sanger for the development of DNA sequencing methods.

Cloning Vectors

The development of recombinant DNA techniques also hinged upon the modification of existing bacterial plasmids to improve their use as cloning vectors. Cohen (1993) has reviewed the important role bacterial plasmids have played in molecular biology.

The general steps of recombinant DNA cloning are the isolation of the DNA to be cloned, the isolation of the DNA to be used as a cloning vector, and the cutting of the two DNAs with a restriction endonuclease that makes sequence-specific cuts in the DNAs. The restriction endonucleases to be used in this cloning experiment generate a four-base-long single-

stranded region known as a "sticky end" or a "cohesive end." Any fragment generated with this enzyme can base pair with any other fragment generated by the same enzyme. The restriction endonuclease subsequently is inactivated. Then the vector DNA and DNA to be cloned are mixed so that the complementary ends of the restriction endonuclease sites can base pair. That is, reannealing of the sticky ends is allowed to occur. These fragments are sealed together using T4 DNA ligase, and the DNA is introduced into a host cell where the cloning vector can replicate. In this experiment the recombinant DNA is introduced into *E. coli* cells by transformation. The *E. coli* are subjected to a process that will select for the presence of the plasmid cloning vector. The presence of the inserted DNA to be cloned is determined by a selection or a screening process, depending on the nature of the cloning vector and the restriction endonuclease used. The fragment inserted into the cloning vector is then further analyzed and characterized.

The following are the general criteria for a cloning vector:

1. There must be an efficient means of introducing the vector DNA into a host cell.
2. The vector must be able to replicate within a host cell so that many copies of the vector can be obtained.
3. There must be a means to isolate vector DNA away from host DNA.
4. There must be restriction endonuclease sites in the vector that can be used for cloning an insert DNA.
5. There should be a selectable marker on the vector to indicate the presence of the vector in the host cell.
6. It is also very convenient if there are unique restriction endonuclease sites within a selectable or screenable marker so that the presence of an insert piece of DNA in the cloning vector can be selected or screened.

For example, the cloning vector pBR322 has two antibiotic resistance markers, tetracycline resistance and ampicillin resistance (see Figure 2.1). In pBR322, there is a unique site for the restriction endonuclease *Pst*I within the ampicillin resistance gene. One can clone *Pst*I fragments into the *Pst*I site of pBR322. After the ligated vector and insert DNA are transformed into *E. coli* that are ampicillin and tetracycline sensitive, the *E. coli* are plated on tetracycline-containing plates to select for the presence of pBR322. Colonies that are tetracycline resistant are then streaked on a rich plate with ampicillin. If a piece of DNA were cloned into the *Pst*I site, the colony would be ampicillin sensitive. If there has been no fragment cloned, the colony still would be ampicillin resistant. The use of two

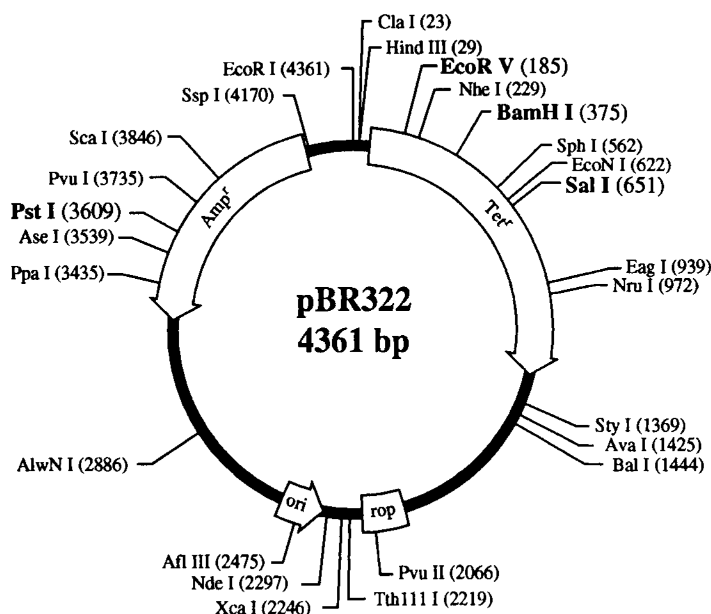


Figure 2.1 A map of the plasmid pBR322. pBR322 is 4361 bp long and contains the antibiotic resistance markers for ampicillin resistance (amp^r) and tetracycline (tet^r) resistance. ori is the origin of plasmid DNA replication. rop is a gene involved in control of plasmid copy number. Numbers indicate the sequence location on the plasmid, where nucleotide 1 is the first T of the EcoRI site.

plates, L_{tet} and L_{amp}, readily shows which colonies contain recombinant DNA plasmids.

pUC18 is another convenient cloning vector. There are many unique restriction endonuclease sites within the polylinker region of pUC (see Figure 2.4). One can clone into the *Pst*I site of pUC18. Ampicillin-sensitive *E. coli* cells transformed with recombinant DNA are plated on $L_{amp\ X\text{-}gal}$ plates. An *E. coli* cell that contains the pUC vector will be ampicillin resistant. The polylinker of pUC is in the gene for the α-donor polypeptide encoded by the first part of *lacZ* gene. If the host is an α-acceptor, functional *lacZ* gene product or β-galactosidase will be produced. X-gal is a colorless substrate for β-galactosidase and becomes an intense blue when cleaved by β-galactosidase. If the pUC vector were still intact, there would be functional β-galactosidase produced and the colony would be blue. If a piece of DNA were cloned into the polylinker, there would not be functional β-galactosidase produced and the colony would be white. When cloning with a pUC vector, the use of one plate, $L_{amp\ Xgal}$, will show which colonies contain recombinant DNA plasmids.

Types of cloning include the following:

1. Sticky end.
2. Blunt end.
3. Linkers: A synthetic sequence of DNA is added to DNA fragments to generate a restriction endonuclease site.
4. Tailing: The use of terminal nucleotide transferase to add A-T or G-C tails to DNA fragments.
5. cDNA: RNA is isolated. This could be a specific mRNA from polyribosomes if the message is sufficiently abundant, and reverse transcriptase is employed to make a cDNA (copy) of the mRNA. This can be useful in determining whether a message is spliced.
6. Libraries: Cosmid or bacteriophage λ vectors to represent all the sequences in the genome of an organism.
7. Cloning of very large DNA fragments: Yeast artificial chromosome (YAC), bacterial artificial chromosome (BAC), or P1 vectors.

pBR322

In the late 1970s, scientists in the laboratories of Boyer and Falkow made improved cloning vectors that contained two antibiotic resistance genes, had unique restriction endonuclease sites, and were, in general, better characterized. One important vector that was made is the plasmid pBR322, which has been called "the most widely used plasmid of molecular biology" (Bolivar, 1988). Although other cloning vectors are used more now, pBR322 was a very important early vector. pBR322 was made by combining segments of DNA from other plasmids. It has the origin of plasmid replication of a clinically isolated plasmid, pMB1, which is closely related to Col E1. pBR322 contains the tetracycline resistance region from a *Salmonella* plasmid, pSC101, and the ampicillin resistance region from the transposon Tn*3* (originally called Tn*A*). Because the region of the transposon that causes transposition was deleted, the ampicillin resistance genes in pBR322 cannot transpose. Bolivar (1988) gives a detailed description of the creation of this plasmid as well as a historical perspective of pBR322. The presence of two antibiotic resistance genes in pBR322 was an important advantage. There were unique restriction endonuclease sites within each resistance gene. It was then possible to clone a DNA segment into one antibiotic resistance gene, thereby inactivating that gene, and still select for the presence of the plasmid using the second antibiotic resistance marker. For example, by cloning a fragment of DNA into the *Bam*HI site of pBR322, which is in the tetracycline resistance gene, the resulting recombinant plasmid will be tetracycline sensitive and ampicillin resistant. After transformation of such recombinant

plasmid DNA into an *E. coli* strain, ampicillin-resistant colonies selected would then be screened for tetracycline sensitivity (see Figure 2.1).

pBR322 was further studied to determine that the plasmid was not self-mobilizable and that the disabled *E. coli* strain χ1776 containing pBR322 could not survive outside laboratory conditions. pBR322 was therefore certified as an EK2 vector, according to the NIH recombinant DNA guidelines. Sutcliffe's (1979) determination of the complete nucleotide sequence of pBR322 by the Maxam and Gilbert technique made it possible to determine the exact location of restriction enzyme sites in the plasmid. This complete characterization of pBR322 helped to make it such a useful and widely used cloning vector. pBR322 has been used to clone many DNA fragments and it has been modified to make other vectors for specific applications. By 1988, 12 years after pBR322 had been made, more than a thousand derivatives of pBR322 had been reported in the literature (Balbas *et al.*, 1986).

pBR325 is a derivative of pBR322, also made by Bolivar *et al.* (1978), and contains a chloramphenicol resistance gene (see Figure 2.2).

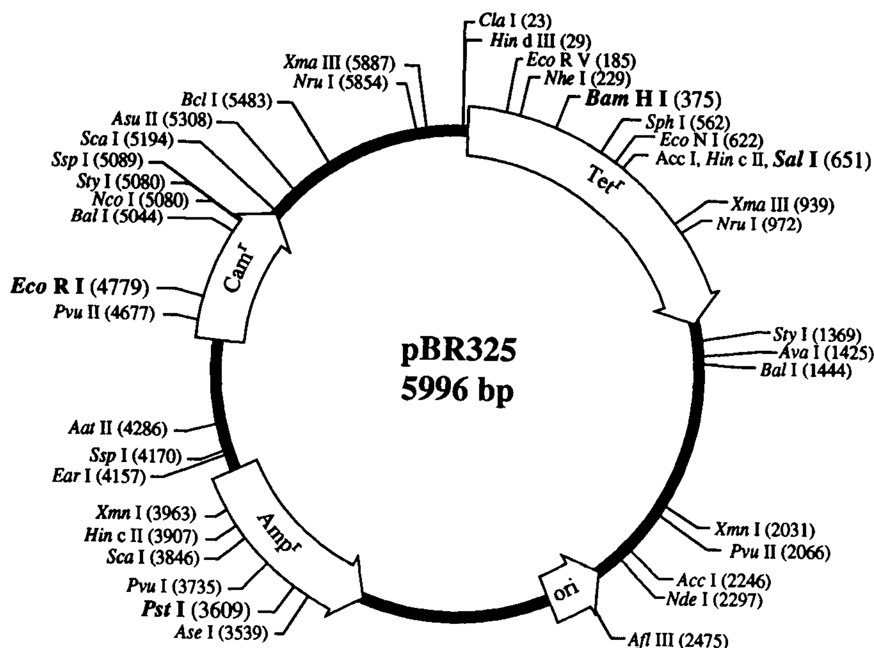


Figure 2.2 A map of the plasmid pBR325. pBR325 is 5996 bp long and contains the genes for ampicillin resistance (amp^r), chloramphenicol resistance (cam^r), and tetracycline resistance (tet^r). ori indicates the origin of plasmid DNA replication. See Figure 2.1.

Vectors That Yield Single-Stranded DNA

Filamentous phages such as M13, fd, and f1 are used to clone single-stranded DNA for use in DNA sequencing. Sequencing reactions using single-stranded DNA have the advantage that information is obtained from one strand at a time. Using phage to clone single-stranded DNA is often easier than using other methods to separate two strands of a double-stranded DNA molecule, such as alkaline CsCl gradients and polyacrylamide gels (Messing and Vieira, 1982). The replicative form of M13 is a circular double-stranded DNA molecule which can be isolated from M13-infected *E. coli* cells. The double-stranded DNA can be used to clone DNA and then to reinfect *E. coli*. The infected cells are not lysed by the M13 phage but continue to extrude M13 phage particles that contain the M13 genome in the form of a single-stranded circular DNA, containing the + strand of the DNA helix. Single-stranded DNA can be readily isolated from the phage particles. Because this is a filamentous phage, it is possible to clone and package additional DNA from a broad range of sizes into the M13 phage vector. Other phages, such as λ, have a limited range of DNA that can be cloned into their genomes because, in those cases, the DNA is packaged by a "headful mechanism"; that is, the DNA must be of a certain size to fit correctly into the assembled phage particle.

Messing created a series of extremely useful cloning vectors based on M13. To make these phage vectors, Messing replaced a nonessential region of the M13 phage genome with a 789-bp *Hin*dII fragment containing part of the *lac* operon (Messing *et al.*, 1977). This *Hin*dII fragment contains part of the *lac* regulatory region (part of the *lacI* gene which codes for the *lac* repressor, the promoter, and operator of the *lac* operon) and the DNA sequence that codes for the first 145 amino acids of the *lacZ* gene. This part of the *lacZ* gene codes for the α-donor polypeptide of β-galactosidase.

α-Complementation of the *lacZ* Gene

In *E. coli*, the *lacZ* gene codes for the polypeptide β-galactosidase, which is 1021 amino acids long. The active form of the β-galactosidase protein is a tetramer of these identical polypeptide chains. Certain nonoverlapping deletions in the *lacZ* gene can complement each other. Deletions in the first part of the *lacZ* gene (near the promoter, or promoter-proximal) produce a polypeptide that does not have β-galactosidase activity. Some deletions such as this can be complemented by nonoverlapping deletions in the latter part (far from the promoter or promoter-distal) of the *lacZ* gene. The amino-terminal peptide is called the α-donor; the carboxy-terminal peptide is called the α-acceptor. This type of complementation, called α-complementation, occurs because the two different types of polypeptides, the α-donor and the α-acceptor, interact to restore β-galactosidase activity.

The M13-derived cloning vectors of Messing contain the part of the *lacZ* gene that codes for the α-donor polypeptide. For α-complementation to occur and β-galactosidase activity to result, the α-acceptor polypeptide must be supplied by the bacterial host. Messing used *E. coli* strains in which all of the *lac* operon was deleted. Then, using an F′ plasmid, a mutated *lacZ* gene coding for an α-acceptor polypeptide was introduced into the *E. coli* strain. The mutation used was deletion M15 (ΔM15), which has a deletion of amino acids 11 to 41 of β-galactosidase, produces a defective enzyme that lacks activity, and can serve as an α-acceptor. It was shown *in vitro* that the α-donor and α-acceptor could complement each other to give β-galactosidase activity. This is known as intracistronic complementation, where mutations in the same gene complement each other. α-Complementation is an example of intracistronic complementation in which the restoration of activity occurs through the reassociation of differently altered subunits (Ullmann *et al.*, 1967; Miller and Reznikoff, 1980). Ullmann (1992) states that the exact mechanism of α-complementation "has not been completely elucidated" and that "the data suggested that the role of α-peptide is conformational rather than structural." One α-acceptor, the M15 protein, lacks amino acids 11 to 41 and is a dimer. It is likely that the role of the α-peptide is to change the three-dimensional structure of the acceptor protein so that a tetramer with enzyme activity is formed.

The *lacZ* gene product, β-galactosidase, is a stable enzyme that can be easily assayed using the chromogenic substrate X-gal (5-bromo-4-chloro-indolyl-β-D-galactoside or galactopyranoside). X-gal is colorless; when the galactoside moiety is cleaved off by β-galactosidase, 5-bromo-4-chloro-indolyl is formed. It then air-oxidizes to produce the intense blue compound, 5-bromo-4-chloro-indigo. Figures 2.3a and 2.3b show the structure of IPTG, an inducer of the *lac* operon, and the reaction of X-gal cleaved by β-galactosidase. See Appendix 5 for the description of the concentration of these materials used in plates. The M13 phage containing this α-donor gene would produce a blue plaque in the presence of X-gal. If a piece of DNA were inserted into the *lacZ* α-donor gene, the α-donor peptide would not be made, there would not be β-galactosidase activity, and the M13 plaque would be colorless, not blue.

Messing designated this modified M13 with a part of the *lacZ* gene that codes for the α-donor peptide (also called *lacZα*) as M13mp1. The M in M13 stands for Munich; the mp stands for Max Planck Institute, where Messing was doing this work.

An important modification of the M13 phage cloning vectors was the addition of a polylinker or multiple cloning site (MCS) into the *lacZα* gene. The polylinker contained unique restriction endonuclease sites suitable for cloning. Sometimes restriction endonuclease sites elsewhere in

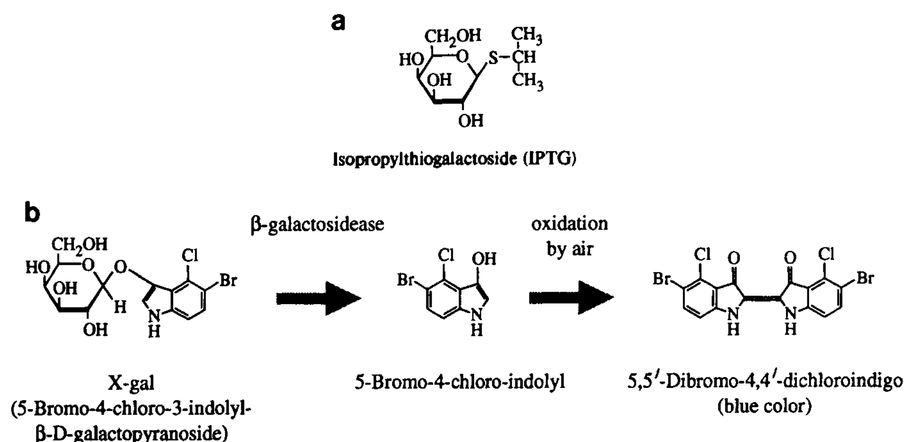


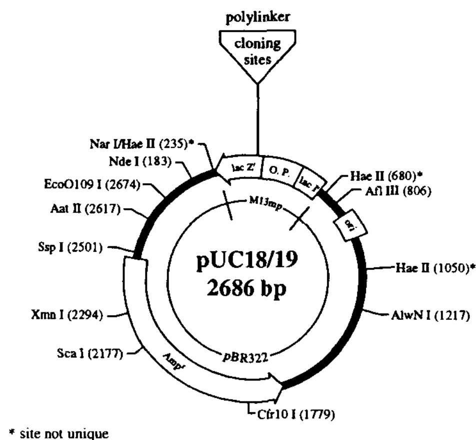
Figure 2.3 Assay for β-galactosidase activity. (a) The structure of IPTG, isopropylthiogalactoside, an inducer of the *lac* operon. (b) The reaction of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), a chromogenic substrate for β-galactosidase. When β-galactosidase cleaves the β-galactoside linkage in X-gal, 5-bromo-4-chloroindolyl is produced and is then oxidized by air to form 5,5′-dibromo-4,4′-dichloroindigo, which is blue.

the vector had to be deleted so that the sites in the polylinker would be unique. It is important to note that the polylinker was inserted in frame with the *lacZα* gene. An altered polypeptide was produced with additional amino acids coded by the polylinker sequence, but the peptide was still functional for α-complementation.

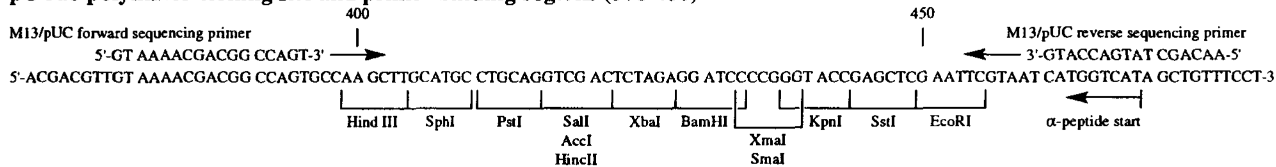
To make the M13mp series of vectors even more useful for sequencing, Messing and co-workers developed a primer for DNA sequencing homologous to part of the M13 vector (Heidecker *et al.*, 1980).

Development of the pUC Plasmids

Messing next developed a set of extremely useful plasmids to serve as cloning vectors, the pUC vectors (Vieira and Messing, 1982). The UC stands for the University of California; Messing initiated this work while at the University of California, Davis, though a large part of the work was done while he was at the University of Minnesota. The basis of the pUC plasmids is a 2297-bp *PvuII* to *EcoRI* fragment of pBR322 which contains the origin of replication and the ampicillin resistance gene but not the tetracycline resistance gene. A 433-bp *HaeII* fragment from M13mp7 which contained the lac region was cloned into one of the *HaeII* sites of the deleted pBR322 derivative. The pUC vectors also contained the polylinker or multiple cloning site inserted in frame with the *lacZα* gene. Vieira and Messing made a related pair of pUC vectors, pUC8 and pUC9, which are identical except that the polylinker is in opposite orientation. This allowed



pUC18 polylinker cloning site and primer binding regions (371-480)



pUC19 polylinker cloning site and primer binding regions (371-480)

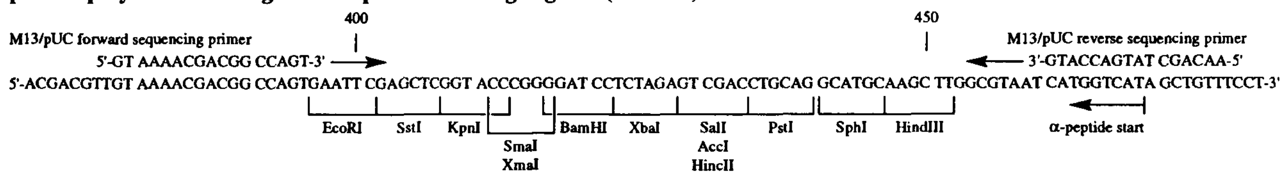


Figure 2.4 A map of the plasmid pUC18 or pUC19. These plasmids are 2686 bp long, contain an ampicillin resistance marker (amp^r), and code for the α-donor of the *lac*Z gene (designated *lac*Z'). ori is the origin of plasmid DNA replication. The sequence below the circular plasmid shows the restriction endonuclease sites in the pUC18 or 19 polylinkers or multiple cloning site regions. pUC18 and 19 contain the same polylinker sequence in reverse orientation relative to each other.

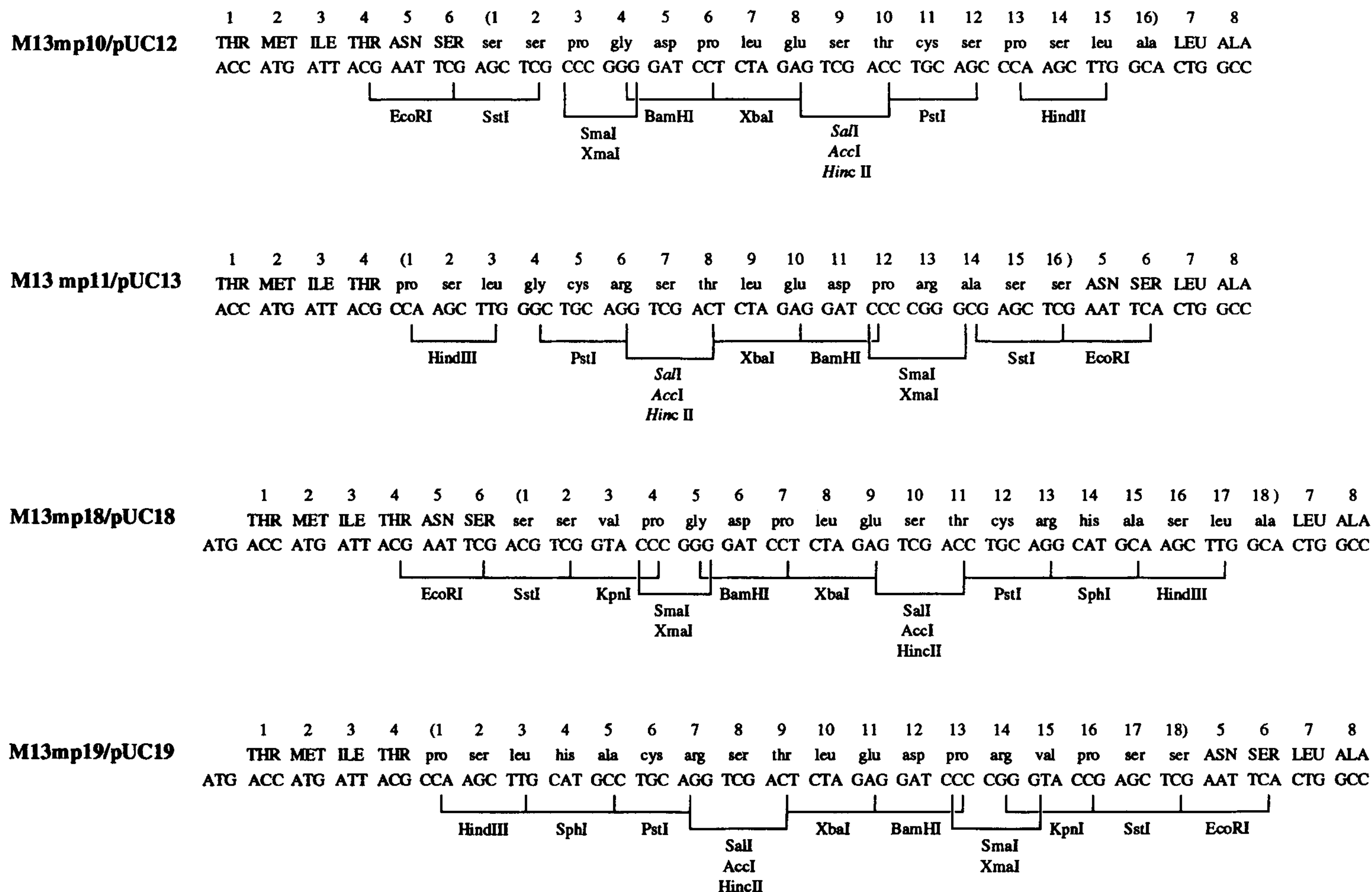


Figure 2.5 The sequences of the polylinkers or multiple cloning site regions of several pUC vectors, pUC12/13 and pUC18/19. The corresponding single-stranded M13 phage has the same polylinker sequence.

a piece of DNA cut with two different restriction enzymes in the polylinker to be cloned in either orientation with respect to the *lac* promoter by using either pUC8 or pUC9. The polylinker of pUC8 and pUC9 contained sites for 11 restriction enzymes.

Messing has continued to improve the pUC and M13 vectors. Polylinkers with additional restriction endonuclease sites were added to the vectors. See the multiple cloning sites indicated for different vectors in Figures 2.4 and 2.5. The vector pUC21 (Vieira and Messing, 1991) contains a multiple cloning site with the recognition sites for 28 restriction endonucleases! New vectors based on the M13 and pUC vectors with new restriction sites continue to be developed (Benes *et al.*, 1993). pUC118/119 and pUC21 also contain the M13 origin of replication. These plasmids can replicate as plasmids using the pBR origin or can use the phage origin to produce single-stranded DNA.

In general, insertion of a fragment of DNA into the multiple cloning site of the pUC vector will inactivate the α-donor polypeptide so that there will be no β-galactosidase activity. However, some fragments of DNA cloned into the MCS still produce β-galactosidase. Close *et al.* (1983) observed that the chloramphenicol acetyltransferase gene (*cat*) from Tn9, when cloned into pUC vectors in one orientation, produced white colonies indicating no α-complementation. However, the *cat* gene cloned in the opposite orientation did show α-complementation and gave blue colonies. Detailed analysis of this clone showed that two polypeptides were produced. The cat sequences had no transcriptional terminator and contained an *E. coli* ribosome binding site near the distal end of the insert. Two fusion peptides were produced, most likely from a polycistronic mRNA from the *lac* promoter. The fusion peptide of sequences from the inserted cat gene and the rest of the *lacZ*α gene produced a functional α-donor peptide. The first few amino acids of the α-donor are not essential for α-complementation. Close *et al.* point out several situations in which a cloned insert may still give β-galactosidase activity. If an insert has no translational stop signal and the DNA fragment is in frame with the *lacZ* α-donor sequence, an insert can still give a blue colony. Also, if the inserted fragment contains its own promoter that can function in *E. coli* and has a ribosome binding site, and the polypeptide produced is in phase with the rest of the *lacZ*α gene sequence, β-galactosidase can be produced.

These series of extremely useful vectors are now used as the basis for other cloning vectors (Benes *et al.*, 1993). Messing (1991) has written an interesting account of the history of the development of the M13 and pUC cloning vectors.

Copy Number of pUC Plasmids

pBR322 plasmids contain the pMB1 replicon and are present at 15–20 copies per *E. coli* cell. pUC vectors also contain the pMB1 replicon but

are present at 500–700 copies per *E. coli* cell. This difference in copy number occurs because pUC plasmids contain a mutation in the *rop* gene, which codes for a protein that is involved in the negative regulation of plasmid replication. (See Sambrook *et al.*, 1989, pp. 1.3–1.5, for more details of this regulation of plasmid replication.)

Mobilization and Conjugation of Plasmids

The factors needed for a plasmid to be transferred from one bacterial cell to another by conjugation include the physical structures for cell-to-cell contact and the mobilization or preparation of the DNA for transfer. Mobilization begins when a plasmid-encoded protein makes a single-stranded nick at a unique sequence at the transfer origin (oriT). In the F plasmid, this is called the oriT, or origin of transfer. Rolling circle replication initiates at the nick. A single-stranded linear DNA molecule is transferred to the recipient cell. In the recipient cell, the second strand of DNA is synthesized to make double-stranded DNA.

For ColE1 type of plasmids, such as the plasmid pMB1 from which the common cloning vectors pBR322, pBR325, and pUC are derived, the site-specific ColE1 nuclease is encoded by the *mob* (for mobility) gene, and the specific cutting site is called the *nic* or *bom* (for basis of mobility) site. pBR322 lacks the mob gene but contains the *nic/bom* site. The *mob* gene nuclease and the transfer apparatus can be supplied by other plasmids in the same bacterial cell. Thus, pBR322 can be mobilized by other plasmids. pUC plasmids lack the *mob* gene and the *nic/bom* site and cannot be mobilized (Sambrook *et al.*, 1989, p. 1.5).

Vectors for Cloning Large DNA Fragments

When cloning DNA from organisms that have large genomes, it is useful to have cloning vectors that can efficiently clone large pieces of DNA. When a library of an organism's DNA is made, a cloning vector with a capacity for large inserts of recombinant DNA is very valuable. Fewer recombinant clones will be needed to clone the entire genome. Such vectors have been essential for the advancement of the human genome project as well. Useful vectors for cloning large pieces of DNA include cosmids, λ phage, P1 phage, yeast artificial chromosomes (YACs), and bacterial artificial chromosomes (BACs).

λ Vectors

Phage λ vectors have been used extensively in recombinant DNA cloning (Murray, 1991; Miller, 1992). Many different types of λ vectors have been developed. These vectors vary in the restriction endonucleases

that can be used for cloning and the size of the fragments that can be cloned (Whittaker *et al.*, 1988). Sambrook *et al.* (1989) describe more than 20 different λ cloning vectors. The sizes that can be cloned into λ vectors range from a few kilobase pairs in some vectors to 10–25 kb in other vectors. Because a large number of λ genes are essential for the propagation of the phage, and DNA of a certain size range can be packaged in the λ phage particle, the DNA fragments that can be cloned in λ vectors are smaller than those that can be cloned in the other vector systems described below. Kohara *et al.* (1987) used λ to clone and order the entire genome of *E. coli*. Kohara chose to do the cloning in λ because, although the inserts were smaller than those using other vectors, the inserts were more stable and there is a rapid procedure for λ DNA isolation.

Cosmids

One problem when cloning large fragments of DNA into plasmid vectors is the very low transformation efficiency of introducing very large DNA molecules into E. *coli*. This problem has been solved by using at least two different methods. Relatively recently, electroporation, a more efficient means of introducing DNA into cells, including bacteria, has been developed. A second way of improving the efficiency of introducing DNA into cells was developed in the late 1970s. This method takes advantage of the ability to package DNA into λ bacteriophage particles *in vitro*, the high efficiency of the bacteriophage λ infection of E. *coli*, and the development of cosmid cloning vectors (Collins and Hohn, 1978; Hohn, 1979). Cosmids are plasmids that contain the λ *cos*, or cohesive ends, sites that are the λ sequences required for the packaging of DNA into a λ phage particle. Cosmids also contain a plasmid origin of DNA replication, at least one selectable marker, and useful restriction endonuclease sites. For cloning into cosmid vectors, cosmid DNA and the DNA to be cloned are digested with restriction endonucleases and joined by ligase. The long concatenated DNA structures that are produced resemble the conformation of the λ DNA that is packaged into λ phage heads. This DNA can be packaged into λ phage heads *in vitro*. Only DNAs that have two *cos* sites the correct distance apart can be packaged into phage heads. This packaged DNA will be transfected into λ-sensitive E. *coli* via the normal λ infection process. After the DNA is introduced into the E. *coli* cell, it will circularize via the *cos* sites and subsequently replicate as a plasmid using the plasmid origin of replication. Those E. *coli* cells that contain cosmids can be identified by selecting for the antibiotic resistance marker on the cosmid. Because λ DNA is packaged by a "headful" mechanism, only those recombinant molecules of cosmid and DNAs to be cloned that are of the appropriate size will be correctly packaged and transduced. This allows size selection of the fragments cloned in a cosmid vector. The total length of the DNA

that is packaged must be approximately 37 to 50 kb. Depending on the size of the cosmid vector itself, specific size ranges of DNAs can be cloned into cosmid vectors. For example, the cosmid pHC79, which is based on the pBR322 origin of replication, is only 6 kb. Therefore, inserts approximately 40 kb can be cloned in this cosmid (Hohn and Collins, 1980). The cosmid pCP13, which is based on the wide-host-range plasmid pRK290, is 21.6 kb and can accept inserted DNAs that are about 23–25 kb long (Darkins and Chakrabarty, 1984; Friedman *et al.*, 1982). pCP13 is useful for moving DNA from *E. coli* to other bacterial species because it can replicate in a variety of different gram-negative bacteria.

Cosmid vectors are definitely an improvement over pBR or pUC cloning vectors for cloning large DNAs. Using cosmid vectors, about 10^6 to 10^7 clones/μg of insert DNA can readily be obtained. However, because only 40–45 kb of DNA can be cloned in cosmid vectors, a very large number of clones are needed to clone an entire complex genome. The task of ordering so many clones is difficult. Other cloning methods were needed to bridge the gap between the cytogenetic or genetic scale of chromosomes and the kilobase pair scale of molecular cloning. Typically, a centimorgan, the unit of recombination frequency used in genetic mapping, is several megabase pairs. Also, for many genetic loci, the functional unit is very large. For example, the human factor VIII gene covers 190 kb. Distances involved in rearrangements of the immunoglobulin supergene family are also very large.

P1 Vectors

This cloning vector system is based on the bacteriophage P1 (Sternberg, 1990a,b, 1992). The capabilities of the P1 cloning system are intermediate between those of cosmids and YACs. The size of fragments cloned into P1 vectors averages from 75 to 95 kb, but fragments as large as 100 kb can be cloned. The efficiency of cloning for P1 is about 10^5 clones per microgram of vector and insert DNA. The P1 cloning vector includes two P1 DNA origins of replication: a plasmid origin of replication that maintains plasmid DNA in *E. coli* at 1 copy per host chromosome and a lytic origin of replication that, when induced, amplifies the copy number of the P1 vector to about 20 copies per cell. In addition to selectable markers, the vector contains the *pac* site and two *loxP* sites of phage P1. Recombinant molecules are packaged *in vitro* using various P1 packaging extracts. Packaging begins with a cut made at the *pac* (packaging) site. DNA is packaged by a headful mechanism, with 100–115 kb of DNA fitting into a P1 phage head. The first cut for packaging is made at the *pac* site; the second cut is not sequence specific but is at a fixed distance from the *pac* site. The linear DNA is injected into the recipient bacterium. Only those DNA molecules that contain two *loxP* sites are able to circu-

larize and replicate in the bacterium. The *loxP* sites are specific DNA sequences from phage P1 upon which a P1 site-specific recombinase, Cre, acts.

YAC Vectors

A yeast artificial chromosome cloning vector (Burke, 1990; Cooke, 1987; Jordan, 1990) consists of two copies of a yeast telomeric sequence (telomers are the sequences at the ends of chromosomes), a yeast centromere, a yeast ars (an autonomously replicating sequence where DNA replication begins), and appropriate selectable markers. The amount of DNA that can be cloned into a YAC is, on average, from 200 to 500 kb. However, as much as 1 Mb (mega, 10^6) can be cloned into a YAC. Difficulties with YAC cloning vectors include the following: (1) The efficiency of cloning is low (about 1000 clones are obtained per microgram of vector and insert DNA). (2) The yield of YAC DNA isolated from a yeast clone containing a YAC is quite low. The YAC DNA is only a few percent of the total DNA in the recombinant yeast cell. It is difficult to obtain even 1 μg of YAC DNA. There is a method for selectively amplifying the YAC (Sternberg, 1990b). (3) YAC clones frequently contain deletions, rearrangements, or noncontiguous pieces of the cloned DNA (C. Anderson , 1993). As a result, each YAC clone must be carefully analyzed to be sure that no rearrangements of the DNA have occurred.

BAC Vectors

Shizuya *et al.* (1992) have developed a high-efficiency cloning system, called bacterial artificial chromosome or BAC, which is based on the single-copy plasmid F factor of *E. coli*. The BAC vectors are capable of maintaining DNA fragments that are larger than 300 kb and are very stable.

Restriction Endonucleases

The existence of restriction modification systems of bacterial viruses was first described in the early 1950s (Luria and Human, 1952; Bertani and Weigle, 1953). A molecular explanation of the phenomenon came 10 years later from the studies of Arber and co-workers (Arber, 1965; Arber and Dussoix, 1962; Dussiox and Arber, 1962). Arber studied the growth of bacteriophage λ on different *E. coli* strains. He observed that λ grown on one *E. coli* strain and then used to infect another strain of *E. coli* produced far fewer plaques than expected. The plating efficiency (the ratio of the number of plaques formed to the number of phage particles used to infect a bacterial strain) was examined.

Plating Efficiency[a]

Bacterial strain	Phage		
	λ grown on K	λ grown on B	λ grown on C
E. coli K	1	10^{-4}	10^{-4}
E. coli B	10^{-4}	1	10^{-4}
E. coli C	1	1	1

[a] Plating efficiency is the ratio of plaque forming units observed to phage particles infecting the bacterium.

Escherichia coli strains that showed this reduced plating efficiency were said to "restrict" the growth of phage grown on another *E. coli* strain. Examination of this phenomenon led to the discovery of restriction/modification systems in *E. coli*. The restriction is due to sequence-specific endonuclease cutting of the infecting λ DNA.

Phage taken from one of the few plaques that formed could now infect the new host strain with a plating efficiency of one. Such phage were said to be "modified" by growth in the new bacterial strain. The modification is caused by a sequence-specific methylation of the phage DNA.

Such restriction/modification systems are a bacterial defense mechanism—a bacterial immune system, of sorts. As DNA enters the bacterial cell by means such as phage infection or transformation, the DNA is rapidly degraded if it is not correctly modified.

The plating efficiency reduction Arber observed is due to the activity of the *E. coli* restriction/modification system encoded by three linked genes that map at minute 98 on the *E. coli* chromosome. The genes are *hsd*R, which codes for an endonuclease; *hsd*M, which is the gene for a methylase; and *hsd*S, whose gene product is needed for sequence recognition.

There are four different restriction/modification phenotypes of *E. coli* possible:

1. $r_k^+m_k^+$: the wild type.
2. $r_k^-m_k^+$: a restriction-deficient strain; such a strain is desirable for transformations because degradation of incoming DNA would be minimized.
3. $r_k^-m_k^-$: such a strain would also be useful for transformation of recombinant DNA.
4. $r_k^+m_k^-$: this genotype would be lethal because the cell would not be able to methylate its own DNA but would cut its own DNA. Such a genotype could be isolated only as a conditional lethal, such as a temperature-sensitive mutant.

Note that the table of plating efficiencies indicates that *E. coli* C lacks the restriction endonuclease but does have the methylase. What would the plating efficiencies be for a strain that is $r_k^-m_k^-$?

Restriction endonucleases have been classified into three or four groups and several subclasses based on the subunit organization of the enzyme, the cofactor requirements of the enzyme, and the recognition and cleavage site of the enzyme. As of January 1990, Kessler and Manta reported that 1284 endonucleases and 130 methylases with known sequence specificity had been isolated and characterized from 1117 different microorganisms. Roberts and Macelis (1992) state that 2103 restriction enzymes are known. These enzymes include 17 different Type I sequence specificities, 179 different Type II sequence specificities, and 4 different Type III sequence specificities. A few eukaryotic restriction modification systems have been identified, including *Cvi*<A-Q>I from virus-infected *Chlorella*-like green algae, CreI from the alga *Chlamydomonas reinhardtii*, and *Hsa*I from embryos of *Homo sapiens*. Regular monthly updates on restriction enzymes are available by electronic mail from REBASE, a restriction enzyme database (see p. 2167 of Roberts and Macelis, 1992).

Restriction and modification system names consist of three italicized letters, which represent the bacteria from which the restriction enzyme was isolated, followed by a letter to identify the particular bacterial strain, if applicable, and ending with a Roman numeral to distinguish different systems from the same bacteria (Wilson, 1988; Smith and Nathans, 1973). For example, *Eco*RI is the first restriction and modification system isolated from *Escherichia coli* strain R. *Hin*dIII refers to the third restriction modification system isolated from *Haemophilus influenzae* strain R_d. *Pst*I is the first system identified from *Providencia stuartii*.

It should be noted that all endonucleases found to cleave DNA at specific sequences are assumed to be part of restriction and modification systems (based on their *in vitro* activity), even though only very few of these systems have actually been shown in the bacteria to restrict and modify phage DNAs (Roberts and Macelis, 1992; Wilson, 1988).

The classification system initially grouped the known restriction endonucleases into three classes of restriction endonucleases and methylases. However, a fourth and perhaps a fifth class of restriction endonucleases have recently been identified. Type II restriction endonucleases have been divided further into subclasses.

Type I or Class I Restriction Endonucleases

Type I restriction endonucleases are large multimeric enzyme complexes with multiple, nonidentical subunits. In Type I or Class I restriction/modification systems, the restriction and modification activities are on

different subunits of a single enzyme complex. Class I restriction modification systems require Mg^{2+}, ATP (the cleavage activity is ATP dependent), and SAM (S-adenosyl-L-methionine) as cofactors of the reactions. Class I enzymes cleave nonspecifically between 100 and 1000 bp downstream from the sequence-specific recognition site. ATP is required to move the restriction endonuclease complex along the DNA to the site where cutting occurs.

Type I restriction endonucleases are found in *E. coli* B (*Eco*B) and *E. coli* K (*Eco*K), and one is produced by the phage P1. In fact, it was the *Eco*B and *Eco*K Type I restriction endonucleases that caused the restriction of phage growth and changes in phage plating efficiency that Arber studied. For example, *Eco*K has a 15-bp-long recognition site, 5'AA*CNNNNNNGTGC3' (where N is any of the four nucleotides), and cuts the DNA approximately 1000 bp away from the recognition site. Note that in Type I restriction endonucleases the recognition site but not the cleavage site shows sequence specificity.

Type II or Class II Restriction Endonucleases

In Class II restriction/modification systems the endonuclease and the methylase activities are separate enzymes, not part of an enzyme complex. Type II restriction endonucleases are smaller than Type I and either are monomeric proteins or have identical subunits. A Class II restriction endonuclease requires only Mg^{2+} as a cofactor. A Class II restriction endonuclease recognizes a specific sequence and cuts at a specific sequence. A Class II methylase is site specific and transfers the methyl group from SAM to the N6, N5, or N4 position of specific adenines or cytosines on both strands of double-stranded DNA.

Class II restriction endonucleases are members of a large complex class that has been further divided into six subclasses based on the specificity of sequence recognition, the cleavage reaction, and methylation sensitivity. Subclass IIP is the most common. The P subclass consists of enzymes whose recognition sequences are 4-, 6-, or 8-nucleotide palindromes. In Subclass IIW, recognition sites are 5- or 7-nucleotide palindromes. Subclass IIN recognition sites are interrupted palindromes that contain a region that can be any nucleotide sequence. The cutting occurs within that region. Subclass IIS enzymes (Szybalski *et al.*, 1991) recognize 4- to 6-bp sequences which are usually asymmetric and make a cut at a precise site 1 to 20 nucleotides to one side of the recognition site. (Unlike Type III enzymes, the cutting is precise and ATP does not stimulate the reaction.) Subclass IIT restriction endonucleases recognize an asymmetric sequence and cut within that sequence. Subclass IIU is a temporary cate-

gory for those restriction endonucleases whose cut site is still undetermined.

It is this DNA sequence specificity that makes restriction endonucleases, especially Type II restriction endonucleases, so valuable for recombinant DNA cloning. Many different Type II restriction endonucleases have been used in many cloning experiments. Indeed, by 1990, more than 1000 Type II enzymes had already been identified, and some are listed in Table 2.1.

Recognition sites for Type II enzymes are 4, 5, 6, or more base pairs long and have twofold symmetry. Type IIP and IIW restriction endonucleases cleave within the recognition sequences and make either blunt or staggered cuts. Those that make staggered cuts produce nucleotide fragments where one strand of the double helix extends beyond the other, forming what is called a "sticky end" or "cohesive end" that can base pair with other similar cohesive ends.

Type IIS restriction endonucleases recognize a specific DNA sequence and cleave the DNA molecule a fixed distance from that recognition site. Note that not every Type IIS restriction fragment generated by a particular Type IIS enzyme can base pair with other fragments generated with the same enzyme.

The restriction endonuclease *Eco*RI, a Type II restriction endonuclease, has been studied in great detail and is part of the best characterized restriction endonuclease/methylase system. The *Eco*RI restriction endonuclease is a dimer of two identical subunits that recognizes the sequence GAATTC, cleaving both strands between the G and the A to produce 3′-hydroxyl-G and 5′-phosphoryl-A ends. The *Eco*RI methylase is a monomeric protein that recognizes the identical sequence GATTC and methylates the N6 amino group of the A's within that sequence. It is curious that although the endonuclease and methylase recognize the same nucleotide sequence, the two proteins do not show any homology with each other.

*Eco*RI is believed initially to bind DNA nonspecifically and then to move along the DNA in one direction until it recognizes the sequence GAATTC. Structural studies suggest that the DNA just outside the recognition sequence on either side of it may be bent or kinked. These bends may be used by the restriction endonuclease to recognize the site. As the endonuclease specifically binds to the recognition site, the DNA conformation is changed somewhat. It has been suggested that methylated DNA does not have these kinks. The methylase may protect DNA by altering the overall DNA structure, and the methyl groups on the adenines may interfere with specific binding of the endonuclease to the recognition sequence (Heitman, 1992).

Using X-ray crystallography, the structure of a complex composed of the *Eco*RI restriction endonuclease and an oligonucleotide with an

Table 2.1
Restriction Endonuclease Cleavage Sites and Information about Heat Inactivation of Restriction Endonucleases

Enzyme	Sequence	Heat inactivation*
HindIII	A ↓ AGCTT	n
BglII	A ↓ GATCT	n
AluI	AG ↓ CT	y
StuI	AGG ↓ CCT	y
ClaI	AT ↓ CGAT	p
DraIII	$CACN_3$ ↓ GTG	ni
PvuII	CAG ↓ CTG	p
NdeI	CA ↓ TATG	n
FokI	CATCC-(13/9)	—
BstXI	$CCAN_5$ ↓ NTGG	p
NcoI	C ↓ CATGG	y
SmaI	CCC ↓ GGG	p
XmaI	C ↓ CCGGG	ni
SacII	CCGC ↓ GG	—
SstII	CCGC ↓ GG	n
HapII	C ↓ CGG	n
HpaII	C ↓ CGG	n
MspI	C ↓ CGG	y
SecI	C ↓ CNNGG	ni
MnlI	CCTC (7/7)	ni
CvnI	CC ↓ TNAGG	y
Eco81I	CC ↓ TNAGG	ni
SauI	CC ↓ TNAGG	ni
EcoNI	CCTNN ↓ N_3AGG	ni
BstNI	CC ↓ WGG	ni
EcoRII	↓ CCWGG	y
StyI	C ↓ CWWGG	y
PvuI	CGAT ↓ CG	n
XorII	CGAT ↓ CG	n
ThaI	CG ↓ CG	n
Eco52I	C ↓ GGCCG	ni
XmaIII	C ↓ GGCCG	p
XhoI	C ↓ TCGAG	n
Eco57I	CTGAAG (16/14)	ni
PstI	CTGCA ↓ G	p
DdeI	C ↓ TNAG	n
Eco57I	CTTCAG-(14/16)	ni
AvaI	C ↓ YCGRG	p
MboII	GAAGA (8/7)	p
EcoRI	G ↓ AATTC	p
SacI	GAGCT ↓ C	ni
SstI	GAGCT ↓ C	y
HinfI	G ↓ ANTC	n

Table 2.1—*Continued*

Enzyme	Sequence	Heat inactivation*
EcoRV	GAT ↓ ATC	y
DpnI	GmA ↓ TC	y
MboI	↓ GATC	y
NdeII	↓ GATC	y
Sau3AI	↓ GATC	y
BglI	$GCCN_4$ ↓ NGGC	y
HhaI	GCG ↓ C	y
NotI	GC ↓ GGCCGC	y
BamHI	G ↓ GATCC	n
HaeIII	GG ↓ CC	n
Sau96I	G ↓ GNCC	n
KpnI	GGTAC ↓ C	y
BstEII	G ↓ GTNACC	n
Eco065I	G ↓ GTNACC	ni
AvaII	G ↓ GWCC	y
SalI	G ↓ TCGAC	n
AccI	GT ↓ MKAC	n
HpaI	GTT ↓ AAC	n
HincII	GTY ↓ RAC	y
HindII	GTY ↓ RAC	ni
HaeII	RGCGC ↓ Y	y
TaqI	T ↓ CGA	n
XbaI	T ↓ CTAGA	n
MboII	TCTTC-(7/8)	p
BalI	TGG ↓ CCA	y
DraI	TTT ↓ AAA	y

* Key: y, yes; n, no; p, partially inactivated; ni, not indicated; N, any of the four bases, W, A or T; Y, C or T; M, A or C; R, A or G; K, G or T. Heat inactivation is 65°C for 10 min. Sequences are 5′ to 3′. ( ) indicates number of bases between the recognition site and the cleavage point. The first number is for the top strand; the second number is for the bottom strand. —, the cleavage point precedes the recognition site. The arrow (↓) indicates the location of the cut (cleavage site).
Source: Gibco BRL Catalog and Reference Guide (1992). Life Technologies, Inc., Gaithersburg, MD. Check the information provided by the supplier for the buffer conditions for each restriction endonuclease digestion and for digestions using more than one enzyme in the same reaction.

*Eco*RI recognition site has been determined. The specific interactions that *Eco*RI uses to recognize its sequence include hydrogen bonds with purines, van der Waals and hydrogen bonds with pyrimidines, sequence-specific phosphate interactions, and DNA conformational distortions. Typically, first one strand is cleaved, then the second strand is cleaved, and the DNA

is released. However, for some sequences and reaction conditions, the DNA is released after only one strand is cut and the enzyme must again bind to the DNA to cut the second strand. The cleavage is believed to involve a hydroxyl ion. Mg^{2+} is required for *Eco*RI activity.

Star activity (*Eco*RI*), where *Eco*RI cuts at altered sequences, can be observed in buffers other than the standard *Eco*RI buffer. Under star activity conditions, sequences differing from GAATTC by one or a few bases are cleaved by *Eco*RI.

Although the substrate of Type II restriction endonucleases is double-stranded DNA, a few restriction enzymes can cut sites in single-stranded DNA (Been and Champoux, 1983). This is generally thought to occur because the restriction enzymes recognize duplex regions formed in the single-stranded DNA. Type II restriction endonucleases that have been shown to cut single-stranded DNA include *Eco*RI (Green *et al.*, 1975), *Hae*III (Koriuchi and Zinder, 1975), *Hha*I (Blakesley and Wells, 1975), *Mbo*I (Blakesley *et al.*, 1977), *Hin*fI (Hofer *et al.*, 1982), *Msp*I (Yoo and Agrawal, 1980), and *Sfa*I (Blakesley and Wells, 1975). The Szybalski *et al.* (1991) review of Type IIS restriction enzymes indicates that *Hga*I, *Mnl* I, and *Ple*I cut single-stranded DNA. Shaw and Mok (1993) developed a procedure to cut single-stranded DNA at any predetermined site by the interaction of a specially synthesized oligodeoxnucleotide and the Class IIN restriction endonuclease *Xcm*I.

Type III or Class III Restriction Endonucleases

In the Class III restriction/modification systems, both the restriction and the modification activities are on an enzyme complex composed of different subunits and do not require ATPase activity or SAM. Class III enzymes recognize specific sequences and cleave at a fairly fixed position 25 to 27 bp outside of the recognition sequence.

The Type III restriction endonucleases are large, multimeric complexes that have both methylation and endonuclease activities. In Type III restriction endonucleases, the cleavage occurs at a specific sequence a fixed distance from the recognition sequence. In many respects Type III enzymes are similar to Type I enzymes except that a specific DNA sequence is recognized and a specific DNA sequence is cleaved in Type III enzymes. To date, only a few examples of Type III restriction endonucleases have been found, including P15I, PI, and *Hin*fIII.

Other Restriction Endonucleases

Petrusyte *et al.* (1988) have proposed a fourth class of restriction endonucleases (Type IV). The prototypical restriction endonuclease of this class is *Eco*57I, which has a single polypeptide chain that contains both methylating and cutting activities; the cutting is stimulated by SAM.

This restriction enzyme is usually classified with Type II restriction endonucleases but it appears to represent a distinct class.

The study of restriction endonuclease systems is still in progress. Recently, a group of restriction endonucleases that cut modified DNA sequences and fail to cut the same unmodified sequences has been characterized. Sutherland *et al.* (1992) detailed the McrBC restriction endonuclease, which appears to represent yet another distinct class of restriction endonucleases. The McrBC (modified cytosine restriction) protein, formerly known as the Rg1B (restricts glucoseless phage) protein, cuts DNA that is methylated. Note that this restriction enzyme has not been named according to the standard rules. The restriction enzyme recognizes $R^{m}C(N_{40\text{-}80})R^{m}C$ and cuts at multiple positions on both strands between the two C's. It is a multisubunit restriction enzyme with GTP required as a cofactor. The system was first observed because of its restriction of T even phage in *E. coli.*

The Use of Restriction Endonucleases: Practical Matters

More than 250 different restriction endonucleases are available from commercial suppliers (see list of suppliers in Appendix 8). Roberts (1990), Roberts and Macelis (1992), and Bhagwat (1992) list all available restriction endonucleases and companies that manufacture the enzymes.

Restriction endonucleases can be purchased from suppliers, typically at concentrations of 10 units/μl. Some manufacturers also have certain restriction enzymes available at high concentrations of 50 units/μl. A unit of restriction enzyme activity is defined as the amount of enzyme needed to digest 1 μg of substrate DNA completely in 60 min under the reaction conditions appropriate for that enzyme. The definition of unit of restriction enzyme used by some manufacturers is the same as above, except that a reaction volume of 50 μl is specified in the definition. Gibco/BRL and Boehringer–Mannheim use the definition that does not specify the reaction volume, but their catalog descriptions of the assay procedure indicate a usual reaction volume of 50 μl. Other manufactures, such as New England Biolabs, Pharmacia, Promega, and United States Biochemical, specify a reaction volume of 50 μl in their definitions of a unit. Manufacturers assay units of activity by setting up restriction digestion reactions with a series of serial dilutions of the enzyme. After the 1-hr digestion under the appropriate conditions, the digestions are examined by gel electrophoresis. The most dilute sample that does not show any partial digestion products on the agarose gel is used to determine the units of activity.

Manufacturers of restriction endonucleases also test the enzymes extensively for contaminating endonuclease and exonuclease activity.

DNAs cut with the restriction enzymes are also checked to determine whether the DNAs can be ligated and recut with the same restriction enzyme. Many suppliers include an expiration date on the restriction enzyme tube. Under optimal storage conditions some manufacturers note that a restriction enzyme preparation can maintain activity for 12 to 18 months.

The manufacturers supply restriction enzymes in glycerol-containing storage buffers, which are different for each enzyme. The majority of restriction endonucleases are stored in buffers that are 50% (v/v) glycerol. However, some exceptions include *Sfi*I, with a storage buffer that is 20% glycerol, and *Spe*I, with a storage buffer containing only 5% glycerol. Restriction endonucleases should be stored at −20°C, but *not* in a frost-free freezer. The lifetime of restriction enzymes stored in frost-free freezers may be shortened due to the warming of the enzymes during the defrost cycle of the freezer. Farmer (1985) suggested that enzymes that must be stored in a frost-free freezer be placed in holes in a brick inside a Styrofoam container to provide more thermal stability. The storage conditions for a few restriction enzymes isolated from thermophilic bacteria may be at temperatures warmer than −20°C. For example, Gibco/BRL recommends storage of *Taq*I restriction endonuclease at 4°C. When restriction endonucleases are removed from the freezer, the enzymes should be kept on ice while being used and returned to the freezer as soon as possible. Small ice chests or storage containers for restriction enzymes can be purchased and will keep the enzymes cold for several hours when the container is left at room temperature.

The manufacturers of restriction endonucleases have determined the optimal assay conditions for each enzyme, and most companies supply a small tube of a concentrated stock of the appropriate reaction buffer with each restriction endonuclease purchased. These buffers are usually supplied as a 10-fold concentrated or 10× stock that must be diluted 10-fold to obtain the appropriate concentration for use with the restriction enzyme. This is convenient because the experimenter does not need to make the reaction buffer. However, the manufacturers of restriction enzymes do not all use the same buffer systems. When enzymes are obtained from more than one supplier, the different buffer systems must be carefully noted. The catalog of each supplier provides detailed information about the buffer systems used, the optimal buffer system for a particular enzyme, and the activity of that enzyme in other buffers. Information about the activity of a restriction enzyme in other buffers is useful when more than one restriction enzyme is used in the same digestion reaction (double or triple digestions use two or three different restriction enzymes in the same reaction). In general, a restriction enzyme from one manufacturer cuts well in the buffer by another manufacturer designated for that same enzyme. See the buffer

charts for different restriction enzymes in the catalogs of companies that sell restriction endonucleases. One company, Pharmacia, uses one buffer system for all its restriction enzymes. This system is called the One-Phor-All Buffer Plus system. Instead of changing to different buffers for different enzymes, one must use different amounts of the one buffer system for different enzymes.

The companies supplying restriction enzymes are continually improving their enzymes and may periodically make changes in the reaction conditions specified for a particular enzyme as new information is learned. When receiving a new restriction enzyme, one should always check the specifications of the assay conditions with each tube of enzyme purchased because changes may have been made as a result of the ongoing experimental process of the manufacturers.

Different Restriction Endonucleases

Isoschizomers (same cut) are restriction endonucleases that recognize the same DNA sequence and make the same cut. Note that isoschizomers may show differences in methylation sensitivities that can be useful to the research scientist. For example:

Endonuclease	1 2	
MspI	5′C↓C GG3′ 3′G G↑CC5′	if C at (1) is methylated, *MspI* does not cleave if C at (2) is methylated as a 5′methycytosine, *MspI* can cleave
HpaII		if (1) or (2) has a 4-methyl C, *HpaII* does not cleave if (2) is a 5-methyl C, *HpaII* does not cleave

Isocaudamers (same tail) are restriction endonucleases that have the same single-stranded overhang region. For example:

Endonuclease	Sequence
BamHI	5′G↓GATC3′
Sau3A	5′-↓GATC 3′

A *Sau3A*-generated fragment can be cloned into a *BamHI* site. What are the chances that the fragment can be cut out again using *BamHI*? Using *Sau3A*?

Setting Up a Restriction Digestion

Examine the supplier's catalog carefully to learn about the restriction enzyme to be used. Does the supplier make any special comments about the enzyme? The following points should be noted.

1. What is the temperature of reaction? Although the vast majority of restriction enzymes used have an optimum temperature of reaction of 37°C, some restriction endonucleases require other temperatures for optimum activity. For example, *Sma*I digestions should be carried out at 30°C, *Bst*EII digestions are done at 60°C, and *Taq*I has a temperature optimum of 65°C. See Tables 2.2 and 2.3.
2. Is the restriction enzyme sensitive to methylation?
3. Does the restriction enzyme have altered specificity of cutting under altered digestion conditions? Many restriction endonucleases have star activity under nonoptimal buffer conditions. Star activity is defined as altered specificity or relaxed specificity of cutting. For example, *Pst*I and *Kpn*I show altered specificity of cutting (star activity) in glycerol concentrations greater than 5%. The enzymes come in a storage buffer that is 50% glycerol. To keep the final glycerol concentration below 5%, restriction enzymes cannot be more than 1/10th the total volume of the reaction. This relaxed specificity or star activity can be observed for some restriction enzymes when changes are made in the reaction buffer, such as replacing Mg^{2+} with Mn^{2+}, lowering the ionic strength, or raising the pH or if the buffer contains too much glycerol or ethylene glycol. The altered specificity is noted by a star (asterisk). For example, *Eco*RI recognizes the sequence 5′-GAATTC-3′, and *Eco*RI* activity recognizes NAATTC and GNATTC, where N is any base. *Bam*HI recognizes GGATCC and *Bam*HI* recognizes GGNTCC, GGANCC, and GNATCC. Even under conditions where star activity occurs, cutting at the normal recognition site is more rapid and occurs the majority of the time; the star cutting products are usually seen as just a fraction of the products (Bhagwat, 1992). Star activity may be observed when the wrong restriction digestion buffers were used, when the buffers were not made

Table 2.2
Restriction Endonucleases: Temperature of Optimum Activity

*Acc*I	55°C, increases activity fivefold
*Apa*I	Optimum temperature 30–32°C, KCl required
*Ava*I	45°C, increases activity fivefold
*Sfi*I	50°C
*Sma*I	30°C recommended; activity reduced by 50% at 37°C
*Spe*I	50°C, increases activity
*Taq*I	65°C optimum, sensitive to pH
*Tha*I	60°C optimum

Note. The majority of restriction endonucleases commercially available have optimal reaction temperatures of 37°C.

Table 2.3
Restriction Endonucleases: Effects of Altered Assay Conditions

*Ava*I	High glycerol may alter specificity
*Bam*HI	High glycerol and low salt may alter specificity
*Dde*I	High glycerol or high salt may alter specificity
Eco47III	Very long digestions or large excess of enzyme may alter specificity
EcoRI	High glycerol, low salt, or high pH may alter specificity
EcoRII	Reaction buffer must have dithiothreitol (DTT) to a concentration of 1 m*M* to give activity
*Nde*II	Reaction buffer requires DTT be added
*Hha*I	High glycerol may alter site specificity
*Hinc*II	High glycerol may alter specificity
*Hpa*I	High glycerol may alter specificity
*Kpn*I	High glycerol may alter specificity
*Mlu*I	High glycerol may inhibit enzyme activity
*Nhe*I	High glycerol or NaCl ≥50 m*M* may inhibit enzyme activity
*Pst*I	High glycerol may alter specificity
*Pvu*II	High glycerol may alter specificity
*Rsr*II	DTT (to 1 m*M*) must be added for activity
*Sal*I	High glycerol may alter specificity
*Sau*3AI	Glycerol concentrations >7.5% (v/v) may alter specificity
*Sca*I	High glycerol, low salt, or high pH may alter specificity
*Sma*I	KCl, not NaCl, is required for enzyme activity
*Ssp*I	High glycerol and NaCl >50 m*M* inhibit enzyme
*Sst*I	High glycerol may alter specificity
*Stu*I	0.1 *M* NaCl inhibits activity by 50%
*Sty*I	0.1 *M* NaCl inhibits activity by 50%
*Sst*II	0.1 *M* NaCl inhibits activity by 50%

Note. Some restriction endonucleases have altered sequence specificity (also called star activity) under different assay conditions. Other restriction endonucleases have a decrease in activity under altered assay conditions. Assay conditions to consider include variation in salt concentration and amount of glycerol present. The above restriction endonucleases are sensitive to assay conditions. High glycerol is defined as a concentration of more than 5% (v/v). Note that restriction enzymes are supplied by the manufacturer in storage buffers that contain glycerol. Most storage buffers are 50% (v/v) glycerol. [Some storage buffers contain less glycerol. For example, *Sfi*I storage buffer is 20% (v/v) glycerol and *Spe*I storage buffer contains only 5% (v/v) glycerol.] To keep the reaction conditions at less than 5% (v/v) glycerol and avoid star activity of the restriction enzymes, the volume of restriction enzyme added should not be more than 1/10 of the final digestion reaction volume. Based on GIBCO-BRL 1992 Catalogue and Reference Guide, Life Technologies, Inc. [phone (800) 828–6686].

correctly, or when too high a volume of restriction enzyme was used in the reaction. Gibco/BRL technical advice staff say that EcoRI can give star activity if digestion times are excessive.

4. How much restriction endonuclease should be used to ensure complete cutting? To ensure complete cutting at all sites for the restriction endonuclease, the amount of enzyme added can be increased (be careful about the effects of excess glycerol on the reaction) or the time of the digestion reaction can be increased. Check the concentration (units/

μl) of the enzyme. Check information about the extent of activity of the restriction endonuclease during extended incubations. Some enzyme suppliers, such as Gibco/BRL, provide a chart of this information. For example, according to the Gibco/BRL assay of activity during extended incubations, *PstI* has full activity in their assay buffer for only 1 hr and all activity is lost after 2 hr. When *PstI* is being used to cut eukaryotic genomic DNA, rather than using a longer digestion time, another aliquot of enzyme should be added after 1–2 hr and an additional incubation time given.

Stopping a Restriction Digestion

There are several ways to stop a restriction digestion reaction; the method of choice depends on what the next manipulations of the sample will be. If the restriction digestion reaction products are simply to be analyzed by running the sample on an agarose gel with no further manipulations, the digestion reaction can be stopped by adding gel loading buffer, also called gel stop buffer or "blue juice." See the protocols for running gels for the recipes. If additional manipulations will be required, for example, addition of another enzyme, such as Klenow fragment or ligase, alternative ways to inactivate the restriction endonuclease must be used. Many restriction endonucleases are sensitive to elevated temperatures and can be inactivated by heating the sample to 65°C for 10 to 20 min. Manufacturers' catalogs frequently contain a chart indicating which restriction endonucleases are susceptible to heat inactivation (see Table 2.1). It is interesting to note that different suppliers report different sensitivities to heat inactivation for the same restriction enzyme. These differences are due in part to different lengths of time used for heat inactivation and may also reflect differences resulting from different salt conditions of the assay buffers used by different suppliers. All but 1 of the 58 restriction endonucleases available from Pharmacia can be heat inactivated in Pharmacia's One-Phor-All Buffer Plus. Pharmacia's heat inactivation protocol is incubation at 65°C for 20 min or at 85°C for 30 min, depending on the enzyme, followed by cooling at room temperature for 20 min. Ethylenediaminetetraacetic acid (EDTA) can be added to inactivate restriction endonucleases because the Type II restriction enzymes used here require Mg^{2+} as a cofactor and each molecule of EDTA can chelate two Mg^{2+} ions. Alternatively, diethylpyrocarbonate (DEPC) can be added to inactivate restriction enzymes. DEPC is added, and the sample is vortexed and heated at 65°C for 10 min. The DEPC reacts with the enzyme, crosslinking and inactivating it. At the elevated temperature DEPC breaks down into ethanol and CO_2. When the next step in sample preparation is ethanol precipitation of the DNA, it is also very likely that the ethanol precipitation step will irrevers-

ibly inactivate the restriction enzymes. A final way of inactivating restriction enzymes is to remove them from the sample by phenol extraction. Proteins separate into the interphase between the aqueous and the organic (phenol) phases when the sample is extracted with phenol. The use of phenol extraction can result in some loss of sample.

Ligase

Ligase, an enzyme that uses ATP to form bonds, is used in recombinant DNA cloning to join restriction endonuclease fragments that have annealed. The ligase commonly used is T4 DNA ligase, which was first isolated from *E. coli* that were infected with the lytic bacteriophage T4. The gene is encoded by the T4 genome and plays a role in the infection cycle of the phage. T4 DNA ligase catalyzes the formation of phosphodiester bonds between adjacent 3′-hydroxyl and 5′-phosphaste termini in double-stranded DNA. The ligase commercially available is now made by recombinant DNA means. T4 DNA ligase is a single polypeptide of 68 kDa; the enzyme requires Mg^{2+} and ATP as cofactors. The enzyme does not act on single-stranded nucleic acids. T4 DNA ligase can join staggered or blunt-ended termini and it can repair nicks in double-stranded DNA. Because T4 DNA ligase requires a 5′ phosphate, it is possible to prevent the rejoining of a vector DNA by removing the phosphate group from the vector using bacterial alkaline phosphatase or calf intestinal alkaline phosphatase. For details, see Sambrook *et al.* (1989, p. 1.56).

Salts to use with ligase to provide the correct conditions for optimum ligase activity are frequently provided by the supplier of ligase. For example, Gibco/BRL supplies a 5× concentrated ligation reaction buffer with its T4 DNA ligase [5× ligation reaction buffer: 0.25 *M* Tris, pH 7.6, 50 m*M* $MgCl_2$, 5 m*M* ATP, 5 m*M* DTT, 25% (w/v) polyethylene glycol (8000)].

Because the *E. coli* cell contains its own ligase, the bacterium can ligate the ends of the recombinant DNA in the cell. However, that process is not as effective as *in vitro* ligation. The use of ligase to join the ends of recombinant DNA molecules before they are introduced into the *E. coli* cell has several advantages over the introduction of recombinant DNA molecules without *in vitro* ligation. The *in vitro* ligation of recombinant DNA molecules greatly increases the frequency of transformation—number of transformants obtained per microgram of DNA—because the ligated DNA is less susceptible to degradation by nucleases within the *E. coli* cell. *In vitro* ligation also preserves the restriction endonuclease cleavage sites used to cut the original DNA. In addition, in the *in vitro* ligation reaction, the concentration of DNAs can be adjusted to favor the formation of circular or linear molecules.

Factors that influence the ligation reaction include the temperature of the reaction, the relative concentration of ends of DNA molecules, and the nature of the ends of the DNA molecules. To determine the temperature of the ligation reaction, both the temperature of optimal ligase activity and the temperature for efficient reannealing of ends of the recombinant DNA molecules are considered. The temperature of optimum ligase activity is 37°C.

The cohesive ends generated by restriction endonuclease digestion are short. For the joining of fragments with cohesive ends, the length of the cohesive ends and the base composition of the ends determine the reannealing temperature of the ends. Note that the restriction endonuclease *Eco*RI generates the single-stranded overhang of AATT. The melting temperature, T_m, of this 4-bp sequence is 5°C. Thus even relatively low temperatures can lead to disruption of the hydrogen bonding between the base pairs.

The recommended temperature for a ligation reaction has varied. BRL assays the unit of ligase activity at 37°C for 20 min. In early uses of T4 DNA ligase to join recombinant DNA fragments, the reaction temperature was 13°C. This temperature was a compromise between the optimum temperature of ligase activity and the temperature of reannealing of the recombinant ends. As more active preparations of ligase have become commercially available, the supplier's suggested temperature of reaction has increased to room temperature. The ligase is so active and used at sufficiently high concentration that when recombinant DNA molecules briefly anneal, the ligase immediately joins the ends.

The ligase is capable of catalyzing the joining of blunt-ended DNA molecules as well, but a higher concentration of ligase is utilized to carry out this reaction efficiently. Recent reports describe the use of an ultrasound treatment at 16°C for 15 min to produce useful ligations (Hengen, 1993).

The Jacobsen Stockmeyer equations can be used to determine the concentrations of ends of DNA molecules to use in ligation reactions. For details of these equations, see Maniatis *et al.* (1982, pp. 286–288) and Sambrook *et al.* (1989, pp. 1.63–1.67).

Gel Electrophoresis

The use of restriction endonucleases allows DNA to be cleaved into sequence-specific fragments. Agarose gel electrophoresis allows the DNA fragments generated by restriction endonuclease digestion to be separated on the basis of size.

Electrophoresis is defined as the migration of particles under the

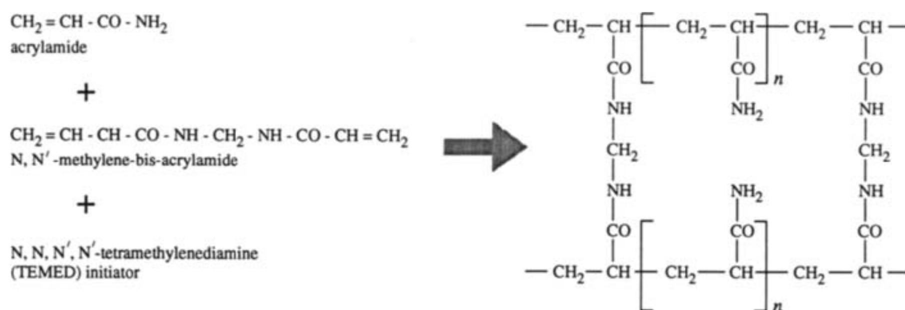


Figure 2.6 The structure of acrylamide, which is polymerized to form a polyacrylamide gel. The bisacrylamide provides crosslinks.

influence of an electric field. In general, how a particle moves in an electric field (the particle's *mobility*) depends on the net electrostatic force acting on the particle, which is determined by the potential gradient and the effective charge of the particle, and the frictional resistance, which is determined by the shape and size of the particle. The mobility of a particle during electrophoresis increases with increasing charge on the particle and decreases with increasing frictional force.

Oxidation occurs at the anode. Reduction occurs at the cathode. Note that ions were originally named after the terminal to which they migrate. The positive ions (cations) flow toward the cathode, where they combine with electrons to be reduced. The negative ions (anions) flow toward the anode, where they donate electrons, which flow out of the solution into the electric circuit. In the electrolytic cell, with an applied external voltage, the +electrode is the anode and the −electrode is the cathode (Day and Underwood, 1967, pp. 222, 224; Dickerson *et al.*, 1970, p. 88.)

In *gel electrophoresis*, there is a supporting medium that acts to prevent mechanical disturbances and convection during the electrophoresis and also acts as a molecular sieve to separate molecules on the basis of size. The matrix material may be starch, polyacrylamide, agarose, or a mixture of agarose–acrylamide.

Acrylamide is one type of matrix that is often used for gel electrophoresis. Figure 2.6 shows the structure of acrylamide monomers and polymerized acrylamide. Table 2.4 lists the concentrations of acrylamide used to separate proteins or small DNAs.

Structure of Agarose

Agarose is a neutral linear polysaccharide purified from agar–agar of red algae. It consists of D-galacose and 3,6-anhydro-L-galactose linked

Table 2.4
Acrylamide Concentrations Used to Separate Certain-Sized DNAs

% Acrylamide (g/100 ml)	Double-stranded DNA size (bp)	Single-stranded DNA (nt)	Double-stranded size of DNA (bp) that comigrates with dyes	
			Bromphenol blue	Xylene cyanole
3.5	100–1000	750–2000	100	460
5.0	80–500	200–1000	65	260
8.0	60–400	50–400	45	160
12.0	40–200		20	70
20.0	6–100		12	45

Note. The base pairs (bp) of DNA indicated above are the size range of linear DNA that can be optimally separated by the indicated concentrations of acrylamide (prepared as acrylamide:bis, 29:1). The comigration chart indicates how the dyes bromphenol blue and xylene cyanole migrate in different acrylamide concentrations (Ogden and Adams, 1987; Brown, 1991, p. 262). For example, in an 8.0% acrylamide gel, bromphenol blue migrates at the same rate as a 45-bp double-stranded DNA molecule. This marker information is useful for monitoring the progress of different sized DNAs during the electrophoresis.

in an alternating fashion by glycosidic bonds. Agarose is soluble in hot water; when the dissolved agarose solution cools, a matrix—a gel— forms through the crosslinking of agarose polymer chains by hydrogen bonds. The formation of the gel is reversible, but the melting and gelling points of agarose are different. Figure 2.7a shows the structure of the repeating unit of agarose.

The structure of agarose in gels has been studied. The X-ray diffraction work of Arnott and co-workers (1974) indicated that the agarose molecules have a double-helical structure. Figure 2.7b shows a model of the double helices formed by agarose. This work suggested the model that an agarose gel is an aggregate of agarose helices that form "long stiff rods" with large spaces between the rods. Using electron microscopy, fibers of agarose can be visualized. Later studies (Waki *et al.*, 1982) used freeze fracture and electron microscopy to examine gel surfaces. These studies determined the number of agarose fibers per unit volume. This work also supported the model that a gel is a network of rods (agarose aggregates) and channels. Waki *et al.* (1982, Table IV) found that for a gel of 0.25 to 4.0% (g/100 ml) agarose, the agarose fibers observed by electron microscopy after freeze fracture had radii of 3 to 6 nm. They estimate that such fibers would contain between 10 and 30 agarose helices. The higher the agarose concentration in the gel, the more fibers per cubic centimeter. They also made the useful observation that agarose gels set in the presence of salt (Waki *et al.*, 1982, Table III) have larger pore sizes than gels of the same agarose concentration set in water. This difference in pore size was

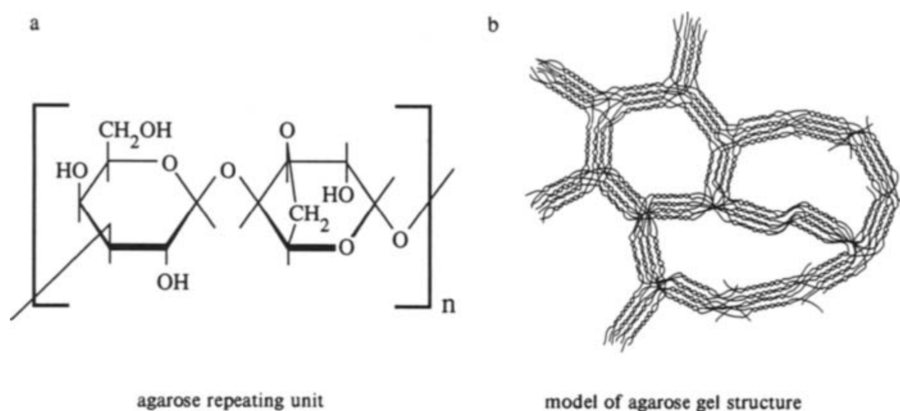


Figure 2.7 (a) The structure of the monomer agarose repeating unit. (b) A model for the structure of agarose in a gel.

also found by examining the electrophoretic mobility of plasmid DNA molecules in gels set in salt or in water. Mickel *et al.* (1977) studied the change in relative mobilities of three conformational forms of DNA as a function of the concentration of buffer, ranging from 20 to 80 m*M*. Mickel *et al.* felt that the changes in relative mobilities observed were due to a decreased stiffness of the nicked circular DNA at higher salt concentrations. Waki *et al.* noted that a salt-dependent change in the structure of the gel may also contribute to the change in relative mobilities that Mickel *et al.* observed.

Advantages of agarose gel electrophoresis include the following:

1. The procedure is rapid and simple to perform.
2. Agarose gel electrophoresis is capable of resolving mixtures of DNA fragments that cannot be resolved by other methods.
3. The location of the DNA within the gel can be determined directly by ethidium bromide (EtBr) staining. EtBr intercalates into double-stranded DNA. The EtBr bound to DNA has an increased fluorescence yield. EtBr absorbs light at 300 and 360 nm and emits light at 590 nm.

Agarose Gel Electrophoresis

DNA is a repeating polymer and therefore has a constant charge/mass ratio. It is not possible to separate DNA molecules by electrophoresis alone; however, gels of a controlled pore size make it possible to separate DNA molecules of different sizes. Figure 2.8 shows the setup of a gel electrophoresis apparatus.

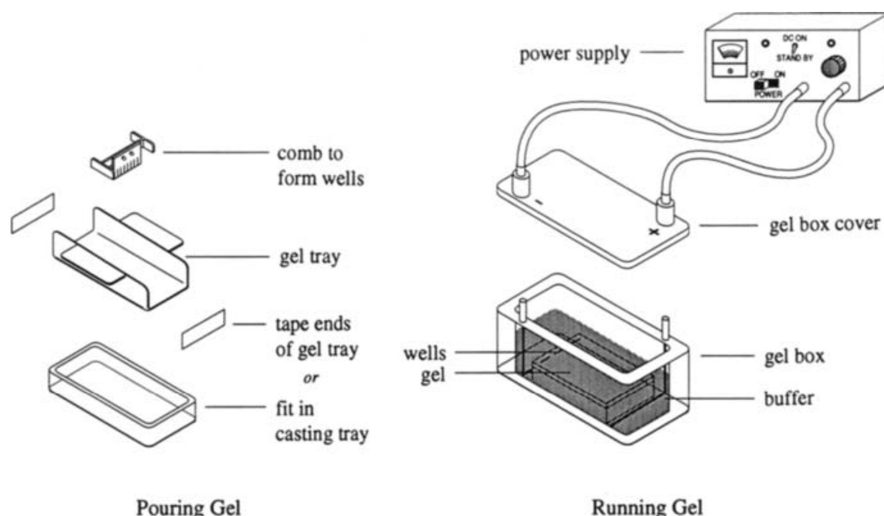


Figure 2.8 Apparatus for gel electrophoresis: gel tray, comb, and casting tray for making a gel; gel box and cover; power supply and leads for running a gel.

The electrophoretic mobility of DNA through agarose largely depends on

1. The molecular size of the DNA; linear DNA travels through the matrix at rates inversely proportional to the $\log_{10}$ of the DNA's molecular weight
2. Agarose concentration (see Table 2.5)
3. Conformation of DNA
4. Applied current

Gel artifacts that are sometimes observed during gel electrophoresis include

1. Overloading
2. Edge effects
3. Salt effects

For photographs of gels showing such artifacts, see Rodriguez and Tait (1983, Chap. 5).

If too much DNA is loaded on a lane, the DNA may migrate as a broad smear rather than as a sharp band. Such overloaded samples do not migrate as far in the gel as does the same DNA sample loaded at a lower concentration. Also, DNA can be seen trailing behind the leading edge of the band if the DNA is overloaded on the gel.

Table 2.5
Agarose Concentration Used to Separate Certain-Sized DNAs

% Agarose (g/100 ml)	Double-stranded DNA size (kb)	Double-stranded size of DNA (kb) that comigrates with dyes	
		Bromphenol	Xylene cyanole
0.3	60–5.0	—	—
0.6	20–1.0	0.7–1.2	9.0–10.0
0.7	10–0.8	0.6–0.8	6.0–7.0
0.9	7–0.5	0.3–0.4	3.0–4.0
1.2	6–0.4	0.18–0.21	1.4–1.9
1.5	4–0.2	0.12–0.14	0.8–1.4
2.0	3–0.1	<0.1	0.8–0.9

Note. The kilobase pairs (kb) of DNA indicated above are the size range of linear DNA that can be optimally separated by the indicated concentrations of agarose gels (Ogden and Adams, 1987). Comigration data were determined by Brad Goodner and students, Department of Biological Sciences, Purdue University.

Sometimes the same DNA sample does not migrate at the same rate in two different parts of the same gel. This difference in migration may occur because the electric field is not constant throughout all parts of the gel. One reason for this effect may be that the gel is heating up due to the voltage applied to it. The edges of the gel in contact with the buffer can dissipate this heat more readily than the center of the gel, which then heats up more than the edges of the gel. As the temperature increases, thus, current increases; the warmer center of the gel has a greater current in it and DNA fragments in the center of the gel migrate more rapidly than those on the cooler edges of the gel, which have a lower current. This can result in a "smiling of bands" in the gel because bands in the center of the gel have migrated farther. This gel artifact can be readily avoided by running the gel at a lower voltage so the gel does not heat up as much or by running the entire gel electrophoresis apparatus in a cold room to avoid overheating.

The concentration of salt present in the sample when the sample is loaded on a gel can change the rate of migration of the DNA sample. This effect can be appreciable for small DNA fragments and can give up to 15% difference in mobility. An increase in ionic strength decreases the rate of migration of a small fragment. This is because the extra ions present at higher ionic strength shield the DNA fragment from the electric field. This artifact can lead to errors if one is attempting to determine the size of small fragments and the molecular weight standards and the unknown samples are loaded in different salt concentrations. Because different restriction endonucleases have different optimum salt concentrations for

activity, samples cut with different restriction endonucleases may have different salt concentrations and therefore migrate differently. This problem can be circumvented by adding excess salt to all the samples to bring them to the same salt concentration before the samples are loaded on the gel.

Pulsed Field Gel Electrophoresis

Agarose gel electrophoresis can separate DNAs up to 20 kb in size, but larger DNAs cannot be separated or do not even enter the gel. Reducing the agarose concentration to 0.1% has allowed the separation of DNAs as large as 500 kb, but such low-percentage agarose gels are very fragile and extremely difficult to handle (Anand, 1986). Schwartz and Cantor (1984) devised a method of separating larger DNAs. They achieved separation of DNAs up to 2 Mb (2000 kb) using pulsed field gel electrophoresis, in which DNA molecules are subjected alternately to two different electric fields at right angles to each other. The basis of separation of DNAs in pulsed field gel electrophoresis is that when molecules are subjected to an electric field perpendicular to the direction of their migration, they reorient themselves along the new field and begin to move under the new field. Smaller molecules will be able to reorient themselves more quickly than larger molecules and will thus have greater mobility. Many different configurations of electrodes have been used in pulsed field gel electrophoresis in addition to the original perpendicular field configuration.

The use of pulsed field gel electrophoresis allows the separation of intact chromosomes of yeast and trypanosomes. When used with restriction endonucleases that cut infrequently, pulsed field gel electrophoresis also makes it possible to construct physical maps of large stretches of DNA of higher eukaryotes.

The migration of individual, fluorescently stained DNA molecules during gel electrophoresis has been observed with a microscope (Schwartz and Koval, 1989; Bendich and Smith, 1990). Such studies of the migration of individual DNA molecules will lead to a clearer understanding of the behavior of DNA during gel electrophoresis and eventually will lead to better models of DNA migration and better DNA separation methods.

Capillary Electrophoresis

Capillary electrophoresis is a promising new technique that is still being developed. Electrophoresis is carried out in a small capillary with an inner diameter of 20–100 μm. The capillary provides a high surface-to-volume ratio and allows very efficient dissipation of the heat generated from applied electric fields. The standard slab gel electrophoresis does

not dissipate much heat. Thus, the maximum electric field that can be used for standard gel electrophoresis is about 15 to 40 V/cm. Any higher electric field would result in overheating of the gels. However, capillary electrophoresis can use electric fields up to 800 V/cm and applied voltages of up to 30,000 V. This reduces separation times to minutes instead of hours. To separate DNAs, gels are made within the capillary tubes. By combining capillary gel electrophoresis with laser-induced fluorescence detection of nucleic acids, fluorescein-labeled DNA can be separated with "unprecedented speed, resolution, efficiency and sensitivity" (Landers, 1993). As little as 10^{-18} mol (attomole range) or about 5×10^{-15} g (femtogram) can be detected by this method. Separations of DNAs in the range 500 to 23,000 bp and the separation of intact chromosomes in the range 3 to 5 Mb (10^6 bp) have been achieved.

Ethidium Bromide Staining of DNA in Gels

Ethidium bromide is used as a stain for DNA in agarose gels. The ethidium bromide molecule intercalates (inserts) between the stacked bases of double-stranded DNA. The structure of ethidium bromide is shown in Figure 2.9. The intercalated ethidium bromide molecules emit fluorescent light when irradiated with ultraviolet light. Three hundred nanometers is the optimal wavelength for exciting fluorescence from the ethidium bromide–DNA complex. Excitation at 254 nm gives only one-third as much fluorescence, while excitation at 366 nm yields only one-seventh as much fluorescence.

Ethidium bromide is made as a stock of 10 mg/ml in H_2O. It is used at concentrations of 0.5 to 1 μg/ml. A stock of ethidium bromide can be made from a powdered form.

Ethidium bromide may be added directly to the agarose gel when the gel is made. Alternatively, after electrophoresis, the gel may be stained in a solution of ethidium bromide (0.5 to 1 μg/ml). Depending on the size of the gel and the percentage agarose content of the gel, it will take from 10 to 20 min to stain a gel with ethidium bromide after the gel is run.

When there is ethidium bromide present during electrophoresis, the rate of migration of a DNA fragment is reduced by 10 to 15%.

A phenomenon called photobleaching occurs when ethidium bromide-stained DNA is illuminated by ultraviolet light. Photobleaching is a decrease in the intensity of the fluorescence of bands. This decreased fluorescence is presumably due to the dissociation of ethidium bromide from the DNA. Photobleaching occurs less rapidly at 300 nm than at 254 nm.

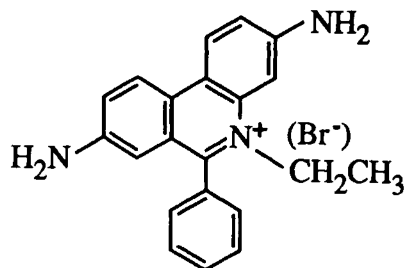


Figure 2.9 The structure of ethidium bromide, a sensitive stain for DNA.

Ethidium Bromide Safety

Ethidium bromide is a valuable aid for visualizing DNA in gels, but it is considered a "powerful mutagen with moderate toxicity" (MacGregor and Johnson, 1977). The greatest safety concern is during the preparation of a stock from the powdered ethidium bromide.

CAUTION: Use great care when weighing out powdered ethidium bromide. Ethidium bromide is a mutagen and probable carcinogen. Ethidium bromide is toxic. Wear gloves and a face mask when working with ethidium bromide in powdered form. Wipe the area with a damp cloth after the work with ethidium bromide powder is complete. A safer alternative is to purchase ethidium bromide in solution. This eliminates the hazards of working with the powdered form. Always wear gloves when working with ethidium bromide and ethidium-stained gels.

Also, while wearing gloves after handling ethidium bromide, be careful not to touch and thereby contaminate other surfaces. Be sure to wash hands with soap and warm water after working with ethidium bromide.

At present, the best way of disposing of ethidium bromide is not clear. There are several studies that suggest different methods to inactivate ethidium bromide. One study (Lunn and Sansone, 1987) compared eight methods of destroying ethidium bromide and found that treatment with sodium nitrite and hypophosphorus acid was most effective in destroying ethidium bromide. A common practice is to add sodium hypochlorite (bleach) to ethidium bromide solutions before disposing of them down the drain. The Lunn and Sansone (1977) study found that bleach treatment yields products that are quite mutagenic and recommends bleach not be used. On the other hand, Quillardet *et al.* (1988) report that bleach treatment reduces ethidium bromide to 20% of its initial mutagenicity or 0.1%

of its initial mutagenicity if an activating mixture is not used in the mutagenicity test. Quillardet recommends using potassium permanganate/hydrochloric acid to destroy ethidium bromide. Bensaude (Quillardet *et al.*, 1988) suggests adsorption of ethidium bromide to activated charcoal followed by incineration, because ethidium bromide decomposes at 262°C.

Some papers recommend that ethidium bromide be treated with $KMnO_4$ to inactivate it. $KMnO_4$ must be handled in a hood. Other studies suggest that the by-product of the $KMnO_4$ treatment of ethidium bromide is even more mutagenic than ethidium bromide. Stained gels should be placed in plastic bags before disposal. Some schools have a solid waste program where ethidium bromide-stained gels are collected and incinerated.

Check with your local safety officer or local waste management control to determine how to dispose of ethidium bromide solutions and stained gels and about the disposal of other DNA stains such as methylene blue.

Sensitivity of Detection with Ethidium Bromide

Under routine staining conditions, about 10 ng of DNA can be detected using ethidium bromide. Sambrook *et al.* (1989) report that as little as 2 ng of DNA in a 0.5-cm-wide band can be detected by the photography of an ethidium bromide-stained gel. Note that if more than 0.5 μg of DNA were loaded in a 0.5-cm-wide well, the gel would be overloaded and trailing and smearing of the DNA in the gel would occur.

Other DNA Stains

Nucleic acids in polyester-backed polyacrylamide gels can be detected using a silver stain. Bands at the picogram (10^{-12}) level can be detected (Caetano-Anolies and Gresshoff, 1994). This has been used in a silver sequencing system from Promega. One concern with the use of silver is the appropriate disposal of the silver.

Santillan-Torres and Ponce-Noyola (1993) report the use of brilliant cresyl blue to stain DNA in agarose gels. They were able to detect as little as 25 ng of DNA in a band. Under the same conditions, other dyes, such as methylene blue, had such a high background staining that bands were difficult to visualize.

Methylene blue is often used as an alternative to EtBr stain. Even though methylene blue is considered a "safer" stain for DNA than ethidium bromide, remember that methylene blue is "moderately toxic."

CAUTION: Wear gloves at all times when handling methylene blue.

Horn (1993, p. 15) compares the risks and benefits of using ethidium bromide or methylene blue to stain agarose gels. Ethidium bromide is more sensitive than methylene blue and requires shorter gel staining and destaining times than does methylene blue. Methylene blue is less expensive and a preparation of methylene blue can be stored for up to 6 months, whereas ethidium bromide preparations for staining gels are light-sensitive, must be stored in the dark, and break down in a few days.

Transformation

Background

Bacterial transformation is defined as the heritable change in the properties of bacteria caused by the uptake of naked DNA. One way of introducing a heritable change into a bacterial genome is bacterial conjugation, in which an F plasmid is transferred to an F^- *E. coli*. Also DNA may be transferred from one bacterium to another via an Hfr (high frequency of recombination) strain, in which an F plasmid has integrated into the chromosome and begins transfer of DNA from that chromosomal location. There is also phage-mediated transfer of DNA from bacterium to bacterium, known as transduction. In restricted or specialized transduction, only specific genes from the bacterial genome are transferred. This occurs because the transducing phage has a lysogenic stage in which the phage integrates into the bacterial host genome. When the lytic cycle is induced, at a low frequency, the prophage is not precisely excised from the genome and adjacent host chromosomal genes are also excised, replicated, and packaged in the phage heads. These chromosomal genes along with the phage DNA are then introduced into another bacterium by the normal phage infection process. Bacteriophage λ is an example of a specialized transducing phage that transduces the genes on either side of the site of normal λ integration into the *E. coli* genome. Bacteriophage ϕ80 is a specialized transducing phage that can transfer the *trp* operon (genes coding for tryptophan biosynthesis). In generalized transduction, any gene of the host can be transferred into another bacterium. This occurs because the bacteriophage-encoded nuclease has degraded the host chromosomal

DNA to the appropriate size (approximately the same size as the phage genome) to be packaged into phage heads. The normal phage infection process brings the host DNA into another bacterium. Bacteriophages P1 and P22 are examples of generalized transducing phage. In generalized transduction, the transducing phage particle contains only bacterial DNA and no phage DNA (Ikeda and Tomizawa, 1965; Ebel-Tsipis *et al.*, 1972).

Transformation is the uptake of naked DNA by an organism. Competence is the state of being able to take up DNA. In certain bacteria, there is a natural transformation system where bacterial cells acquire competence—the ability to take up DNA—as they grow. For example, with the gram-positive *Streptococci*, when the bacteria are grown to a cell density of between 10^7 and 10^8 bacteria/ml, the bacteria become competent. In the gram-negative bacterium *H. influenza*, competency is induced by starvation that does not block protein synthesis. When *Azotobacter* are grown to stationary phase, the cells become competent.

Studies of natural transformation systems in bacteria have led to separate models of how transformation takes place for gram-negative and for gram-positive bacteria. Note that in these systems, the incoming DNA can transform the recipient bacterium only if the DNA is homologous to the recipient bacterium's DNA and homologous recombination occurred. In the studies to be discussed later with artificial transformation systems, the incoming DNA is often a plasmid DNA which can replicate independently within the recipient bacterial cell and does not demand homologous recombination to be maintained in the host cell (Smith *et al.*, 1981).

The model of natural transformation for a gram-positive bacterium is as follows:

1. The bacterium is exposed to competence factor. In gram-positive bacteria, soluble factor taken from the media of cells in the competent state and added to noncompetent bacteria can induce the competent state in the noncompetent bacteria.
2. Hydrolysis of patches of the cell wall exposes a receptor for DNA.
3. The receptor binds DNA; 30- to 50-kb DNA seems to bind best.
4. A nuclease associated with the receptor puts double-stranded nicks into the DNA.
5. The double-stranded DNA is converted to single-stranded DNA by hydrolysis of one strand of the DNA. The resulting single-stranded DNA pieces are about 10 kb long.
6. As the single-stranded DNA enters the cytoplasm, it is sequestered and protected by proteins.
7. The transforming single-stranded DNA replaces the homologous DNA in the recipient genome.

The gram-positive bacteria appear to take up any DNA with the same efficiency. This was shown for *Bacillus subtilis* transformed with *B. subtilis* DNA or with *B. subtilis* DNA plus an excess of *E. coli* DNA. The rate of transformation with *B. subtilis* DNA decreased with the addition of *E. coli* DNA, indicating that the *E. coli* DNA can compete with *B. subtilis* DNA for uptake into *B. subtilis*. Recombination depends on homology, so the incoming *E. coli* DNA could not recombine and would not be scored as a transformation event.

For the gram-negative bacteria natural transformation process, a similar model can be made. Three major differences between gram-negative and gram-positive natural transformation systems are that no competence factor has been observed with gram-negative bacteria, a specific sequence of DNA is recognized by the receptor, and the single-stranded DNA cannot be readily isolated, suggesting that recombination may be closely associated with the membrane receptor. In natural transformation studies in *H. influenza*, excess *E. coli* DNA could not complete with *H. influenza* DNA. Danner *et al.* (1980) found that *H. influenza* uses a specific 11-bp sequence to recognize DNA as its own DNA. A piece of DNA with this sequence will be taken up by *H. influenza*. There are approximately 600 copies of this specific sequence in *H. influenza* DNA.

Stewart and Carlson (1986) suggest that the differences in the natural transformation systems of gram-positive and gram-negative bacteria may not be significant. In both *B. subtilis* and *H. influenza*, "the transforming DNA causes single strand replacement of homologous regions in recipient genome accompanied by simultaneous degradation of replaced recipient strand and non-integrated portions of transforming strand."

For other bacteria, there are no known natural transformation systems. However, the cells can be made competent by artifical means, often by noxious treatments such as treatment at 0°C with $CaCl_2$, which may decrease the cell viability but result in the cells being able to take up DNA.

For *Agrobacterium tumefaciens*, freezing and thawing the bacteria in polyethylene glycol (PEG) allow transformation. For *E. coli*, a combination of calcium ions, low temperature (0–5°C), and a heat pulse of 42°C allows *E. coli* cells to take up plasmid DNA.

Transformation Procedures

Researchers investigating transformation efficiencies in *E. coli* have noted variability in the transforming ability of different preparations of the same plasmid as well as variability in different batches of *E. coli* cells (Conley and Saunders, 1984). Conley and Saunders (1984) showed that a

linear pBR322 plasmid transforms *E. coli* at a 10^2 to 10^3 lower frequency than does covalently closed supercoiled pBR322 plasmid DNA. The plasmid was linearized by cutting with a restriction endonuclease that cuts once in the plasmid. Dagert and Ehrilich (1979), using 0.1 *M* $CaCl_2$ to make competent *E. coli* cells, observed that the transformation efficiency increased as cells were stored on ice, although the cell viability decreased with period of storage on ice. In their experiments, they determined a transformation frequency of 2×10^7 transformants/μg of pBR322 using freshly prepared competent *E. coli* cells. The transformation frequency of cells stored on ice for 24 hr was six times greater and a higher percentage of viable cells (20%) were competent. The cell viability decreased steadily throughout the storage on ice. The transformability of cells reached a maximum at 24 hr of storage on ice and declined with longer incubation on ice.

Inoue *et al.* (1990) carefully examined the factors that influence transformation efficiencies in *E. coli*. They found that growth of *E. coli* cells at a lower temperature resulted in a higher transformation efficiency. They determined an optimum temperature of growth of cells to be 18°C when grown to an A_{600} of 0.75. The time to reach such a density at the lower temperature may be 2 days. Using their procedure for making competent cells, they obtain transformation frequencies of 1–3 $\times$ 10^9 transformants/μg pBR322 DNA. Such frequencies are comparable to those obtained with electroporation. (Electroporation uses a pulse of electricity to create pores in the cell membrane. DNA can enter the cell in this way.) These high efficiencies are needed for making a complete cDNA library of a complex genome.

Hanahan sought to understand the nature of the transformation process in *E. coli* in more detail and to improve the efficiency of transformation in *E. coli*. Hanahan devised transformation procedures which, for certain strains of *E. coli*, increased the efficiency of transformation 100- to 1000-fold more than the efficiency with the standard $CaCl_2$ treatment. Hanahan's modified transformation procedures included the growth of cells in 10–20 m*M* Mg^{2+}, followed by combining DNA and cells in the presence of one of these additional components: 45 m*M* Mn^{2+}; 10 m*M* Ca^{2+}, 100 m*M* Rb^{2+}, or K^+; 7% dimethyl sulfoxide (DMSO), 75 m*M* dithiothreitol (DTT), or 3 m*M* hexamine cobalt(III) chloride. Hanahan notes that the culture conditions of the bacteria (growth in a large flask, with a large surface-to-volume ratio, and vigorous agitation without foaming) were important to achieving high transformation efficiencies, but these procedures did not enhance the transformation efficiencies of all *E. coli* strains he studied. He was able to obtain 5×10^8 transformants/μg of plasmid pBR322 or 1 transformed cell per 400 plasmid molecules.

Hanahan's studies suggested three roles divalent cations may play in aiding transformation: (1) The divalent cations shield the negative charges on DNA (from the phosphate groups) and on the outside of the cell (from cell-surface phospholipids and lipopolysaccharides) so the DNA can come in close association with the cell more readily. (2) The combination of low temperature and divalent cations may aid in the crystallization of regions of the membrane to make channels for DNA uptake more accessible. (3) The divalent cations may help to reorganize the lipopolysaccharides away from the channels they normally guard.

The use of these protocols to transform *E. coli* cells has been a valuable tool in making recombinant DNA cloning possible. Alternative methods of introducing DNA into the bacterial cell and into eukaryotic cells have been developed. For a review of some of these methods see Karcher (1994).

Recombinant DNA Cloning: Overview

In this experiment, the student will learn the procedures of recombinant DNA cloning of *Hin*dIII, *Eco*RI, or *Pst*I fragments of a cosmid (cos203), containing chromosomal DNA of *A. tumefaciens*, into the cloning vector pUC13 or pUC19. Any other large DNA of interest can be subcloned by the same procedures.

The objectives of this experiment are as follows:

1. To understand, from hands-on experience, the steps of recombinant DNA cloning.
2. To understand the advantages of cloning DNA fragments.
3. To learn the uses of recombinant DNA clones.
4. To use the recombinant DNA clones generated in this experiment in a follow-up experiment as hybridization probes for restriction endonuclease site mapping (Chapter 3).

The steps of a recombinant DNA cloning experiment are to isolate DNA to be cloned, cut DNA with the appropriate restriction enzymes, check an aliquot of each restriction digestion reaction for complete cutting on a gel, inactivate restriction enzymes, precipitate the appropriate amounts of insert and vector DNAs together, ligate DNAs, transform recombinant DNA molecules into *E. coli*, analyze recombinants using a DNA rapid isolation procedure called a "mini-prep," and restriction digestion and gel electrophoresis of mini-prep DNA.

Description of pUC Vectors

The pUC vectors, such as pUC13 and pUC19 created by Messing and co-workers (Vieira and Messing, 1982; Norander *et al.*, 1983; and Yanisch-Perron *et al.*, 1985), are very useful for cloning.

pUC13 contains the origin of replication and ampicillin (*amp*^{r}) resistance gene of the plasmid pBR322, a ColE1-type plasmid. It is a small (2.7 kb), high-copy-number plasmid. pUC13 also contains a portion of the *lac* regulatory region plus the first 145 amino acids of the β-galactosidase gene, known as the α-donor. (In order to have functional β-galactosidase, the host *E. coli* cell must contain the α-acceptor part of the β-galactosidase gene.) pUC13 also contains a "polylinker" with many unique restriction enzyme sites inserted between the O and Z′ genes of *lac*. The *lac*O gene is the operator region where the *lac* repressor can bind to prevent transcription of the *lac* operon. Z′ indicates that only a portion of the *lac*Z gene is present. This approximately 60-bp sequence is inserted in such a way that is does not interfere with the α-complementing ability of the α-peptide. If a piece of DNA were cloned into one of the restriction endonuclease sites within the polylinker, the α-complementing peptide would be disrupted.

pUC19 differs from pUC13 in the restriction endonuclease sites in the polylinker.

Might some pieces of DNA be cloned into pUC that would not completely inactivate the α-complementation?

β-Galactosidase

In *E. coli* the β-galactosidase protein is active as a tetramer, with identical monomers of 1021 amino acids. Certain deletions in the *lacZ* gene near the promoter-proximal end (α-acceptor) produce inactive enzyme that can be complemented by nonoverlapping deletions in promoter-distal regions (α-donor). α-Complementation occurs when α-donor and α-acceptor peptides interact to restore β-galactosidase activity. See the Introduction to this chapter for more details.

Note that *E. coli* strains JM83 and DH5α are appropriate hosts for pUC13 and pUC19 because they supply the α-acceptor peptide. *lac*ZΔM15 is a deletion of the operator-proximal region of β-galactosidase that lacks amino acids 11 to 41 and produces a defective protein that can function as an α-acceptor.

Why would Messing design a plasmid using the α-donor/acceptor system instead of the whole *lacZ* gene? This is a smaller piece of DNA; it adds fewer restriction endonuclease sites and allows the cloning vector to be smaller.

Strain List

E. coli strain	Reference	Genotype
DH1	1	F^-, *rec*A1, *end*A1, *gyr*A96, *thi*, *hsd*R17, *sup*E44, *rel*A1,λ^-
DH5α	2	F^-, *end*A1, *hsd***R**17 (r_{K-},m_{K+}), *sup*E44, *thi*-1, λ^-, *rec*A1, *gyr*A96, *rel*A1, ϕ80d*lac***ZΔM**15 Δ(*lac***ZYA**-*arg***F**) U169
JM83	1,2	F^-, *ara*,**Δ**(*lac*-*pro***AB**), *rps***L**(=**strA**), ϕ80d *lac*ZΔM15
E646	3	*E. coli* DH1 containing cosmid 203 (tet^r)

References: (1) C. Yanisch-Perron *et al.* (1985). (2) BRL's *Focus* **8**, 9. (3) Rong *et al.*, 1990.

Genetic Markers

Marker	Description	Usefulness
*end*1	Endonuclease mutation	Absence of endonuclease improves quality of DNA from mini-preps.
*hsd*R17(r_K-m_{K+})	Restriction negative and modification positive	r_{K-} allows cloning of DNA without cleavage by endogenous restriction enzymes. m_{K+} DNA grown in this host is methylated, and then can efficiently transform r_{K+} *E. coli* hosts.
gyrA	Mutation in DNA gyrase	Confers resistance to to nalidixic acid.
*lac*ZΔM15	Partial deletion of *lac*Z, remaining part (called α-acceptor) can allow α-complementation if α-donor provided (as on a pUC plasmid), thereby having functional β-galactosidase	Required for use with cloning vector pUC or M13. On X-gal plates, can give blue and white colonies or plaques for recombinant clone selection.

Genetic Markers—*Continued*

Marker	Description	Usefulness
*recA*1	Recombination deficient	Increases stability of inserts, i.e., transformed DNA will not recombine with host DNA.
relA	Mutation eliminating stringent factor	
supE	Amber suppressor	

Restriction Endonucleases

Endonuclease	Recognition sequence	
*Eco*RI	↓ 5′ . . . G AATT C . . . 3′ 3′ . . . C TTAA G . . . 5′ ↑	Note: Star activity may occur if used in too high a glycerol concentration or too high an enzyme:DNA ratio. As a general rule, do not have more than 10% of the final reaction volume as restriction enzyme.
*Hin*dIII	↓ 5′ . . . A AGCT T . . . 3′ 3′ . . . T TCGA A . . . 5′ ↑	
*Pst*I	↓ 5′ . . . C TGCA G . . . 3′ 3′ . . . G ACGT C . . . 5′ ↑	Glycerol concentrations >5% (v/v) may alter the specificity of cutting.

The Insert DNA to Be Cloned: Origin and Significance of Cosmid 203

Agrobacterium tumefaciens causes the tumorous disease crown cell on many species of plants. The molecular mechanism of *Agrobacterium* tumorigenesis involves the transfer of bacterial DNA to plant cells, where it stably integrates into the plant nuclear genome and is expressed. The region of DNA that is transferred is called the T (transferred) DNA and is located on a plasmid, the Ti (tumor inducing) plasmid, harbored by virulent strains of the bacterium.

The infection cycle of *Agrobacterium* involves a complex series of chemical signals that pass between the susceptible plant and the bacterium. Wounded plant cells secrete a number of phenolic compounds, including acetosyringone and related molecules. *Agrobacterium* perceives these signals via the *virA* gene product. It is thought that this event leads to the modification of the virG protein, resulting in its activation. The activated virG protein, in turn, activates the transcription of Ti-plasmid-located loci, other *vir* genes, that code for proteins that process the T-DNA from the Ti-plasmid and form the molecular apparatus that allows the passage of the transferred DNA from the bacterium to the plant cell. See the reviews by Nester *et al.* (1984), Binns and Thomashow (1988), Ream (1989), Winans (1992), and Zambryski (1988, 1992).

In addition to the activation of the *vir* loci by phenolic molecules, some bacterial chromosomal genes are induced by other compounds from plant cells. One plant-inducible gene that has been cloned is *picA* (Rong *et al.*, 1990, 1991). One of several cosmid clones isolated containing this plant-inducible gene is cosmid 203. A pectic substance that can induce the promoter of *picA* gene has been identified (Rong *et al.*, 1994).

Details of Cosmid 203

Cosmid 203 contains about 20 kb of *A. tumefaciens* chromosomal DNA cloned into the 21.6-kb vector pCP13 (Darkins and Chakrabarty, 1984; Friedman *et al.*, 1982). Cosmid 203 contains a kanamycin resistance gene.

pCP13 is derived from the plasmid pLAFR1, a wide-host-range plasmid that can be mobilized *in trans* among many gram-negative bacteria. Note that pLAFR1 has the *cos* sites from phage λ, allowing the packaging in the λ phage coat protein *in vitro* and subsequent transfection of *E. coli*. This cosmid has the advantage of size selection of inserts, so all inserts in the library will be of approximately the same size.

Inserts into pLAFR1 are about 23 kb. For an *E. coli*-size genome (4100 kb) only 820 clones in pLAFR1 would give a 99% probability that the library was complete.

Alternative DNAs to Clone

The details of a specific large cosmid that can be subcloned are presented above. Other large DNAs, for example, λ DNA, could be used. Other cosmid DNAs could be used in this experiment. The DNA to be subcloned should not contain an ampicillin resistance marker, however, because the pUC cloning vector contains the amp^r gene.

Recombinant DNA: P1 Level of Physical Containment—Laboratory Practices

1. Keep laboratory doors closed while experiments are in progress.
2. Decontaminate work surfaces daily and immediately after spills of organisms.
3. Decontaminate (autoclave or treat with bleach) all glassware before washing, reuse, or disposal.
4. Do not pipet by mouth. Use mechanical pipetting devices.
5. Do not eat, drink, or store food in the lab.
6. Wash hands after handling recombinant DNA-containing organisms. Wash hands before leaving the laboratory.
7. Work to minimize the creation of aerosols.
8. Autoclave bacterial plates before discarding them.
9. Keep the laboratory neat and clean.

NOTE

Several optional exercises involving restriction endonuclease digestion of DNA and gel electrophoresis of DNA are presented. These exercises should be done before the cloning experiment if students have little experience in these areas.

PROTOCOL 2.1a (Optional): Restriction Digestion of DNA Samples and Gel Electrophoresis of DNA Samples

1. Set up restriction digestion of DNA sample. For example, in a microcentrifuge tube, put
   - 5 μl (=0.5 μg) of a plasmid DNA (Typically, 0.5 μg of a plasmid DNA would be digested and run on one lane of a gel.)
   - 1 μl 10× restriction enzyme digestion buffer (Note the buffer is a 10× concentrated stock that must be diluted 10-fold to provide the appropriate salt conditions for optimum restriction endonuclease activity. That is, use 1/10th of final volume of reaction mixture.)
   - 3 μl distilled H_2O (to bring reaction mixture to desired final volume)
   - 1 μl restriction enzyme (Typically, restriction enzymes are supplied at concentrations of 10 units/μl. Usually at least 1 unit of restriction

enzyme/μg of DNA is used to cut a plasmid DNA, and 5 to 10 units/μg of DNA is used for bacterial DNA or eukaryotic genomic DNA.)
- Final volume 10 μl

2. Allow digestion to proceed at desired temperature, for most restriction enzymes, 37°C for 1 hr.
3. After the digestion time, add 3 μl stop buffer to inactivate the restriction enzyme and to prepare the sample for loading onto a gel. (Gel loading dye, stop mix, or stop buffer: 50% glycerol, 0.7% SDS (sodium dodecyl sulfate, a detergent); 0.1% BPB (bromophenol blue, a dye).)

PROTOCOL 2.1b (Optional): Restriction Enzyme Digestion of DNA to Be Cloned

This exercise is used to begin to determine the restriction endonuclease site map of the cosmid used in the recombinant DNA cloning experiment. In a later experiment, Southern blotting is used to add details to this restriction map.

Different groups may use different restriction enzymes.

1. Cut the cosmid DNA (0.5–1.0 μg) with the restriction enzymes in single (using one restriction enzyme) and in double (using two restriction enzymes) digestions. Determine the correct buffers to use for each enzyme for single and double digestions by examining the suppliers' catalogs.
2. Run the samples on an 0.8% agarose gel, along with λ *Hind*III size markers. (See Protocol 2.2 for details.)
3. After the gel has been run, stained, and photographed, determine the sizes of the fragments generated. Indicate a rough map.
4. By convention, the largest fragment observed is called fragment number 1; the next largest fragment is called fragment number 2, etc. List the sizes generated as follows: *Eco*RI fragment 1 is 20 kb, etc. Indicate whether the data suggest any restriction sites within that fragment. *Eco*RI fragment I contains *Xho*I site, etc.
5. Pool the data from all groups to create a working restriction map of this cosmid.

In the later Southern experiment, each group will use one of the fragments cloned as a probe to obtain further restriction mapping information.

PROTOCOL 2.2 (Optional): Gel Electrophoresis

This exercise is performed to gain experience with preparing, loading, and running agarose gels. The migration of DNA fragments in different percentages of agarose gels is examined. The migration of two dyes in different percentages of agarose gels is also examined to determine how these dyes can be used to monitor the migration of DNA in the gels.

Read the section on ethidium bromide safety above.

Materials

- Agarose
- 10× TBE: 0.89 *M* Tris (Trizma base); 0.89 *M* boric acid; 0.02 *M* EDTA to be used as 1× TBE
- Gel loading buffer: gel loading dye, stop mix, or stop buffer:50% glycerol; 0.7% sodium dodecyl sulfate (SDS, a detergent); 0.1% bromophenol blue (BPB, a dye)
- Bacteriophage λ DNA, digested with *Hin*dIII, or other DNA of known size as a standard
- Aliquots of the DNA to be used in cloning experiment, cut with several different restriction enzymes (designated as unknown DNA in this exercise)
- Gel electrophoresis setup: power supply, gel box, gel tray, comb to make wells, casting tray, leads
- Balance
- Microwave or boiling water bath
- Ethidium bromide staining solution
- UV transilluminator
- Camera and film to photograph gels on the UV transilluminator
- Microfuge
- Microfuge tubes
- Micropipettors and micropipettor tips

Procedure

1. Weigh out the amount of agarose needed to make 100 ml of a 0.5, 0.6, 0.7, 0.8, 1, 1.5, or 2% gel. % is g/100 ml.

CAUTION: When using the microwave, be sure to use autoclave gloves to pick up hot flasks. Do not swirl a flask to mix the contents of the flask until the flask has cooled briefly. Point the mouth of the flask away from

you. Swirling an overheated flask may cause the liquid inside to "boil out" of the hot flask.

2. Place the correct amount of agarose for the percentage of gel to be made in a 250-ml Erlenmeyer flask. Add 100 ml of 1× TBE gel running buffer. Mark the height of the level of liquid in the flask with a mark on the outside of the flask. Wad up a Chemwipe to make a loose plug. Place the plug loosely in the opening of the flask. Microwave the solution to dissolve all the agarose; swirl flask occasionally to mix. Check the height of the liquid level in the flask. If necessary, add sterile distilled water to bring the liquid back to the original level. Allow the dissolved agarose to cool to about 45–50°C before pouring the agarose into a gel casting tray containing the gel tray and comb to make the gel wells. Allow the gel to solidify. This can take 15–20 min, depending on the agarose concentration.
3. Once the gel is solidified, remove the comb from the gel. When removing the comb, do not tear holes in the wells formed. To aid in removing the comb, pour a small amount of 1× TBE running buffer around the comb before loosening the comb.
4. Transfer the gel on the gel tray to the gel box. Add 1× TBE running buffer to cover the gel completely.
5. Prepare DNA samples in microfuge tubes to be loaded on the gel. Add 3 μl of gel loading buffer for every 10 μl of DNA sample. Mix the sample and loading buffer completely. This can be done by vortexing the capped microfuge tube or inverting the capped microfuge tube several times to mix. Spin the tube for 5 sec in a microfuge to force droplets of solution clinging to the sides of the tube into the bottom of the tube.
6. Carefully add DNA to the gel wells. Record which samples are loaded in which lanes.
7. Connect the current and allow electrophoresis to occur at 100 V. *Which lead of the power supply goes to which connection on the gel box?* Remember that DNA is a polyanion.
8. When enough time has elapsed, turn off the power. Typically for a 1% agarose mini-gel, the conditions are 100 V for about 1 hr.
9. Measure the distance the two dyes migrated from the bottom of the gel wells.
10. Prepare ethidium bromide staining solution: Dilute 100–200 μl of stock 10 mg/ml EtBr (dissolved in H_2O) into 200 ml of H_2O.
11. Carefully move the gel to the ethidium bromide staining tray. Rock the tray. Check the gel on a UV transilluminator after 10 to 15 min to determine if the gel is well stained.

12. When gel is stained, photograph the gel using a UV transilluminator.

CAUTIONS
1. **High voltage is used in gel electrophoresis. Use great care!**
2. **Ethidium bromide is a mutagen, perhaps a carcinogen. Wear gloves when handling ethidium bromide and avoid spills.**
3. **The ultraviolet light from the UV transilluminator can burn skin and eyes! Wear protective eye goggles, gloves, and lab coat. Minimize exposure to UV light.**

NOTES

1. A more accurate way to replace the water lost from the flask of agarose while microwaving is to weigh the flask and contents before and after microwaving. Add sterile distilled water to the flask to bring the flask and contents back to the original weight.
2. Instead of covering a flask with a Chemwipe, a piece of aluminum foil may be used. Many microwaves can function properly with a small piece of aluminum foil covering the flask. This covering helps reduce the extent of evaporation.

CAUTION: **If using aluminum foil, do not swirl an overheated flask.**

3. If a microwave is not available, agarose can be melted in a boiling water bath over a Bunsen burner.
4. If gels are poured a long time before they will be used, the top of the solidified gel can be covered with 1× TBE running buffer to prevent the gel from drying out. Alternatively, a gel can be prepared in advance, wrapped well in plastic wrap, and refrigerated until needed. If storing a gel before use, be careful when handling the gel, so that the wells are not broken.
5. For some applications, such as the separation of DNA fragments by gel electrophoresis, nonnutrient agar instead of agarose may be used as the gel matrix (Santiago-Blay and Battaion, 1993). The cost of nonnutrient agar is about 22% of the cost of agarose. DNA separated in nonnutrient agar can be stained well with ethidium bromide, but not with methylene blue.

Analysis of Gels

For each gel determine the size of unknown fragments relative to λ phage markers by plotting the molecular weight versus the distance migrated on semilog paper (MW on the log scale).

1. Measure the distance each DNA fragment migrated from the origin (the bottom of gel wells). If a band is very wide, measure to the bottom of the band.
2. Plot the data from the size standards on semilog paper. Plot the λ *Hin*dIII (and λ *Hin*dIII and *Eco*RI, double digest, if used) data on semilog paper, with the molecular weight on the log scale and centimeters migrated on the linear scale.
3. Using the points just plotted, draw a standard curve. How can the best line be drawn? Points at very high or very low molecular weight may not fit the curve well. (See Russell, 1992, p. 455.)
4. Using this standard line, determine the sizes of the unknown DNA bands. Determine the size of each DNA fragment. Report data measurements in a table of % agarose, BPB dye ran (cm), and xylene cyanol dye ran (cm). Also report a table of

λ Hind fragment number (largest fragment is number 1)	Size (kb)	Distance migrated (cm)

Report the same information for λ (*Hin*dIII and *Eco*RI, if used) and for the unknown DNAs.

5. Using the standard curve made above and the distance the two dyes migrated, find the "apparent size" of the two dyes.
6. Compare the apparent sizes of the two dyes in different percentage agarose gels. Knowing the apparent size of BPB and xylene cyanol in different percentage agarose gels is very useful. The migration of these dyes, also known as tracking dyes, on gels is used to monitor the progress of the electrophoresis.

PROTOCOL 2.3
Large-Scale Plasmid Isolation Using Alkaline Lysis

This procedure is used to isolate a large quantity of DNA subcloning. If desired, the instructor can prepare this DNA in advance for the student. In an alkaline lysis, DNA is denatured by the high pH of Solution II. Solution III returns the lysis mixture to a neutral pH. The supercoiled plasmid DNA renatures relatively rapidly. (The two strands of the supercoiled covalently closed circular DNA remain intertwined.) The chromosomal DNA fragments do not renature as rapidly and are pelleted along with denatured protein. The supercoiled plasmid DNA remains in the supernatant solution.

Materials for Alkaline Lysis Plasmid Prep

- Solution I

  50 m*M* glucose

  25 m*M* Tris [tris(hydroxymethyl)aminomethane, Trizma base]

  10 m*M* EDTA (ethylenediaminetetraacetic acid, also called ethylenedinitrilo tetraacetic acid)

Adjust to pH 8.0 with NaOH or HCl.

- Solution II

  0.2 *M* NaOH

  1% SDS (sodium dodecyl sulfate, a detergent; also called lauryl sulfate)

Make Solution II fresh each time. For 100 ml of Solution II, mix 10 ml of 2 *M* NaOH, 5 ml of 20% SDS, 85 ml of sterile-distilled H_2O.

- Solution III

  3 *M* KOAc (potassium acetate), pH 5.5

Adjust to pH 5.5 with glacial acetic acid.

- PEG, 13%: polyethylene glycol; average molecular weight, 8000. Dissolve 1.3 g PEG in sterile H_2O. Adjust the final volume to 10 ml.
- L broth (See Chapter 1 for recipe.)
- Lysozyme (10 mg/ml in Solution I)
- Phenol:chloroform (1:1): Use phenol crystals, such as Baker reagent grade phenol crystals. When melted, the phenol should be colorless or have only a slight amount of color. Phenol which is colored deep yellow or deep pink should not be used.

Melt 1 pound phenol at 65°C. (This is about 440 ml.)

Add 440 ml of chloroform. Mix.

Add 26.4 g of NaCl. Mix.

Add 440 ml of 2 *M* Tris, pH 7.0. Mix.

Remove the aqueous (top) phase.

Add 440 ml of 50 m*M* Tris, pH 8.0.

The solution is now ready for use. Store at 4°C.

- 3 *M* NaOAc (sodium acetate)
- Ethanol
- RNase A (10 mg/ml, preboiled)
- Isopropanol saturated with 20× SSC (1× SSC is 0.15 *M* NaCl, 0.015 *M* Na citrate)
- 1 m*M* Tris–HCl, 0.1 m*M* EDTA, pH 8.0

Procedure

1. Using sterile technique, place 2 ml L broth with appropriate antibiotics for the strain to be grown in a sterile culture tube. Inoculate the tube with the bacterial strain from which plasmid DNA will be isolated. Incubate the tube with shaking at 37°C for 6–16 hr.
2. Use the 2-ml culture grown in step 1 to inoculate a larger volume of L broth containing the appropriate antibiotic. Use volumes of 100 ml to 1 liter, depending on the amount of plasmid to be isolated. Incubate the inoculated flask for 8–16 hr at 37°C with vigorous shaking.
3. Harvest the *E. coli* cells by centrifugation at 8000 rpm for 5 min. Decant the medium. The medium should be decanted into a flask with bleach before the used medium is discarded into the sink. *Note:* The pellet of cells may be frozen at −20°C at this stage.
4. Resuspend the cells in 7 ml Solution I. Pipet the solution up and down to resuspend the cells completely.
5. Transfer the resuspended cells to a sterile 40-ml screw-capped centrifuge tube (an Oakridge tube).
6. Dissolve 10 mg lysozyme in 1 ml Solution I.
7. Add 1 ml of lysozyme in Solution I to the cell suspension. Mix well by inverting the capped centrifuge tube. Incubate at room temperature for 10 min.
8. Add 16 ml Solution II to the cell suspension. Mix Solution II into the cell suspension gently by inverting the capped centrifuge tube.
9. Incubate the tube on ice for 5 min.
10. Add 12 ml *ice-cold* Solution III. Mix Solution III into the cell suspension gently by inverting the capped centrifuge tube.
11. Incubate the tube on ice for at least 15 min.
12. Centrifuge the tube at 12,000 rpm for 5 min.
13. Carefully avoiding the white precipitate, transfer the supernatant solution into a 250-ml centrifuge bottle. To avoid transferring the white precipitate, the supernatant solution can be poured through three or four layers of sterile cheesecloth.
14. Add 0.6 volume of cold isopropanol to precipitate the nucleic acids. Invert the capped centrifuge bottle several times to mix the isopropanol and solution completely.
15. Centrifuge the bottle at 8000 rpm for 5 min.
16. Drain the pellet completely. If necessary to dry the pellet better, place the bottle in a desiccator chamber under vacuum for 5 to 10 min to remove the last traces of isopropanol. Resuspend the precipitant in 5 ml sterile-distilled H_2O, and transfer the solution to a 15-ml corex centrifuge tube.

17. Add 0.5 ml 3 M NaOAc and 50 μl RNase A (10 mg/ml, preboiled). Mix well. Incubate at 37°C for 15 to 30 min.

CAUTION: Use great care when handling phenol. Phenol is caustic and toxic. Wear gloves, safety glasses or goggles, and a laboratory coat. Dispense the phenol in the area designated for working with phenol, ideally, in a fume hood. Dispose of waste phenol in a designated waste container. Use caution when mixing the phases in a phenol extraction.

18. Add 5 ml phenol:chloroform. Mix well. Centrifuge at 5000 rpm for 5 min to separate phases completely. There will be a layer of white denatured proteins at the interphase between the aqueous (top) phase and the phenol (bottom) phase. Carefully remove and save the aqueous phase in a clean 15-ml corex tube. Avoid any of the white material at the interphase between the two phases. Repeat the phenol:chloroform extraction procedure two or three times, until the interface between the two phases is clear.
19. Add 10 ml cold ethanol. Mix well.

PEG is used to remove RNA from the plasmid DNA. Under the conditions used, the plasmid DNA precipitates in the presence of PEG, while RNA remains in solution. If a PEG precipitation is to be done:

20. Centrifuge at 10,000 rpm for 10 min to pellet the nucleic acid. Decant the ethanol and dry the pellet completely. The pellet can be dried under vacuum. Alternatively, the pellet can be allowed to air-dry.
21. Dissolve the pellet in 367 μl of H_2O and transfer the solution to a sterile microfuge tube. Add 133 μl of 3 M NaCl and mix. Add 500 μl of freshly prepared 13% PEG (av. mol. wt. 8000) and mix. Incubate in an ice water bath for 1 hr.
22. Centrifuge in a microfuge for 10 min at maximum speed. Discard the supernatant solution.
23. Wash the pellet with 50 μl of cold 70% ethanol. Centrifuge the sample for 5 min. Decant the ethanol. Dry the pellet in a Speed-vac (a microfuge under vaccum) under vacuum.
24. Resuspend the DNA in the appropriate volume of 1 mM Tris and 0.1 mM EDTA, pH 8.0. If 1 liter of cells was used, for a high-copy-number plasmid, such as pUC and pBR plasmids, use 0.5 ml. For a low-copy-number plasmid, such as pRK plasmids, use 0.1 ml.
25. Label the microfuge tube with the date and name of the plasmid. Store the DNA sample at 4°C (in the refrigerator).

If a PEG precipitation will not be done:

20. Centrifuge at 10,000 rpm for 10 min to pellet the nucleic acid. Decant the ethanol.
21. Wash the pellet with 50 μl of cold 70% ethanol. Centrifuge the sample

for 5 min. Decant the ethanol and dry the pellet completely. The pellet can be dried under vacuum. Alternatively, the pellet can be allowed to air-dry.

22. Resuspend the DNA in the appropriate volume of 1 m*M* Tris and 0.1 m*M* EDTA, pH 8.0. If 1 liter of cells was used, for a high-copy-number plasmid, such as pUC and pBR plasmids, use 0.5 ml. For a low-copy-number plasmid, such as pRK plasmids, use 0.1 ml.
23. Transfer the dissolved DNA to a sterile microfuge tube. Label the microfuge tube with the date and name of the plasmid. Store the DNA sample at 4°C (in the refrigerator).

If a cesium chloride–ethidium bromide density gradient will be used:

Do the protocol above through step 15. Then proceed as described below:

16. Drain the pellet completely. Place the bottle in a desiccator chamber under vacuum for 5 to 10 min to dry the pellet. Dissolve the pellet in 5 ml 1 m*M* Tris and 0.1 m*M* EDTA, pH 8.0. Measure the volume of solution while transferring the solution into a 15-ml corex centrifuge tube.

CAUTION: Wear gloves when handling CsCl.

17. Add 1.1 g CsCl per milliliter of solution measured in step 16. Make sure the CsCl dissolves completely. If desired, use a refractometer to check that the refractive index is $\eta = 1.3925$.

CAUTION: Use great care when handling ethidium bromide. Ethidium bromide is toxic and may be carcinogenic. It is mutagenic in the Ames test. Wear gloves and a laboratory coat when handling ethidium bromide.

18. Add 0.5 ml of 10 mg/ml ethidium bromide solution. *Once the ethidium bromide has been added, keep the DNA solution in low light.* DNA may be nicked in the presence of ethidium bromide and light. A nick in a supercoiled plasmid DNA molecule will cause the now relaxed plasmid to band with bacterial chromosomal DNA in the CsCl–EtBr gradient. Thus exposure to light could decrease the yield of plasmid DNA obtained.
19. If debris is observed in the solution after the addition of CsCl and ethidium bromide, centrifuge the solution at 10,000 rpm for 10 min to pellet the debris.
20. Put the solution into VTi 65 vertical rotor ultracentrifuge tubes. Use a heat sealer to seal the tubes.
21. Centrifuge the tubes for 16 hr at 45,000 rpm at 15°C in an ultracentrifuge.

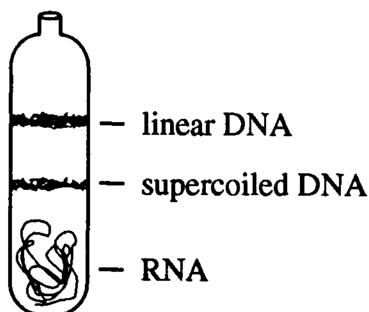


Figure 2.10 Sketch of an ultracentrifuge tube showing a CsCl–EtBr density gradient to isolate supercoiled plasmid DNA from linear plasmid or chromosomal DNA and RNA.

CAUTION: Wear UV protective goggles when working around UV light. Wear gloves when handling the centrifuge tubes with ethidium bromide in them. Cover the work area with an absorbent material. Have a container at hand for waste CsCl–EtBr. Dispose of waste CsCl–EtBr according to the prescribed procedure of your institution.

22. After the centrifugation run, carefully remove the tubes from the ultracentrifuge rotor. Do not disturb the gradient in the tube. Observe the centrifuge tube under long-wavelength ultraviolet light. Identify the upper (chromosomal) and lower (supercoiled plasmid) bands. Figure 2.10 shows the positions of these DNAs in a quick-seal centrifuge tube after the CsCl–EtBr density gradient has been centrifuged. The supercoiled plasmid DNA binds a limited amount of EtBr, has a limited density decrease due to the presence of EtBr, and bands at a greater density (lower in the centrifuge tube) than does chromosomal DNA.

    As each EtBr molecule intercalates into DNA, the overall buoyant density of the DNA molecule decreases. Supercoiled DNA binds EtBr molecules until the DNA can no longer be twisted. Linear DNA molecules are able to rotate about the axis of the helix as EtBr binds. Therefore, a linear DNA molecule can bind more EtBr than can a supercoiled DNA molecule and has a greater density decrease. (See Rodriguez and Tait, 1983, p. 39.)

CAUTION: Use great care when puncturing the ultracentrifuge tube with a syringe needle.

23. Clamp the ultracentrifuge tube securely onto a ring stand.

24. Carefully use a 21-gauge needle to poke a hole in the top of the ultracentrifuge tube. The hole will break the seal and allow air into the tube when the plasmid band is drawn out.
25. Attach a 21-gauge needle to a 3-ml syringe. Pull the plunger out of the syringe to break the seal and reinsert the plunger in the syringe.
26. Observe the bands in the tube by UV light. Position the opening of the 21-gauge needle attached to the syringe so that the widest part of the needle opening is pointed up. Carefully insert the needle attached to the syringe into the centrifuge tube slightly below the plasmid band. Slowly pull the plasmid band out of the centrifuge tube and into the syringe. Be careful to avoid any of the upper band.
27. Remove the needle from the centrifuge tube and cover the hole with a gloved finger while moving the tube into the container for waste CsCl–EtBr.
28. Put the plasmid just withdrawn from the centrifuge tube into a 15-ml corex tube.
29. Extract the ethidium bromide from the sample: Add an equal volume of isopropanol saturated with 20× SSC. Mix thoroughly. Let the phases separate. Discard the upper red layer into a bottle for organic waste. Repeat the procedure until the lower layer is clear.
30. Precipitate the DNA with ethanol: Add an equal volume of 1 m*M* Tris and 0.1 m*M* EDTA, pH 8.0, and 2 volumes of 100% ethanol. Mix completely.
31. Store the DNA in ethanol overnight at −20°C.
32. Collect the DNA by centrifugation at 10,000 rpm for 15 min. Decant the ethanol.
33. Rinse the sample with 2 ml of 70% ethanol. Centrifuge at 5000 rpm for 5 min. Decant the alcohol. Dry the pellet completely. (This can be done by applying a vacuum or allowing the sample to air-dry.)
34. Redissolve the DNA pellet in the appropriate volume of 1 m*M* Tris and 0.1 m*M* EDTA, pH 8.0. If 1 liter of cells was used, for a high-copy-number plasmid, such as pUC and pBR plasmids, use 0.5 ml. For a low-copy-number plasmid, such as pRK plasmids, use 0.1 ml.
35. Transfer the dissolved DNA to a sterile microfuge tube. Label the microfuge tube with the date and name of the plasmid. Store the DNA sample at 4°C (in the refrigerator).

NOTES

1. Terrific broth (TB) may be used in place of L broth. The recipe for TB (Tartof and Hobbs, 1987) is:

For 1 liter TB
Add 100 ml sterile 0.17 *M* KH_2PO_4
0.72 *M* K_2HPO_4
to 900 ml sterile 12 g Bacto-tryptone
24 g Bacto-yeast extract
4.0 ml glycerol

TB allows the *E. coli* culture to grow to a higher cell density and therefore can give higher plasmid yields.

2. A flask that is considerably larger than the volume of medium should be used to ensure good aeration of the culture during growth. For example, for 100 ml of medium, use a 500-ml Erlenmeyer flask; for 1 liter of medium, use a 3-liter culture flask.
3. If there is not enough time to finish the whole procedure in one laboratory period, the procedure can be stopped at an alcohol precipitation step. After the addition of isopropanol (step 14) or ethanol (step 19), store the sample at −20°C until the next laboratory period.
4. Often, even after RNase treatment, RNA will be found in the plasmid DNA isolated with this procedure. The presence of RNA does not interfere with most further procedures, such as restriction enzyme digestions, cloning, or ligation (see Davis *et al.*, 1986, p. 104). To remove RNA, the PEG treatment can be used or the DNA can be purified in a cesium chloride–ethidium bromide gradient.
5. In the ultracentrifuge tube used for a CsCl–EtBr density banding to separate chromosomal from supercoiled plasmid DNA, protein is a dark purple color and is seen at the top of the tube. RNA is a dark red color and is found in a pellet at the bottom of the centrifuge tube (Davis *et al.*, 1986).
6. There are alternative procedures for very rapid isolation of plasmid DNA. Such procedures are generally more expensive and the materials are available in kit form. For example, Qiagen sells a resin that is useful for rapid plasmid DNA isolation. In such a procedure, bacterial cells are lysed by an alkaline method. The bacterial lysate is put over a column containing the Qiagen ion-exchange resin. RNA and proteins are washed off the column first, and then the purified plasmid DNA is eluted from the column.

Determination of DNA Concentration

The concentration of DNA can be determined spectrophotometrically. Dilute DNA in H_2O or a buffer. (Be sure to use the same buffers as a blank for the spectrophotometer.) Read the absorbance at 260 and 320 nm. In a 1-cm-wide cuvette, 50 μg/ml double-stranded DNA has an

absorbance at 260 nm of 1.00. Note that for single-stranded DNA, 1 A_{260} unit is 33 μg/ml. For single-stranded RNA, 1 A_{260} unit is 40 μg/ml. This difference is because the more ordered the structure, the less light absorbed by the bases. Single-stranded nucleic acids are less ordered than double-stranded DNAs and absorb more light. Double-stranded DNA is said to be hypochromic compared to single-stranded nucleic acids. The A_{320} is subtracted from the A_{260} to correct for the light-scattering of dust or cell debris.

$$\text{Concentration of DNA } (\mu\text{g/ml}) = (A_{260} - A_{320}) \times \frac{50\ \mu\text{g/ml}}{1\ \text{absorbance unit}} \times \text{dilution.}$$

Alternatively, the concentration of DNA can be estimated by comparing the intensity of bands of the unknown DNA with that of similar-sized DNAs of known concentration on an ethidium bromide-stained gel or spotted onto a membrane that is then illuminated under UV light.

What other macromolecules might interfere with our DNA concentration determination? Examining the entire spectrum of a DNA sample is often useful to determine the presence of other molecules. Take readings every 10 nm from 230 to 320 nm.

How could RNA be eliminated?

Peak absorbance (nm)	Substance absorbing at that wavelength
230	EDTA, polysaccharides, ethanol
260	DNA, RNA
270	Phenol
280	Proteins
320	Cell debris scatters light

A very pure sample of double-stranded DNA that is free of proteins will have an A_{260} to A_{280} ratio of about 1.8. See Hengen (1994) for a discussion of spectrophotometric, fluorometric, and ethidium bromide methods of determining DNA concentration.

PROTOCOL 2.4: Recombinant DNA Cloning

Schedule for Recombinant DNA Cloning Experiment

DAY 1 (2–3 hr): Cut insert and vector DNA, check an aliquot of digestion reaction on gel, inactivate restriction enzyme, precipitate the DNAs together.

DAY 2 (1/2 to 1 hr): Set up ligation of samples.

DAY 3 (2–3 hr): Transform ligated DNA into *E. coli*.

DAY 4 (1/2 to 1 hr): Check results, streak white colonies from $L_{amp\ X\text{-}gal}$ plates. Inoculate 2-ml cultures for mini-prep analysis of insert DNA.

This recombinant DNA cloning experiment uses a large DNA cosmid that will be subcloned. Subclones will be used as hybridization probes in Chapter 3. The procedures can readily be applied to other DNAs to be cloned.

Restriction Digestions for Cloning

1. Set up restriction endonuclease digestions of vector DNA and insert DNA. Different groups will clone with different restriction endonucleases. Use a restriction endonuclease that has a unique site within the pUC19 cloning vector. Because the cosmid is a large DNA (about 40 kb), there are many restriction endonuclease sites for the enzymes used within the cosmid. Remember to check that the appropriate 10× buffer for the restriction endonuclease is used.
   a. Set up restriction endonuclease digestion of the DNA to be cloned, called insert DNA. An example is given below.

      For insert DNA, cosmid 203 DNA, *labeled "I" for insert* DNA—in a microfuge tube, carefully place the following:
      - 3.5 μg = *X* μl DNA; this will vary, depending on the concentration of that particular DNA preparation. In this example 3.5 μg = 20 μl of cosmid 203 DNA.
      - 22 μl of sterile distilled H_2O; add water to bring the sample to the desired final volume.
      - 5 μl of 10× restriction endonuclease buffer for the restriction endonuclease to be used. The volume of 10× buffer used must be 1/10 of the final reaction volume.
      - 3 μl of restriction endonuclease (30 units of enzyme). Add the enzyme last. Mix gently. Keep enzyme on ice. Return to −20°C freezer as soon as possible.

        The total volume of reaction is 50 μl. The concentration of DNA is now 0.07 μg/μl.
   b. Set up the digestion of the vector DNA, *labeled "V"*. For the vector DNA, pUC19:
      - 1.0 μg = 2 μl DNA.
      - 12 μl H_2O; add sterile distilled water.
      - 3 μl 10× restriction endonuclease buffer. The vector and insert DNA are cut with the same restriction endonuclease so that they will have "compatible sticky ends."

- 3 μl restriction endonuclease.

  The total volume of reaction is 30 μl. The concentration of this DNA is now 0.033 μg/μl.

2. Mix each reaction by inverting the closed microfuge tube several times and spinning the tube for a few seconds in a microfuge to remove droplets of solution from the sides of the microfuge tube.
3. Incubate the restriction digestion reactions (vector digestion and insert digestion) in a 37°C water bath for approximately 1 hr.
4. After approximately 30 min of incubation of the restriction digestions, remove a small aliquot from each tube into microfuge tubes. Be sure to return the rest of the restriction digestions to the 37°C water bath to continue incubating for the full hour. For example, for the insert restriction digestion, take 7 μl of the digestion reaction; for the vector reaction, take 15 μl. To each aliquot, add 3–5 μl of gel loading dye.
5. Check the extent of restriction endonuclease digestion by gel electrophoresis. Load the samples in the wells of an 0.8% agarose mini-gel. Set the power supply at 100–125 V. Watch the gel; once the blue dye front has migrated about 1/4 the length of the gel, stop the electrophoresis and stain the gel in ethidium bromide. (A gel containing ethidium bromide could be run. However, the student must be extremely careful about handling the large volume of gel buffer that contains ethidium. Also, the presence of ethidium bromide slows the rate of migration of a DNA fragment approximately 10–15%. Occasionally, when a gel with ethidium bromide is run, the staining does not work well and the gel must be restained anyway. Generally, it is simpler to stain the gel after electrophoresis. Typically, 10 or 15 min of staining is sufficient.)
6. Check the gel to see if the restriction digestions are complete. If digestion is not complete, add an additional aliquot of restriction endonuclease and incubate for an additional 30 min. Generally, an excess of restriction endonuclease should be used to ensure complete cutting in a reasonable period of time. See step 1. A large amount of vector DNA (approximately 0.5 μg) is run on a gel to make it easier to detect any uncut vector. Because uncut vector transforms more efficiently than the recombinant molecules and because nothing can be cloned into an uncut vector, it is essential that the cutting of the vector be complete.
7. After determining that the digestions are complete, inactivate the restriction enzymes before ligating the two DNAs together. See the section on stopping a restriction digestion. The simplest way to inactivate a restriction endonuclease is by a heat treatment at 65°C for 10–20 min. Check the information from the supplier of the restriction enzyme to determine if the enzyme used can be heat inactivated (see Table 2.1). An alternative method for inactivating restriction endonucleases is to use diethylpyrocarbonate (DEPC).

CAUTION: Diethylpyrocarbonate is toxic. The container of DEPC may be under pressure. Wear gloves when handling DEPC. Use a fume hood. Do not inhale the vapors. Read the supplier's information about safety precautions for the use of DEPC.

Dilute the DEPC liquid from the reagent bottle: Place 2 μl of concentrated DEPC into 198 μl of 100% ethanol. Mix well. Add 1/10 volume of the diluted DEPC to each restriction digestion microfuge tube. Vortex to mix well. Incubate the tubes at 65°C for 10 min. At 65°C DEPC breaks down into CO_2 and ethanol.

CAUTION: Use only the amount of DEPC prescribed. A large excess of DEPC may not be destroyed by the 65°C treatment. If too much DEPC is used, it may interfere with the ligation reaction.

8. Once the cutting is complete and the restriction enzymes have been inactivated, combine the vector and insert DNAs.
   - Add 0.1 μg of vector to all of the remaining insert DNA. This is a ratio of vector to insert of 0.1 μg vector to 3 μg insert DNA (1:30 ratio), which has been found empirically useful in cloning cosmid fragments. Depending on the sample volume, it may not be necessary to precipitate the sample. In this case, it is necessary. Add 3 μl of vector to all of the insert DNA.
   - Add 1/10 volume of 3 *M* NaOAc
   - Add 2 volumes of cold ethanol.
9. If a ligation reaction is to be set up immediately, place the precipitated DNAs in a dry ice/ethanol bath for 10 min. Thaw the sample and centrifuge in a microfuge for 10 min. Then proceed with setting up the ligation, steps 11–14.
10. If a ligation reaction will be set up at a later time, store the precipitated DNAs in ethanol at −20°C. Centrifuge the sample for 10 min in a microfuge.

Ligation

11. Decant the ethanol. Wash the pellet with 70% ethanol. Centrifuge the sample again. Decant the 70% ethanol. Dry the sample completely in a Speed-vac.
12. To the dry sample, add 7 μl sterile-distilled water. Resuspend the DNA completely. Add 2 μl 5× ligation buffer. (5× ligation buffer is 0.25 *M* Tris, pH 7.6; 50 m*M* $MgCl_2$; 5 m*M* ATP; 5 m*M* DTT; 25% (w/v) polyethylene glycol 8000; from Gibco/BRL.) Add 1 μl (4–6 units/μl) ligase. Mix thoroughly using the Pipetman tip. Let the ligation reaction incubate at 16°C overnight, at room temperature overnight, or at 37°C for 1 hr.

13. To check the extent of ligation, remove a 1-μl aliquot of the ligation sample before the ligase is added. Place the aliquot in 10 μl of water and 3 μl of gel loading dye. This is a ligation time "zero" sample.
14. At the end of the ligation, take an additional 1-μl aliquot of the ligation sample. Place the aliquot in 10 μl of water and 3 μl of gel loading dye. This is a ligation time "infinity" sample.

Bacterial Transformation

1. When the ligation reaction is over, proceed with the bacterial transformation procedure. Alternatively, the ligated sample can be stored at −20°C until the transformation. During the waiting periods of the transformation protocol, check the ligation reaction by running the time zero and time infinity ligation samples and a molecular weight standard on an 0.8% agarose gel. It is expected that at least some lower molecular weight bands on the gel will disappear and that higher molecular weight bands or smears will appear in the sample that was ligated.
2. Let frozen competent DH5α *E. coli* cells thaw on ice. Protocol 2.5a describes the preparation of frozen competent *E. coli* cells.
3. Add 92 μl of ice-cold 0.1 *M* $CaCl_2$ to the remaining 8 μl of the ligation reaction. (Two 1-μl aliquots were removed to check the ligation reaction products on a gel.) Mix using a micropipettor. Transfer the DNA and $CaCl_2$ solution to a thin-walled 5-ml clear plastic tube on ice.
4. Gently add 200-μl thawed competent cells. Let the cells and DNA incubate on ice for 45–60 min.
5. Heat shock the cells by taking the tube rapidly from the ice to a 42°C water bath. Keep the tube at 42°C for 2 min.
6. Using aseptic technique, add 2.7 ml prewarmed (37°C) L broth. (Transformation protocols vary at this step. In some protocols the sample is put back on ice for a few minutes after the heat shock.)
7. Incubate the cells at 37°C for 45–60 min. (Transformation protocols again vary. Some protocols specify shaking the cells during the incubation at 37°C; other protocols do not. The cells are somewhat fragile at this point and should be handled gently.) This incubation period allows time for the plasmids that have been taken up to become established and the antibiotic resistance marker to be expressed. (When selecting for amp^r, the incubation period is not essential.)
8. Plate 0.1-ml aliquots of the transformed *E. coli* cells on $L_{amp\ X\text{-}gal}$ plates. (Ampicillin in the plates is at 50–100 μg/ml; X-gal is at 40 μg/ml. To make the plates, the appropriate amount of X-gal is dissolved in a minimal amount of dimethylformamide.

CAUTION: When handling DMF, use a glass, not a plastic, tube for the DMF.

Alternatively, a stock of X-gal can be made. Make a 20 mg/ml solution of X-gal in DMF. Store the solution wrapped in aluminum foil to protect it from the light, at −20°C.) When plating out cells, place an aliquot of cells in the center of the agar on the plate. Dip a bent glass rod (called a "hockey stick") into a beaker of alcohol. Remove the rod, let excess alcohol drain, and pass the rod through a Bunsen burner flame to ignite the alcohol. Let the rod cool and then use the rod to spread the cells evenly across the surface of the agar.

CAUTION: Be careful when flaming the bent glass rod! Do not set fire to the alcohol in the beaker. Have a beaker large enough to cover the alcohol containing beaker nearby. In the event of an alcohol fire, the larger beaker can be placed over the burning beaker to smother the fire. Review how to respond to a fire before beginning this part of the lab.

Transformed cells that were not plated may be stored at 4°C until the next day. More cells may be plated out the next day if needed.

9. Incubate the plates, inverted, at 37°C for 16–24 hr. Colonies containing the uninterrupted pUC vector will be blue on $L_{amp\ X\text{-}gal}$ plates. Colonies containing a pUC vector with a piece of DNA interrupting the gene for the α-donor peptide will not make β-galactosidase and will be white. Note that L_{amp} plates kept at 37°C for more than 24 hr may show growth of satellite colonies around an ampicillin-resistant colony because the ampicillin-resistant colony has degraded the antibiotic in the medium around the colony. Satellite colonies are not ampicillin resistant. See Figure 2.11 and the description of ampicillin in Appendix 4.
10. When performing a transformation, include several controls:
    a. Supercoiled, uncut vector DNA. Set up a transformation as described above using 0.1 μg of supercoiled vector DNA. (This is the same amount of vector that was used in the cloning sample.) This is expected to give a large number of transformants. Plate 0.1-ml aliquots and 0.1-ml aliquots of 1 : 10 and 1 : 100 dilutions.
    b. Linear, cut vector DNA. Set up as above.
    c. Cut and religated vector DNA. Set up as above.
    d. A viable cell count. After the transformation procedure, plate serial dilutions of one of the control transformations onto L plates (no antibiotic in the plates). Based on studies of transformation frequencies summarized in the introductory material of this chapter, what results might be expected for each of the controls?

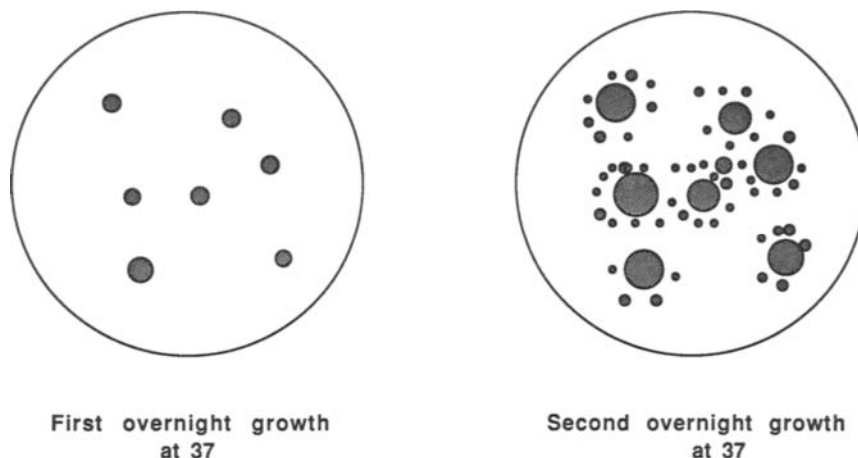


Figure 2.11 Sketch of the growth of bacterial colonies on L_{amp} plates after the bacteria were transformed with an ampicillin-resistant plasmid. After one overnight growth period at 37°C, only those bacteria than contain the ampicillin-resistant plasmid are able to grow on the ampicillin-containing plate. However, after an additional 24 hr at 37°C, more colonies are able to grow on the plate. The original ampicillin-resistant bacterial colonies are producing β-lactamase, the enzyme that inactivates ampicillin. β-Lactamase is secreted into the medium, where it inactivates the ampicillin in the vicinity of an ampicillin-resistant colony. This allows ampicillin-sensitive colonies to grow. Colonies that grow near an ampicillin-resistant colony after a long incubation are called satellite colonies.

PROTOCOL 2.5a: Competent *Escherichia coli* Cells

Preparing and Freezing Competent Cells

Materials

- Prepare in advance for making competent *E. coli* cells

1. Take *E. coli* strain out of permanents by streaking from vial onto a plate using a sterile toothpick. Check that strain has the appropriate antibiotic resistance markers.
2. Autoclave 500 ml of L broth in a 2-liter culture flask.
3. Autoclave and prechill the following:
   a. 125 ml 0.1 *M* $MgCl_2$ (2.54 g $MgCl_2$ in water to 125 ml)
   b. 125 ml 0.1 M $CaCl_2$ (prepare 1.66 g $CaCl_2$ in water to 150 ml; use some in step c below)
   c. 21.5 ml 0.1 *M* $CaCl_2$ plus 3.5 ml glycerol
   d. A canister of 10-ml pipets

Procedure

Modified from Morrison (1979):

1. Set up an inoculum of cells in 20 ml of L broth plus appropriate antibiotics. Grow the culture overnight with shaking at 37°C.
2. Inoculate 500 ml of L broth plus appropriate antibiotics with 1 ml of the overnight culture and grow with vigorous shaking until the A_{550} (absorbance at 500 nm) is between 0.5 and 0.8. Typically, this takes about 5 hr.
3. When the cells have reached the correct density, rapidly cool the cells by vigorously swirling the flask in an ice water bath for 3 min.
4. Pour the chilled cells into two 250-ml centrifuge bottles.
5. Centrifuge at 7000 rpm for 5 min in a Sorvall GSA rotor.
6. After centrifugation, drain the supernatant fluid from the pellets. Combine the two pellets by resuspending the two pellets in a total volume of 125 ml ice-cold 0.1 *M* $MgCl_2$.
7. Centrifuge at 7000 rpm for 5 min in a Sorvall GSA rotor.
8. Drain the pellet. Resuspend the pellet in 125 ml cold 0.1 *M* $CaCl_2$. Put on ice for 20 min.
9. Centrifuge at 7000 rpm for 5 min in a Sorvall GSA rotor.
10. Drain the pellet. Resuspend the pellet in a 21.5-ml sterile solution of 0.1 *M* $CaCl_2$ and 3.5 ml glycerol.
11. Set up a dry ice–ethanol bath. Prechill a microfuge rack and twenty-five 1.5-ml microfuge tubes in the dry ice–ethanol bath.
12. Working rapidly, and using the prechilled 10-ml pipets, dispense 0.5-ml aliquots of the resuspended cells into the prechilled 1.5-ml microfuge tubes in the dry ice–ethanol bath.
13. Leave tubes in dry ice–ethanol bath until the cell suspension is frozen solid. Store the frozen competent cells at −70°C.

PROTOCOL 2.5b: Preparing Fresh Competent *Escherichia coli* Cells for Transformation

1. Inoculate a tube containing 2 ml of L broth with the *E. coli* strain to be used in the transformation. Grow the culture overnight at 37°C with shaking.
2. Dilute the overnight culture 1:40 into L broth prewarmed to 37°C. Grow the culture with shaking at 37°C until the culture reaches an absorbance measured at 600 nm of 0.5–0.6. For example, add 1 ml of

an overnight culture into 39 ml of prewarmed L broth in a 250-ml side-arm flask. Use the side-arm to monitor the absorbance of the culture without removing cells from the flask. Generally, the culture will reach an A_{600} = 0.5–0.6 in 1.5 to 2 hr.

3. When the bacterial cells have reached the correct density, remove the culture flask from 37°C and place the flask on ice for 5 min.

NOTE

During all the following manipulations, keep the cells cold by using a refrigerated centrifuge and keeping the tube with cells on ice while resuspending the cell pellet.

4. Pour the cells into a sterile screw-capped centrifuge tube, such as an Oakridge tube. Centrifuge the tube in a Sorvall preparative centrifuge set at 4°C at 7000 rpm for 5 min.
5. After centrifugation, decant the medium from the cell pellet. Resuspend the cell pellet from the original 40-ml culture in 20 ml ice-cold 10 m*M* NaCl. Gently resuspend the cells by gently pipetting the solution up and down onto the cell pellet.
6. Centrifuge the cells at 7000 rpm for 5 min.
7. Decant the supernatant solution. Gently resuspend the cell pellet in ice-cold 30 m*M* $CaCl_2$.
8. Place the cells on ice for 20 min.
9. After the 20-min incubation on ice, centrifuge the cells at 7000 rpm for 5 min.
10. Decant the supernatant fluid from the cell pellet. Gently resuspend the cell pellet in 1 ml ice-cold 30 m*M* $CaCl_2$. Keep the cells on ice until used in a transformation.

Using Competent Cells for Transformation

1. In a sterile, plastic-capped 4-ml tube combine the DNA to be transformed and enough 30 m*M* $CaCl_2$ to bring the volume of the solution to 100 μl. For example, if a ligated DNA is to be transformed, mix 10 μl of a ligation reaction with 90 μl of 30 m*M* $CaCl_2$. Chill the tube with the DNA sample on ice for 5 min.
2. Add the 200 μl of competent cells prepared earlier. Tightly close the cap of the plastic tube and invert the tube to mix the DNA and cells gently.
3. Place the tube with cells and DNA on ice for 1 hr.
4. Rapidly take the tube with cells and DNA from ice and plunge the tube

into a 42°C water bath for 2 min. Time the 2 min exactly. It is during this heat shock step that the DNA is taken into the bacterial cells.

5. Remove the tube from 42°C and place it on a rack at room temperature. Using sterile technique, add 2.7 ml L broth prewarmed to 37°C. Invert the capped tube twice to mix the cells and L broth.
6. Incubate the tube at 37°C for 1 hr. This incubation allows time for the incoming plasmid to be established in the bacterial cell and time for the antibiotic resistance marker of the plasmid to be expressed in the bacterial host cell.
7. Spread 100- or 200-μl aliquots of the transformed cells on the appropriate selective medium.
8. Incubate the plates, inverted, at 37°C overnight.

PROTOCOL 2.5c:
A Rapid Colony Transformation Procedure

Materials

- TFB buffer (transformation buffer):
  10 m*M* Mes–KCl, pH 6.2 (morpholinoethanesulfonic acid)
  100 m*M* RbCl or KCl
  45 m*M* $MnCl_2$
  10 m*M* $CaCl_2$
  3 m*M* $HACoCl_3$ (hexamine cobalt(III) chloride)

Filter through a 0.22-μm membrane to sterilize. Store TFB buffer at 4°C.

Procedure

This procedure easily produces low-efficiency competent *E. coli* cells and is useful for the reintroduction of isolated plasmid DNA into an *E. coli* strain. This protocol is based on the method of Hanahan (1983).

1. Streak out the recipient *E. coli* strain on an L agar plate. Incubate the plate overnight at 37°C.
2. Place 200 μl of TFB buffer in a 4-ml sterile plastic tube.
3. Carefully pick two to four colonies from a freshly grown plate. Resuspend the colonies completely in TFB buffer.
4. Incubate the resuspended *E. coli* cells on ice about 10 min.
5. Add approximately 10 μl of DNA.
6. Incubate the cells and DNA on ice for 10 min.
7. Heat shock the cells by placing the plastic tube at 42°C for 2 min.

8. Add approximately 2.8 ml of L broth prewarmed to 37°C.
9. Incubate the cells for about 15 min at 37°C.
10. Plate 0.1-ml aliquots on selective plates.

PROTOCOL 2.6a: Boiling Mini-Prep Isolation of Plasmid DNA

DNA from the recombinant DNA clones isolated in Protocol 2.4 can be obtained by Protocol 2.6a or 2.6b. Isolated mini-prep DNA is cut with the same enzyme that was used in recombinant DNA cloning. Examine these cut DNAs by gel electrophoresis. Include on the gel a molecular weight standard and an aliquot of the DNA that was subcloned (the insert DNA), cut with the same restriction enzyme. By comparing the size of the fragments of the clones and the original DNA, the DNA fragments that were subcloned can be identified.

1. Using a sterile toothpick, pick a bacterial colony from an agar plate. Spot the colony onto a reference plate. Inoculate 1 ml L broth plus appropriate antibiotics in a test tube or 1.5-ml microfuge tube. Make sure the tubes are numbered to correspond to the reference plate.
2. Shake the culture tubes vigorously at 37°C overnight.
3. If necessary, transfer the cultures to numbered microfuge tubes. The bacterial cultures can be poured into the microfuge tubes. Centrifuge the cultures for 1 min in a microfuge to pellet the bacteria. Decant and discard the supernatant medium. *Note:* The supernatant medium should be collected in a beaker and treated with bleach before it is discarded.
4. Resuspend the bacterial pellet in 200 μl of lysis buffer. Use a pipet aid to resuspend the cells. Do not vortex the cells.
   - Lysis buffer:

     8% sucrose

     0.5% Triton X-100

     50 m*M* EDTA

     10 m*M* Tris, pH 8.0

     plus 5 mg/ml lysozyme added just before use.
5. Place the tube in a boiling water bath for 40 sec.
6. Centrifuge the tube in a microfuge for 5 min.
7. (Optional) If the uncut plasmid is not to be checked, skip step 7; go directly to step 8. If the uncut plasmid DNA is to be checked to see if an insert is present, remove 5 μl of the supernatant fluid. Mix the aliquot of supernatant fluid with 5 μl of gel electrophoresis loading

buffer and load the sample and a sample of uncut cloning vector on an agarose gel. This rapid check can be used to detect the addition of an insert which is 10% the size of the vector or greater, up to a total plasmid size of about 8 kb.

8. Carefully transfer the supernatant fluid to a new microfuge tube. Do not disturb the pellet. Add 21 μl of 3 *M* NaOAc and 250 μl of cold isopropanol. Mix the solutions thoroughly and set the tubes on ice for an hour.
9. Pellet the DNA by centrifugation for 15 min in a microfuge. Decant the supernatant solution.
10. Wash the DNA pellet with 70% ethanol by adding 50 μl of 70% ethanol. Centrifuge the sample for another 5 min and drain the alcohol from the pellet. Dry the sample completely using a Speed-vac.
11. Resuspend the dried DNA pellet in 25 μl 1 m*M* Tris and 0.1 m*M* EDTA, pH 7.0–8.0. Use 8-μl aliquots of the DNA for restriction endonuclease digests to characterize the plasmid DNA.

PROTOCOL 2.6b: Alkaline Mini-Prep Procedure for Isolating Plasmid DNA

1. Inoculate 1 to 2 ml of L broth plus appropriate antibiotics in culture tubes with individual bacterial colonies. Number the test tubes and the corresponding colonies for identification. Shake the culture tubes at 37°C overnight.
2. Pour the bacterial cells into appropriately numbered microfuge tubes. Centrifuge the bacterial cells in a microfuge for 1 min. If the cells are centrifuged for a long time, it will be more difficult to resuspend the cell pellet. Decant and discard the supernatant medium. Collect the supernatant medium in a beaker. Add bleach to the used medium before discarding the used medium.
3. Resuspend the cell pellets in 100 μl alkaline extraction Solution I. Be sure the pellet is resuspended completely before continuing.

   Solution I

   50 m*M* glucose

   25 m*M* Tris, pH 8.0

   10 m*M* EDTA
4. Prepare fresh Solution II by combining 1 ml 2 *M* NaOH, 0.5 ml 20% SDS, and 8.5 ml H_2O.

   Solution II

0.25 NaOH
1% SDS

5. Immediately add 200 μl of freshly prepared Solution II. Mix well by inverting the microfuge tubes several times. Place the microfuge tubes on ice for 5 min. Upon addition of Solution II, the samples begin to clear because the cells have lysed.
6. Add 150 μl of ice-cold Solution III to each tube. Mix well by inverting the microfuge tubes several times. Place the microfuge tubes on ice for 15 min.

   Solution III
   3 *M* KOAc
   Bring to pH 5.5 by adding glacial acetic acid.
7. Centrifuge the microfuge tubes 5 min in microfuge.

   [Solution III is added to bring the sample back to a neutral pH and to precipitate an insoluble potassium–SDS salt. A white precipitate of denatured proteins and cell debris is formed. Much of the chromosomal DNA (being larger and still single-stranded following the addition of Solution II) is trapped in this material and is removed from the solution. The supercoiled plasmid DNA and small RNAs remain in the supernatant solution.]
8. Decant the supernatant solution into new microfuge tubes.

CAUTION: Wear gloves, a laboratory coat, and goggles or glasses when working with phenol. Dispose of waste phenol in a container designated for organic waste.

9. Add 200 μl phenol:chloroform (1:1) to each tube. Invert closed microfuge tubes several times to mix the phases well. The phenol:chloroform step extracts additional proteins from the solution.
10. Centrifuge the samples 1 min in a microfuge.
11. Using a pipettor or a Pasteur pipet, transfer the upper (aqueous) phase to new microfuge tubes. Do not transfer any of the white precipitate from the interface or any of the phenol phase.
12. Add 300 μl cold isopropanol to each tube. Mix well. DNA and RNA are precipitated in this step.
13. Centrifuge the samples in a microfuge for 5 min.
14. Decant the isopropanol. Drain the pellet well. Blot excess liquid onto clean tissues or paper towels.
15. Prepare fresh TES plus RNase by adding 20 μl 5 mg/ml RNase to 1 ml TES.

    TES
    50 m*M* Tris, pH 8.0

20 mM NaCl
5 mM EDTA

16. Resuspend the pellet in 50 μl TES plus RNase. This brief RNase treatment will remove RNA from the samples.
17. Add 5 μl 3 M NaOAc and 125 μl cold ethanol to each sample to precipitate DNA. Mix samples by inverting the microfuge tubes. Once ethanol is added, the samples can be stored at $-20°C$ indefinitely.
18. Centrifuge the samples for 5 min in a microfuge. Decant the ethanol. Dry pellets completely by using a Speed-vac or allowing the samples to air-dry. Resuspend the pellets in 40 μl 1 mM Tris and 0.1 mM EDTA, pH 7.5. Mix the samples with a pipet tip to help resuspend the DNA pellet.

For restriction digestions, use a 10-μl aliquot of the sample per restriction enzyme reaction.

NOTES

1. Steps 1 through 3 above can be eliminated as follows: Streak bacterial colonies to be analyzed in long streaks or patches on an agar plate. Incubate the plate overnight and then store the plate wrapped with Parafilm until ready to begin the mini-prep protocol. Colonies can be stored on plates for several days before being used in this protocol. Using a sterile toothpick or inoculating loop, remove large clumps of the bacterial cells from the plate and resuspend the cells in 100 μl alkaline extraction Solution I. Resuspend the cell clumps completely before continuing. Continue the protocol above starting at step 4.
2. Preparation of phenol:chloroform (1 : 1). Use phenol crystals, such as Baker reagent grade phenol crystals. When melted, the phenol should be colorless or have only a slight amount of color. Phenol which is colored deep yellow or deep pink should not be used. Melt 1 pound phenol at 65°C. (This is about 440 ml.) Add 440 ml of chloroform. Mix. Add 26.4 g NaCl. Mix. Add 440 ml 2 M Tris, pH 7.0. Mix. Remove the aqueous (top) phase. Add 440 ml 50 mM Tris pH 8.0. The solution is now ready for use. Store at 4°C. Alternatively, molecular biology grade phenol can be purchased.

References

Anand, R. (1986). Pulsed field gel electrophoresis: A technique for fractionating large DNA molecules. *Trends Genet.* **2**, 278–283.

Anderson, C. (1993). Genome shortcut leads to problems/YAC troubles run deep. *Science* **259**, 1684–1687.

Anderson, J. E. (1993). Restriction endonucleases and modification methylases. Curr. *Opinion Struct. Biol.* **3**, 24–30.
Arber, W. (1965). Host specificity of DNA produced by *Escherichia coli.* V. The role of methionine in the production of host specificity. *J. Mol. Biol.* **11**, 247–256.
Arber, W., and Dussoix, D. (1962). Host specificity of DNA produced by *Escherichia coli.* I. Host controlled modification of bacteriophage lambda. *J. Mol. Biol.* **5**, 18–36.
Arnott, S., Fulmer, A., and Scott, W. E. (1974). The agarose double helix and its function in agarose gel structure. *J. Mol. Biol.* **90**, 269–284.
Balbas, P., Soberon, X., Merino, E., Zurita, M., Lomeli, H., Valle, F., Flores, N., and Bolivar, F. (1986). Plasmid vector pBR322 and its special-purpose derivatives—a review. *Gene* **50**, 3–40.
Balbas, P., Soberon, X., Bolivar, F., and Rodriguez, R. L. (1988). The plasmid, pBR322. *In* "Vectors: A Survey of Molecular Cloning Vectors and Their Uses" (R. L. Rodriguez, and D. T. Denhardt, eds.). Butterworths, Boston.
Barlow, D. P., and Lehrach, H. (1987). Genetics by gel electrophoresis: The impact of pulsed field gel electrophoresis on mammalian genetics. *Trends Genet.* **3**, 167–171.
Been, M. D., and Champoux, J. J. (1983). Cutting of M13mp7 phage DNA and excision of cloned single-stranded sequences by restriction endonucleases. *In* "Methods in Enzymology" (R. Wu, L. Grossman, and K. Moldave, eds.), Vol. 101, pp. 90–98. Academic Press, San Diego.
Bendich, A. J., and Smith, S. B. (1990). Moving pictures and pulsed field gel electrophoresis show linear DNA molecules from chloroplasts and mitochondria. *Curr. Genet.* **17**, 421–425.
Benes, V., Hostomsky, Z., Arnold, L., and Paces, V. (1993). M13 and pUC vectors with new unique restriction sites for cloning. *Gene* **130**, 151–152.
Berg, P., Baltimore, D., Boyer, H. W., Cohen, S. N., Davis, R. W., Hogness, D. S., Nathans, D., Roblin, R., Watson, J. K., Weissman, S., and Zinder, N. D. (1974). Potential biohazards of recombinant DNA molecules. *Science* **185**, 303.
Berg, P., Baltimore, D., Brenner, S., Roblin III, R. O., Singer, M. F. (1975). Asilomar conference on recombinant DNA molecules. *Science* **188**, 991–994.
Bertani, G., and Weigle, J. J. (1953). Host controlled variation in bacterial viruses. *J. Bacteriol.* **65**, 113–121.
Bhagwat, A. S. (1992). Restriction enzymes: Properties and use. *In* "Methods in Enzymology" (R. Wu, ed.), Vol. 216, pp. 199–224. Academic Press, San Diego.
Bickle, T. A., and Kruger, D. H. (1993). Biology of DNA restriction. *Microbiol. Rev.* **57**, 434–450.
Binns, A. N., and Thomashow, M. F. (1988). Cell biology of *Agrobacterium* infection and transformation of plants. *Annu. Rev. Microbiol.* **42**, 575–606.
Blakesley, R. W., and Wells, R. D. (1975). "Single-stranded" DNA from ϕX174 and M13 is cleaved by certain restriction endonucleases. *Nature (London)* **257**, 421.
Blakesley, R. W., Dodgson, J. B., Nes, I. F., and Wells, R. D. (1977). Duplex regions in "single-stranded" ϕX174 are cleaved by a restriction endonuclease from *Haemophilus aegyptius. J. Biol. Chem.* **252**, 7300.
Bolivar, F. (1978). Construction and characterization of new cloning vehicles. III. Derivatives of plasmid pBR322 carrying unique EcoRI sites for selection of EcoRI generated recombinant molecules. *Gene* **4**, 121–136.
Bolivar, F. (1988). Plasmid pBR322: The multipurpose cloning vector. *Focus* **10**(4), 61–64.
Bolivar, F., Rodriguez, R. L., Betlach, M. C., and Boyer, H. W. (1977a). Construction and characterization of new cloning vehicles. I. Ampicillin-resistant derivatives of the plasmid pMB9. *Gene* **2**, 75–93.
Bolivar, F., Rodriguez, R. L., Greene, P. J., Betlach, M. C., Heyneker, H. L., and Boyer, H. W. (1977b). Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. *Gene* **22**, 95–113.

Brooks, J. E. (1987). Properties and uses of restriction endonucleases. *In* "Methods in Enzymology" (S. L. Berger and A. R. Kimmel, eds.), Vol. 152, pp. 113–129. Academic Press, San Diego.

Brown, D. D. (1994). Some genes were isolated and their structure studied before the recombinant DNA era. *Bioessays* **16,** 139–143.

Brown, T. A. (1991). "Molecular Biology Lab Fax," Academic Press, San Diego.

Burke, D. T. (1990). YAC cloning: Options and problems. *Genet. Anal. Tech. Appl.* **7,** 94–99.

Burkhart J. G., Burkhart, B. A., Sampson, K., and Malling, H. V. (1992). Evidence for a previously undetected CpG methyl-directed restriction system in *E. coli. Nucleic Acids Res.* **20**(Suppl), 4368.

Caetano-Anolies, G., and Gresshoff, P. M. (1994). Staining nucleic acids with silver: An alternative to radioisotopic and fluorescent labeling. *Promega Notes* **45,** 13–18.

Carle, G. F., and Olson, M. V. (1987). Orthogonal-field-alternation gel electrophoresis. *In* "Methods in Enzymology" (R. Wu, ed.), Vol. 155, pp. 468–482. Academic Press, San Diego.

Carle, G. F., Frank, M., and Olson, M. V. (1986). Electrophoretic separations of large DNA molecules by periodic inversion of the electric field. *Science* **232,** 65–68.

Chung, C. T., Niemela, S. L., and Miller, R. H. (1989). One-step preparation of competent *Escherichia coli:* Transformation and storage of bacterial cells in the same solution. *Proc. Natl. Acad. Sci. U.S.A.* **86,** 2172–2175.

Clark, S. M., and Mathies, R. A. (1993). High-speed parallel separation of DNA restriction fragments using capillary array electrophoresis. *Anal. Biochem.* **215,** 163–170.

Close, T. J., Christmann, J. L., and Rodriguez, R. L. (1983). M13 bacteriophage and pUC plasmids containing DNA inserts but still capable of β-galactosidase α-complementation. *Gene* **23,** 131–136.

Cohen, S. N. (1993). Bacterial plasmids: Their extraordinary contribution to molecular genetics. *Gene* **135,** 67–76.

Cohen, S. N., and Chang, A. C. Y. (1977). Revised interpretation of the origin of the pSC101 plasmid. *J. Bacteriol.* **132,** 734–737.

Cohen, S. N., Chang, A. C. Y., and Hsu, L. (1972). Nonchromosomal antibiotic resistance in bacteria: Genetic transformation of *Escherichia coli* by R-factor DNA. *Proc. Natl. Acad. Sci. U.S.A.* **69,** 2110–2114.

Cohen, S. N., Chang, A. C. Y., Boyer, H. W., and Helling, R. B. (1973). Construction of biologically functional bacterial plasmids *in vitro. Proc. Natl. Acad. Sci. U.S.A.* **70,** 3240–3244.

Cohn, B. L. (1987). Ethidium bromide safety. *Trends Genet.* **3,** 308.

Collins, J., and Hohn, B. (1978). Cosmids: Type of plasmid gene cloning vector that is packageable *in vitro* in bacteriophage lambda heads. *Proc. Natl. Acad. Sci. U.S.A.* **75,** 4242–4246.

Conley, E. C., and Saunders, J. R. (1984). Recombination-dependent recircularization of linearized pBR322 plasmid DNA following transformation of *Escherichia coli. Mol. Gen. Genet.* **194,** 211–218.

Cooke, H. (1987). Cloning in yeast: An appropriate scale for mammalian genomes. *Trends Genet.* **3,** 173–174.

Dagert, M., and Ehrlich, S. D. (1979). Prolonged incubation in calcium chloride improves the competence of *Escherichia coli* cells. *Gene* **6,** 23–28.

Danner, D. B., Deich, R. A., Sisco, K. L., and Smith, H. O. (1980). An eleven-base-pair sequence determines the specificity of DNA uptake in *Haemophilus* transformation. *Gene* **11,** 311–318.

Darkins, A., and Chakrabarty, A. M. (1984). Cloning of genes controlling alginate biosynthesis from a mucoid cystic fibrosis isolate of *Pseudomonas aeruginosa. J. Bacteriol.* **159,** 9–18.

Davis, L. G., Dibner, M. D., and Battey, J. F. (1986). "Basic Methods in Molecular Biology." Elsevier, New York.
Day, R. A., Jr., and Underwood, A. L. (1967). "Quantitative Analysis." Prentice–Hall, Englewood Cliffs, NJ.
Denniston, K. J., and Enquist, L. W. (1981). Recombinant DNA. *In* "Benchmark Papers in Microbiology." Vol. 15. Dowden, Hutchinson, & Ross, Stroudsburg, PA.
Deutsch, J. M. (1988). Theoretical studies of DNA during gel electrophoresis. *Science* **240,** 922–924.
Dickerson, R. E., Gray, H. B., and Haight, G. P., Jr. (1970). "Chemical Principles." W. A. Benjamin, Menlo Park, CA.
Dixon, B. (1993). Gene flow and bacterial transformation. *Biotechnology* **11,** 767.
Dugaiczyk, A., Boyer, H. W., and Goodman, H. M. (1975). Ligation of *EcoRI* endonuclease-generated DNA fragments into linear and circular structures. *J. Mol. Biol.* **96,** 171–184.
Dussoix, D., and Arber, W. (1962). Host specificity of DNA produced by *Escherichia coli.* II. Control over acceptance of DNA from infecting phage lambda. *J. Mol. Biol.* **5,** 37–49.
Ebel-Tsipis, J., Botstein, D., and Fox, M. S. (1972). Generalized transduction by phage P22 in *Salmonella typhimurium.* I. Molecular origin of transducing DNA. *J. Mol. Biol.* **71,** 433–448.
Edmondson, S. P., and Gray, D. M. (1984). Analyis of the electrophoretic properties of double-stranded DNA and RNA in agarose gels at a finite voltage gradient. *Biopolymers* **23,** 2725–2742.
Farmer, J. L. (1985). Enzyme storage in frost-free freezer. *Focus* **7,** 5.
Flavell, R. V., Dart, E., Fuchs, R. L., and Fraley, R. T. (1992). Selectable marker genes: Safe for plants? *Biotechnology* **10,** 141–144.
Food and Agriculture Organization/World Health Organization (1991). "Strategies for Assessing the Safety of Foods Produced by Biotechnology." World Health Organization.
Friedman, A. M., Long, S. R., Brown, S. E., Buikema, W. J., and Ausubel, F. M. (1982). Construction of a broad host range cosmid cloning vector and its use in the genetic analysis of *Rhizobium* mutants. *Gene* **18,** 289–296.
Gefter, M. L., Becker, A., and Hurwitz, J. (1967). The enzymatic repair of DNA. I. Formation of circular λ DNA. *Proc. Natl. Acad. Sci. U.S.A.* **58,** 240–247.
Greene, P. J., Poonian, M. S., Nussbaum, A. L., Tobias, L., Garfin, D. E., Boyer, H. W., and Goodman, H. M. (1975). Restriction and modification of a self-complementary octanucleotide containing the *Eco*RI substrate. *J. Mol. Biol.* **99,** 237–261.
Grobstein, C. (1977). The recombinant-DNA debate. *Sci. Am.* **237,** 22–33.
Grobstein, C. (1979). "A Double Image of the Double Helix: The Recombinant-DNA Debate." Freeman, San Francisco.
Hanahan, D. (1983). Studies on Transformation of *E. coli* with plasmids. *J. Mol. Biol.* **166,** 557–580.
Harada, K., and Orgel, L. E. (1993). Unexpected substrate specificity of T4 DNA ligase revealed by *in vitro* selection. *Nucleic Acids Res.* **21,** 2287–2291.
Heidecker, G., Messing, J., and Gronenborn, B. (1980). A versatile primer for DNA sequencing in the M13mp2 cloning system. *Gene* **10,** 69–73.
Heitman, J. (1992). How the *Eco*RI endonuclease recognizes and cleaves DNA. *Bioessays* **14,** 445–454.
Helling, R. B., Goodman, H. M., and Boyer, H. W. (1974). Analysis of endonuclease R·*Eco*RI fragments of DNA from lambdoid bacteriophages and other viruses by agarose-gel electrophoresis. *J. Virol.* **14,** 1235–1244.
Hengen, P. N. (1993). Methods and reagents: Bummer buffers and lightning ligations. *Trends Biochem. Sci.* **18,** 446–448.
Hengen, P. N. (1994). Methods and reagents: Determining DNA concentrations and rescuing PCR primers. *Trends Biochem. Sci.* **19,** 93–94.

Hofer, B., Ruhe, G., Koch, A., and Koster, H. (1982). Primary and secondary structure specificity of the cleavage of "single-stranded" DNA by endonuclease *HinfI*. *Nucleic Acids Res.* **10,** 2763–2773.

Hohn, B. (1979). *In vitro* packaging of λ and cosmid DNA. *In* "Methods in Enzymology" (R. Wu, ed.) Vol. 68, pp. 299–309. Academic Press, San Diego.

Hohn, B., and Collins, J. (1980). A small cosmid for effective cloning of large DNA fragments. *Gene* **11,** 291–298.

Holzwarth, G., Platt, K. J., McKee, C. B., Whitcomb, R. W., and Crater, G. D. (1989). The acceleration of linear DNA during pulsed-field gel electrophoresis. *Biopolymers* **28,** 1043–1058.

Horiuchi, K., and Zinder, N. D. (1975). Site-specific cleavage of single-stranded DNA by a *Hemophilus* restriction endonuclease. *Proc. Natl. Acad. Sci. U.S.A.* **72,** 2555.

Horn, T. M. (1993). "Working with DNA and Bacteria in Precollege Science Classrooms." National Association of Biology Teachers, Reston, VA.

Ikeda, H., and Tomizawa, J. (1965). Transducing fragments in generalised transduction by phage P1. I. Molecular origin of the fragments. *J. Mol. Biol.* **14,** 85–109.

Inoue, E., Nojima H., and Okayama, H. (1990). High efficiency transformation of *Escherichia coli* with plasmids. *Gene* **96,** 23–28.

Jackson, D. A., and Stich, S. P. (1979). "The Recombinant DNA Debate." Prentice–Hall, Englewood Cliffs, NJ.

Jackson, D. A., Symons, R. H., and Berg, P. (1972). Biochemical method for inserting new genetic information into DNA of Simian virus 40: Circular SV40 DNA molecules containing lambda phage genes and the galactose operon of *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* **69,** 2904–2909.

Jordan, B. (1990). YAC power. *BioEssays* **12,** 183–187.

Karcher, S. J. (1994). Getting DNA into a cell: A survey of transformation methods. *Am. Biol. Teacher* **56,** 14–20.

Kato, K.-I., Concalves, J. M. N., Hots, G. E., and Bollum, F. J. (1967). Deoxynucleotide-polymerizing enzymes of calf thymus gland. II. Properties of the terminal deoxynucleotidyl transferase. *J. Biol. Chem.* **242,** 2780–2789.

Kessler, C., and Holtke, H.-J. (1986). Specificity of restriction endonucleases and methylases—a review (Ed. 2). *Gene* **47,** 1–153.

Kessler, C., and Manta, V. (1990). Specificity of restriction endonucleases and DNA modification methyltransferases—a review (Ed. 3). *Gene* **92,** 1–248.

Kessler, C., Neumaier, P. S., and Wolf, W. (1985). Recognition sequences of restriction endonucleases and methylases—a review. *Gene* **33,** 1–102.

Kohara, Y., Akiyama, K., and Isono, K. (1987). The physical map of the whole *E. coli* chromosome: Application of a new strategy for rapid analysis and sorting of a large genomic library. *Cell (Cambridge, Mass.)* **50,** 495–508.

Krimsky, S. (1982). "Genetic Alchemy: The Social History of the Recombinant DNA Controversy." The MIT Press, Cambridge, MA.

Kwan, H., Li, C., Chiu, S., and Cheng, S. (1991). A simple method to prepare intact yeast chromosomal DNA for pulsed field gel electrophoresis. *Nucleic Acids Res.* **19,** 1347.

Lalands, M., Noolandi, J., Turmel, C., Brousseau, R., Rosseau, J., and Slater, G. W. (1988). Scrambling of bands in gel electrophoresis of DNA. *Nucleic Acids Res.* **16,** 5427–5437.

Landers, J. P. (1993). Capillary electrophoresis: Pioneering new approaches for biomolecular analysis. *Trends Biochem.* **18,** 409–414.

Lautenberger, J. A., Kan, N. C., Lackey, D., Linn, S., Edgell, M. H., and Hutchison, C. A., III. (1978). Recognition site of *Escherichia coli* B restriction enzyme on φX174 and SV40 DNAs: An interrupted sequence. *Proc. Natl. Acad. Sci. U.S.A.* **75,** 2271–2275.

Lawrence, S. K., Smith, C. L., Srivastava, R., Canton, C. R., and Weissman, S. M. (1987).

Megabase-scale mapping of the HLA gene complex by pulsed field gel electrophoresis. *Science* **235**, 1387–1390.

Lerman, L. S., and Silverstein, K. (1987). Computational simulation of DNA melting and its application to denaturing gradient gel electrophoresis. *In* "Methods in Enzymology" (R. Wu, ed.), Vol. 155, pp. 482–501. Academic Press, San Diego.

Li, L., Wu, L. P., and Chandrasegaran, S. (1992). Functional domains in *Fok*I restriction endonuclease. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 4275–4279.

Lobban, P. E., and Kaiser, A. D. (1973). Enzymatic end-to-end joining of DNA molecules. *J. Mol. Biol.* **78**, 453–471.

Lumpkin, O. J., DéJardin, P., and Zimm, B. H. (1985). Theory of gel electrophoresis of DNA. *Biopolymers* **24**, 1573–1593.

Lunn, G., and Sansone, E. B. (1987). Ethidium bromide: Destruction and decontamination of solutions. *Anal. Biochem.* **162**, 453–458.

Luria, S. E., and Human, M. L. (1952). A nonhereditary, host-induced variation of bacterial viruses. *J. Bacteriol.* **64**, 557–569.

MacGregor, J. T., and Johnson, I. J. (1977). *In vitro* metabolic activation of ethidium bromide and other phenanthridinium compounds: Mutagenic activity in *Salmonella typhimurium*. *Mutat. Res.* **48**, 103–108.

Mandel, M., and Higa, A. (1970). Calcium-dependent bacteriophage DNA infection. *J. Mol. Biol.* **53**, 159–162.

Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982). "Molecular Cloning: A Laboratory Manual." Cold Spring Harbor Press, Cold Spring Harbor, NY.

Martin, F., Vairelles, D., and Henrion, B. (1993). Automated ribosomal DNA fingerprinting by capillary electrophoresis of PCR products. *Anal. Biochem.* **214**, 182–189.

McClelland, M., and Nelson, M. (1992). Effect of site-specific methylation on DNA modification methyltransferases and restriction endonucleases. *Nucleic Acids Res.* **20**(Suppl.), 2145–2157.

McClelland, M., Jones, R. J., Patel, Y., and Nelson, M. (1987). Restriction endonucleases for pulsed field mapping of bacterial genomes. *Nucleic Acids Res.* **15**, 5985–6005.

McEvoy, T. G., Robinson, J. J., and Sreenan, J. M. (1992). A role for transgenic animals in food production? *Trends Food Sci. Technol.* **3**, 294–302.

McPeek, F. D., Jr., Coyle-Morris, J. F., and Gemmill, R. M. (1986). Separation of large DNA molecules by modified pulsed field gradient gel electrophoresis. *Anal. Biochem.* **156**, 274–285.

Mertz, J. E., and Davis, R. W. (1972). Cleavage of DNA by R_I restriction endonuclease generates cohesive ends. *Proc. Natl. Acad. Sci. U.S.A.* **69**, 3370–3374.

Messing, J. (1983). New M13 vectors for cloning. *In* "Methods in Enzymology" (R. Wu, L. Grossman, and K. Moldave, eds.), Vol. 101, pp. 20–78. Academic Press, San Diego.

Messing, J. (1988). M13, the universal primer and the polylinker. *Focus* **10**(2), 21–26.

Messing, J. (1991). Cloning in M13 phage or how to use biology at its best. *Gene* **100**, 3–12.

Messing, J., and Vieira, J. (1982). A new pair of M13 vectors for selecting either DNA strand of double-digest restriction fragments. *Gene* **19**, 269–276.

Messing, J., Gronenborn, B., Muller-Hill, B., and Hofschneider, P. H. (1977). Filamentous coliphage M13 as a cloning vehicle: Insertion of a *Hin*dII fragment of the *lac* regulatory region in M13 replicative form *in vitro*. *Proc. Natl. Acad. Sci. U.S.A.* **74**, 3642–3646.

Messing, J., Crea, R., and Seeburg, P. H. (1991). A system for shotgun DNA sequencing. *Nucleic Acids Res.* **9**, 309–321.

Michiels, F., Burmeister, M., and Lehrach, H. (1987). Derivation of clones close to *met* by preparative field inversion gel electrophoresis. *Science* **236**, 1305–1308.

Mickel, S., Arena, V., Jr., and Bauer, W. (1977). Physical properties and gel electrophoretic behavior of R12-derived plasmid DNAs. *Nucleic Acids Res.* **4**, 1465–1482.

Miller, H. I. (1992). Putting the bST (bovine somatotropin) human-health controversy to rest. *Biotechnology* **10**, 147.

Miller, J. H. (1992). "A Short Course in Bacterial Genetics: A Laboratory Manual and Handbook for *Escherichia coli* and Related Bacteria," Section 9. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Miller, J. H., and Reznikoff, W. S. (1980). "The Operon." Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Morrison, D. A. (1979). Transformation and preservation of competent bacterial cells by freezing. *In* "Methods in Enzymology" (R. Wu, ed.), Vol. 68, pp. 326–331. Academic Press, New York.

Murray, N. E. (1991). Special uses of lambda phage for molecular cloning. *In* "Methods in Enzymology" (J. H. Miller, ed.), Vol. 204, pp. 280–301. Academic Press, San Diego.

Nelson, M., Raschke, E., and McClelland, M. (1993). Effect of site-specific methylation on restriction endonucleases and DNA modification methyltransferases. *Nucleic Acids Res.* **21**, 3139–3154.

Nester, E. W., Gordon, M. P., Amasino, R. M., and Yanofsky, M. F. (1984). Crown gall: A molecular and physiological analysis. *Annu. Rev. Plant Phys.* **35**, 387–413.

Neumann, E., Schaefer-Ridder, M., Wang, Y., and Hofschneider, P. H. (1982). Gene transfer into mouse lyoma cells by electroporation in high electric fields. *EMBO J.* **1**, 841–845.

Norrander, J., Kempe, T., and Messing, J. (1983). Construction of improved M13 vectors using oligodeoxynucleotide-directed mutagenesis. *Gene* **26**, 101–106.

Ogden, R. C., and Adams, D. A. (1987). Electrophoresis in agarose and acrylamide gels. *In* "Methods in Enzymology" (S. L. Berger and A. R. Kimmel, eds.), Vol. 152, pp. 61–87. Academic Press, San Diego.

Olivera, B. M., and Lehman, I. R. (1967). Linkage of polynucleotides through phosphodiester bonds by an enzyme for *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* **57**, 1426–1433.

Petrusyte, M., Bitinaite, J., Menkevicius, S., Klimasauskas, S., Butkus, V., and Janulaitis, A. (1989). Restriction endonucleases of a new type. *Gene* **74**, 89–91.

Prentki, P., Karch, F., Iida, S., and Meyer, J. (1981). The plasmid cloning vector pBR325 contains a 482 base-pair-long inverted duplication. *Gene* **14**, 289–299.

Quillardet, P., Hofnung, M., and Bensaude, O. (1988). Ethidium bromide and safety—readers suggest alternative solutions. *Trends Genet.* **4**, 89–90.

Ream, W. (1989). *Agrobacterium tumefaciens* and interkingdom genetic exchange. *Annu. Rev. Phytopathol.* **27**, 583–618.

Revie, D., Smith, D. W., and Yee, T. W. (1988). Kinetic analysis for optimization of DNA ligation reactions. *Nucleic Acids Res.* **16**, 10,301–10,321.

Roberts, R. J. (1990). Restriction enzymes and their isoschizomers. *Nucleic Acids Res.* **18**(Suppl.), 2331.

Roberts, R. J., and Macelis, D. (1992). Restriction enzymes and their isoschizomers. *Nucleic Acids Res.* **20**(Suppl.), 2167–2180.

Roberts, R. J., and Macelis, D. (1993). REBASE—restriction enzymes and methylases. *Nucleic Acids Res.* **21**, 3125–3137.

Rodriguez, R. L., and Tait, R. C. (1983). "Recombinant DNA Techniques: An Introduction." Addison-Wesley, Reading, MA.

Rong, L., Karcher, S. J., O'Neal, K., Hawes, M. C., Yerkes, C. D., Jayaswal, R. K., Hallberg, C. A., and Gelvin, S. B. (1990). *picA*, a novel plant-inducible locus on the *Agrobacterium tumefaciens* chromosome. *J. Bacteriol.* **172**, 5825–5836.

Rong, L., Karcher, S. J., and Gelvin, S. B. (1991). Genetic and molecular analyses of *picA*, a plant-inducible locus on the *Agrobacterium tumefaciens* chromosome. *J. Bacteriol.* **173**, 5110–5120.

Rong, L., Carpita, N. C., Mort, A., and Gelvin, S. B. (1994). Soluble cell wall components from carrot roots induce the *picA* and *pgl* loci of *Agrobacterium tumefaciens*. *Mol. Plant/Microbe Interactions* **7**, 6–14.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). "Molecular Cloning: A Laboratory Manual," 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Santiago-Blay, J. A., and Battaion, S. (1993). Non-nutrient agar: A cheap alternative matrix for electrophoresis. *Trends Genet.* **9,** 158.

Santillan-Torres, J. L., and Ponce-Noyola, P. (1993). A novel stain for DNA in agarose gels. *Trends Genet.* **9,** 40.

Schwartz, D. C., and Cantor, C. R. (1984). Separation of yeast chromosome-sized DNAs by pulsed field gradient gel electrophoresis. *Cell (Cambridge, Mass.)* **37,** 67–75.

Schwartz, D. C., and Koval, M. (1989). Conformational dynamics of individual DNA molecules during gel electrophoresis. *Nature (London)* **338,** 520–522.

Serwer, P., Easom, R. A., Hayes, S. J., and Olson, M. S. (1989). Rapid detection and characterization of multimolecular cellular constituents by two-dimensional agarose gel electrophoresis. *Trends Biochem. Sci.* **14,** 4–7.

Sgaramella, V. (1972). Enzymatic oligomerization of bacteriophage P22 DNA and of linear Simian virus 40 DNA. *Proc. Natl. Acad. Sci. U.S.A.* **69,** 3370–3374.

Sharp, P. A., Sugden, B., and Sambrook, J. (1973). Detection of two restriction endonuclease activities in *Haemophilus parainfluenza* using analytical agarose ethidium bromide electrophoresis. *Biochemistry* **12,** 3055–3063.

Shizuya, H., Birren, B., Kim, U-J., Mancino, V., Slepak, T., Tachiri, Y., and Simon, M. (1992). Cloning and stable maintenance of 300-kilobase-pair fragments of human DNA in *Escherichia coli* using an F-factor-based vector. *Proc. Natl. Acad. Sci. U.S.A.* **89,** 8794–8797.

Singer, M., and Soll, D. (1973). Guidelines for DNA hybrid molecules. *Science* **181,** 1114.

Sinsheimer, R. (1975). Troubled dawn for genetic engineering. *In* "The Weekly Review of Science and Technology," Vol. 68. New Scientist, London.

Smith, C. L., and Cantor, C. R. (1987). Purification, specific fragmentation, and separation of large DNA molecules. *In* "Methods in Enzymology" (R. Wu, ed.), Vol. 155, pp. 449–467. Academic Press, San Diego.

Smith, H. O., and Nathans, D. (1973). A suggested nomenclature for bacterial host modification and restriction systems and their enzymes. *J. Mol. Biol.* **81,** 419–423.

Smith, H. O., and Wilcox, K. W. (1970). A restriction enzyme from *Haemophilus influenzae.* I. Purification and general properties. *J. Mol. Biol.* **51,** 379–391.

Smith, H. O., Danner, D. B., and Deich, R. A. (1981). Genetic transformation. *Annu. Rev. Biochem.* **50,** 41–68.

Smith, S. B., Aldridge, P. K., and Callis, J. B. (1989). Observation of individual DNA molecules undergoing gel electrophoresis. *Science* **243,** 203–206.

Smith, S. S., Gilroy, T. E., and Ferrari, F. A. (1983). The influence of agarose-DNA affinity on the electrophoresis separation of DNA fragments in agarose gels. *Anal. Biochem.* **128,** 138–151.

Sternberg, N. L. (1990a). Bacteriophage P1 cloning system for the isolation, amplification, and recovery of DNA fragments as large as 100 kilobase pairs. *Proc. Natl. Acad. Sci. U.S.A.* **87,** 103–107.

Sternberg, N. L. (1990b). Alternatives to YACs. *Genet. Anal. Tech. Appl.* **7,** 126–132.

Sternberg, N. L. (1992). Cloning high molecular weight DNA fragments by the bacteriophage P1 system. *Trends Genet.* **8,** 11–16.

Stewart, G. J., and Carlson, C. A. (1986). Biology of natural transformation. *Annu. Rev. Microbiol.* **40,** 211–235.

Stich, S. P. (1988). The recombinant DNA debate. *Philosophy Public Affairs* **7,** 187–205.

Sutcliffe, J. G. (1979). Complete nucleotide sequence of the *Escherichia coli* plasmid pBR322. *Cold Spring Harbor Symp. Quant. Biol.* **43,** 77–90.

Sutherland, E., Coe, L., and Raleigh, E. A. (1992). McrBC: A multisubunit GTP-dependent restriction endonuclease. *J. Mol. Biol.* **225,** 327–348.

Szybalski, W., Kim, S. C., Hasan, N., and Podjajska, A. J. (1991). Class-IIS restriction enzymes—a review. *Gene* **100,** 13–26.

Tartof, K. D., and Hobbs, C. A. (1987). *Focus* **9**(2), 12.

Ullmann, A. (1992). Complementation in β-galactosidase: From protein structure to genetic engineering. *Bioessays* **14**, 201–205.
Ullmann, A., Jacob, F., and Monod, J. (1967). Characterization by *in vitro* complementation of a peptide corresponding to an operator-proximal segment of the β-galactosidase structural gene of *Escherichia coli*. *J. Mol. Biol.* **24**, 339–343.
Van Brunt, J. (1990). Affairs of the heart. *Biotechnology* **8**, 21–23.
Vandaele, W. (1992). bST and the EEC: politics vs. science. *Biotechnology* **10**, 148–149.
Vieira, J., and Messing, J. (1982). The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene* **19**, 259–268.
Vieira, J., and Messing, J. (1987). Production of single-stranded plasmid DNA. *In* "Methods in Enzymology" (R. Wu, and L. Grossman, eds.), Vol. 153, pp. 3–11. Academic Press, San Diego.
Vieira, J., and Messing, J. (1991). New pUC-derived cloning vectors with different selectable markers and DNA replication origins. *Gene* **100**, 189–194.
Waki, S., Harvey, J. D., and Bellamy, A. R. (1982). Study of agarose gels by electron microscopy of freeze-fractured surfaces. *Biopolymers* **21**, 1909–1926.
Watson, J. S., and Tooze, J. (1982). "The DNA Story: A Documentary History of Gene Cloning." Freeman, San Francisco.
Weiss, B., and Richardson, C. C. (1967). Enzymatic breakage and joining of deoxyribonucleic acid. I. Repair of single-stranded breaks in DNA by an enzyme system from *Escherichia coli* infected with T4 bacteriophage. *Proc. Natl. Acad. Sci. U.S.A.* **57**, 315–325.
Whittaker, P. A., Campbell, A. J. B., Southern, E. M., and Murray, N. E. (1988). Enhanced recovery and restriction mapping of DNA fragments cloned in a new λ vector. *Nucleic Acids Res.* **16**, 6725–6735.
Wilson, G. G. (1988). Type II restriction-modification systems. *Trends Genet.* **4**, 314–318.
Winans, S. C. (1992). Two-way chemical signaling in *Agrobacterium*–plant interactions. *Microbiol. Rev.* **56**, 12–31.
Wrubel, R. P., Krimsky, S., and Wetzler, R. E. (1992). Field testing transgenic plants. *BioScience* **42**, 280–289.
Yanisch-Perron, C., Vieira, J., and Messing, J. (1985). Improved M13 phage cloning vectors and host strains: Nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**, 103–119.
Yoo, O. J., and Agrawal, K. L. (1980). Cleavage of single strand oligonucleotides and bacteriophage φX174 DNA by MspI endonuclease. *J. Biol. Chem.* **255**, 10,559–10,562.
Yuan, R. (1981). Structure and mechanism of multifunctional restriction endonucleases. *Annu. Rev. Biochem.* **50**, 285–315.
Zambryski, P. (1988). Basic processes underlying *Agrobacterium*-mediated DNA transfer to plant cells. *Annu. Rev. Genet.* **22**, 1–30.
Zambryski, P. C. (1992). Chronicles from the *Agrobacterium*–plant cell DNA transfer story. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **43**, 465–490.
Zilinskas, R. A., and Zimmerman, B. K. (1986). "The Gene-Splicing Wars." Macmillan, New York.
Zimmerman, S. B., Little, J. W., Oshinsky, C. K., and Gellert, M. (1967). Enzymatic joining of DNA strands: A novel reaction of diphosphopyridine nucleotide. *Proc. Natl. Acad. Sci. U.S.A.* **57**, 1841–1848.

Suggested Reading

Gel Electrophoresis

Aaij, C., and Borst, P. (1972). The gel electrophoresis of DNA. *Biochim. Biophys. Acta* **269**, 192–200.

Dunbar, B. S. (1987). Basic theories and principles of electrophoresis. *In* "2-Dimensional Electrophoresis and Immunological Techniques," pp. 1–23. Plenum, New York.
Freifelder, D. (1976). Electrophoresis. *In* "Physical Biochemistry: Application to Biochemistry and Molecular Biology," Chap. 9, pp. 211–234. Freeman, San Francisco.
Johnson, P. H., and Grossman, L. I. (1977). Electrophoresis of DNA in agarose gels. Optimizing separations of conformational isomers of double and single stranded DNAs. *Biochemistry* **16,** 4217–4224.
Lerman, L. S., and Frisch, H. L. (1982). Why does the electrophoretic mobility of DNA in gels vary with the length of the molecule? *Biopolymers* **21,** 995–997.
Lumpkin, O. J., and Zimm, B. H. (1982). Mobility of DNA in gel electrophoresis. *Biopolymers* **21,** 2315–2316.
Lyamichev, V. I., Panyutin, I. G., and Lyubchenko, V. L. (1982). Gel electrophoresis of partially denatured DNA. Retardation effect: Its analysis and application. *Nucleic Acids Res.* **10,** 4813–4826.
Schindler, C. W., Krolewski, J. J., and Rush, M. G. (1982). Selective trapping of circular double-stranded DNA molecules in solidifying agarose. *Plasmid* **7,** 263–270.
Southern, E. (1980). Gel electrophoresis of restriction fragments. *In* "Methods in Enzymology" (A. San Pietro, ed.), Vol. 69, p. 152. Academic Press, San Diego.

Gel Electrophoresis to Size Nucleic Acids

Lee, F. S., and Bauer, W. B. (1985). Temperature dependence of the gel electrophoretic mobility of superhelical DNA. *Nucleic Acids Res.* **13,** 1665–1682.
Lehrach, H., Diamond, D., Wozney, J. M., and Boedtker, H. (1977). RNA molecular weight determination by gel electrophoresis under denaturing conditions, a critical reexamination. *Biochemistry* **16,** 4743–4751.
Maniatis, M., Jeffrey, A., and van de Sande, H. (1975). Chain length determination of small double- and single-stranded DNA molecules by polyacrylamide gel electrophoresis. *Biochemistry* **14,** 3787–3794.
McDonell, M. W., Simon, M. N., and Studier, F. W. (1977). Analysis of restriction fragments of T7 DNA and determination of molecular weights by electrophoresis in neutral and alkaline gels. *J. Mol. Biol.* **110,** 119–146.
Serwer, P. (1980). Electrophoresis of duplex deoxyribonucleic acid in multiple-concentration agarose gels: Fractionation of molecules with molecular weights between 2×10^6 and 110×10^6. *Biochemistry* **19,** 3001–3004.
Serwer, P. (1981). Improvements in procedures for electrophoresis in dilute agarose gels. *Anal. Biochem.* **112,** 351–356.

Cloning

Armstrong, K. A., Hershfield, V., and Helinski, D. R. (1972). Gene cloning and containment properties of plasmid ColE1 and its derivatives. *Science* **196,** 172–174.
Bahl, C. P., Marians, K. J., Wu, R., Slavinsky, J., and Narang, S. A. (1976). A general method for inserting specific DNA sequences. *Gene* **1,** 81–92.
Barnes, W. M. (1977). Plasmid detection and sizing in single colony lysates. *Science* **195,** 393–394.
Cami, B., and Kourilsky, P. (1978). Screening of cloned recombinant DNA in bacteria by *in situ* colony hybridization. *Nucleic Acids Res.* **5,** 2381–2390.
Charnay, P., Perricaudet, M., Galibert, F., and Tiollais, P. (1978). Bacteriophage lambda and plasmid vectors, allowing fusion of cloned genes in each of three translational phases. *Nucleic Acids Res.* **5,** 4479–4494.
Clarke, L., and Carbon, J. (1976). A colony bank containing synthetic ColE1 hybrid plasmids representative of the entire *E. coli* genome. *Cell (Cambridge, Mass.)* **9,** 91–99.
Clarke, L., Hitzeman, R., and Carbon, J. (1979). Selection of specific clones from colony

banks by screening with radioactive antibody. *In* "Methods in Enzymology" (R. Wu, ed.), Vol. 68, pp. 436–442. Academic Press, San Diego.

Clevell, D. B. (1972). Nature of ColE1 plasmid replication in *E. coli* in presence of chloramphenicol. *J. Bacteriol.* **110,** 667–676.

Covey, C. D., Richardson, D., and Carbon, J. (1976). A method for the deletion of restriction sites in bacterial plasmid DNA. *Mol. Gen. Genet.* **145,** 155–158.

Dugaiczk, A., Boyer, H. W., and Goodman, H. M. (1975). Ligation of ***Eco*R1** endonuclease-generated DNA fragments into linear and circular structures. *J. Mol. Biol.* **96,** 171–184.

Goff, S., and Berg, P. (1978). Excision of DNA segments introduced into cloning vectors by the poly dA dT joining method. *Proc. Natl. Acad. Sci. U.S.A.* **75,** 1763–1767.

Guarente, L., Roberts, T. M., and Ptashner, M. (1980). A technique for expressing eukaryotic genes in bacteria. *Science* **209,** 1428–1430.

Hershfield, V., Boyer, H. W., Yanofsky, C., Lovett, M. A., and Helinski, D. R. (1974). Plasmid ColE1 as a molecular vehicle for cloning and amplification of DNA. *Proc. Natl. Acad. Sci. U.S.A.* **71,** 3455–3459.

Hohn, B. (1979). *In vitro* packaging of λ and cosmid DNA. *In* "Methods in Enzymology" (R. Wu, ed.), Vol. 68, pp. 299–309. Academic Press, San Diego.

Ish-Horowicz, D., and Burke, J. F. (1981). Rapid and efficient cosmid cloning. *Nucleic Acids Res.* **9,** 2989–2998.

Kahn, M., Kolter, R., Thomas, C., Figursky, D., Meyer, R., Remaut, D., and Helinski, D. R. (1979). Plasmid cloning vehicles derived from plasmids ColE1, F, R6K, RK2. *In* "Methods in Enzymology" (R. Wu, ed.), Vol. 68, pp. 268–280. Academic Press, San Diego.

Okayama, H., and Berg, P. (1982). High-efficiency cloning of full-length cDNA. *Mol. Cell Biol.* **2,** 161–170.

Wartell, R. M., and Reznikoff, W. S. (1980). Cloning DNA restriction endonuclease fragments with protruding, single-stranded ends. *Gene* **9,** 307–319.

Sequencing

Anderson, S., Gait, M. J., Mayol, L., and Young, I. G. (1980). A short primer for sequencing DNA cloned in the single-strand phage vector M13mp2. *Nucleic Acids Res.* **8,** 1731–1743.

Church, G. M., and Gilbert, W. (1984). Genomic sequencing. *Proc. Natl. Acad. Sci. U.S.A.* **81,** 1991–1995.

Church, G. M., and Kieffer-Higgins, S. (1988). Multiplex DNA sequencing. *Science* **240,** 185–188.

Frischauf, A. M., Garoff, H., and Lehrach, H. (1980). A subcloning stradegy for DNA sequence analysis. *Nucleic Acids Res.* **8,** 5541–5549.

Hayaski, K. (1980). A cloning vehicle suitable for strand separation. *Gene* **11,** 109–115.

Hayward, G. S. (1972). Gel electrophoretic separation of complementary strands of bacteriophage DNA. *Virology* **49,** 342–344.

Yang, M. M., and Youvan, D. C. (1989). A prospectus for multispectral-multiplex DNA sequencing. *Biotechnology* **7,** 576–580.

Transformation

Chung, C. T., Niemela, S. L., and Miller, R. H. (1989). One-step preparation of competent *E. coli:* Transformation and storage of bacterial cells in the same solution. *Proc. Natl. Acad. Sci. U.S.A.* **86,** 2172–2175.

Dagert, M., and Ehrlich, S. D. (1979). Prolonged incubation in calcium chloride improves the competence of *E. coli* cells. *Gene* **6,** 23–28.

Danner, D. B., Deich, R. A., Sisco, K. L., and Smith, H. O. (1980). An eleven-base-pair sequence determines the specificity of DNA uptake in *Haemophilus* transformation. *Gene* **11,** 311–318.

Hanahan, D. (1983). Studies on transformation of *E. coli* with plasmids. *J. Mol. Biol.* **166,** 557–580.
Liu, H., and Rashidbaigi, A. (1990). Comparison of various competent cell preparation methods for high efficiency DNA transformation. *Biotechniques* **8,** 21–23.
Sisco, K. L., and Smith, H. O. (1979). Sequence specific DNA uptake in *Haemophilus* transformation. *Proc. Natl. Acad. Sci. U.S.A.* **76,** 972–976.
Smith, H. O., Danner, D. B., and Deich, R. A. (1981). Genetic transformation. *Annu. Rev. Biochem.* **50,** 41–68.
Stewart, G. J., and Carlson, C. A. (1986). The biology of natural transformation. *Annu. Rev. Microbiol.* **40,** 211–235.

Restriction Endonucleases

Nathans, D., and Smith, H. O. (1975). Restriction endonucleases in the analysis and restructuring of DNA molecules. *Annu. Rev. Biochem.* **44,** 273–293.
Roberts, R. (1976). Restriction endonucleases. *CRC Rev. Biochem.* **4,** 123–164.
Winnacker, E. L. (1987). Restriction endonucleases. *In* "Genes to Clones: Introduction to Gene Technology" (Translated by Horst Ibelgaufts), Chap. 2.1, pp. 3–32. VCH Publishers, New York.
Wilson, G. G. (1988). Type II restriction-modification systems. *Trends Genetics* **4,** 314–318.

Restriction Site Mapping

Gottlieb, J. K., and Duckworth, D. H. (1983). Location of the *abi, col* and *imm* genes on pHU011, a colicen Ib plasmid derivative. *Gene* **26,** 301–302.
Jorgensen, R. A., Berg, D. E., Allet, B., and Reznikoff, W. S. (1979). Restriction enzyme cleavage map of Tn*10*, a transposon which encodes tetracycline resistance. *J. Bacteriol.* **137,** 681–685.
Smith, H. O., and Bernstiel, M. L. (1976). A simple method for DNA restriction site mapping. *Nucleic Acids Res.* **3,** 2387–2399.
Yoneda, Y., Graham, S., and Young, F. E. (1979). Restriction-fragment map of the template *Bacillus subtilis* bacteriophage SPO2. *Gene* **7,** 51–52.

Genomic Mapping

Carle, G. F., Frank, M., and Olson, M. V. (1986). Electrophoretic separations of large DNA molecules by periodic inversion of the electric field. *Science* **232,** 65–68.
Coulson, A., Sulston, J., Brenner, S., and Karn, J. (1986). Toward a physical map of the genome of the nematode. *Caenorhabditis elegans. Proc. Natl. Acad. Sci. U.S.A.* **83,** 7821–7825.
Daniels, D. L., and Blattner, F. R. (1987). Mapping using gene encyclopaedias. *Nature (London)* **325,** 831–832.
Olson, M. V., Dutchik, J. E., Graham, M. Y., Brodeur, G. M., Helms, C., Frank, M., MacCollin, M., Scheinman, R., and Frank, T. (1986). Random-clone strategy for genomic restriction mapping in yeast. *Proc. Natl. Acad. Sci. U.S.A.* **83,** 7826–7830.
Rackwitz, H.-R., Zehetner, G., Frischauf, A.-M., and Lehrach, H. (1984). Rapid restriction mapping of DNA cloned in lambda phage vectors. *Gene* **30,** 195–200.
Smith, C. L., and Kolodner, R. D. (1988). Mapping of *Escherichia coli* chromosomal Tn5 and F insertions by pulsed field gel electrophoresis. *Genetics* **119,** 227–236.

3

SOUTHERN BLOT ANALYSIS

Southern Blot Introduction

The detection of homologous DNA sequences through the method of Southern blotting (Southern, 1975) has made a tremendous contribution to molecular biology and recombinant DNA technology. Southern blotting technology is essential for understanding basic problems such as gene structure, gene expression, and genome organization (Meinkoth and Wahl, 1984). Increasingly, Southern blotting plays a role in the diagnosis of heritable diseases and in the detection of microbial and viral pathogens (see Lerman, 1986 and Wiley, 1988). Southern blot analysis also has forensic applications (Neufeld and Colman, 1990). This chapter presents the methods for Southern blotting using a nonradioactive biotinylated nucleic acid detection system or a radioactively labeled DNA detection system.

A simple form of the Southern blot is a plasmid-to-plasmid hybridization, where plasmid DNA digested with restriction enzymes is subjected to electrophoresis on a gel and blotted. A piece of the plasmid (a cloned fragment or a fragment isolated from a gel) is used as a hybridization probe. Such experiments are used to map the location of the fragment and to construct maps of restriction endonuclease sites.

In Chapter 4, a plant genomic Southern blot analysis is presented. In such an experiment, genomic (chromosomal) DNA is isolated from an organism, cut with restriction endonucleases, subjected to electrophoresis on a gel and blotted. A cloned piece of DNA from the organism or a related organism can then be used as a probe for hybridization. Such experiments give information about genomic organization. Questions such as the number of copies and the location of the gene homologous to the probe can be answered.

Using Southern Blot Analysis to Map Restriction Endonuclease Sites

In this experiment, a DNA fragment subcloned from a larger DNA will be mapped to the original, larger DNA. The subcloned fragment is used as a hybridization probe to probe a Southern blot of the original, larger DNA cut with different restriction endonucleases.

Other aids to restriction site mapping include:

1. Isolate a restriction endonuclease fragment from an agarose gel. Cut the fragment with other restriction endonucleases. Size the fragments produced on an agarose gel. This method can identify restriction sites within the large fragment.
2. Label one end of a linear DNA to be mapped, as done by Smith and Birnstiel (1976). Cut the DNA with a restriction endonuclease under conditions that will give partial digestion. (That is, the DNA fragment will not be cut at all the sites for that restriction enzyme.) Separate the fragments produced by gel electrophoresis. The labeled end is detected. A ladder of fragment sizes generated is used to determine the sizes of adjacent restriction endonuclease sites for that enzyme.
3. See Danna (1980) and Rakwitz *et al.* (1984) for other mapping strategies.

Nonradioactive Labeling of Nucleic Acids

Nonradioactive labeling of nucleic acids to be used as hybridization probes has several advantages over radioactive labeling of nucleic acids. Nonradioactive labeling methods eliminate the need to deal with the licensing, waste disposal, and safety concerns associated with the use of radioactive material. The probes generated in nonradioactive labeling systems are more stable than probes labeled with ^{32}P. The detection sensitivities of the radioactive and nonradioactive probes are comparable. Nonradioactive detection methods typically require shorter exposure times to detect the labeled probe (Beck and Koster, 1990; Beck, 1992; Kessler, 1992; Kricka, 1992; Pollard-Knight, 1990).

Types of nonradioactive labeling and detection systems used include the horseradish peroxidase system, the digoxigenin–anti-digoxigenin system, and the biotin–streptavidin system. With all three of these systems the hybridized probe may be detected with chromogenic (colorimetric) substrates (substrates that produce a colored product) or chemiluminogenic substrates (substrates that produce products that give off light) for the enzymes involved in each system. See Figure 3.1.

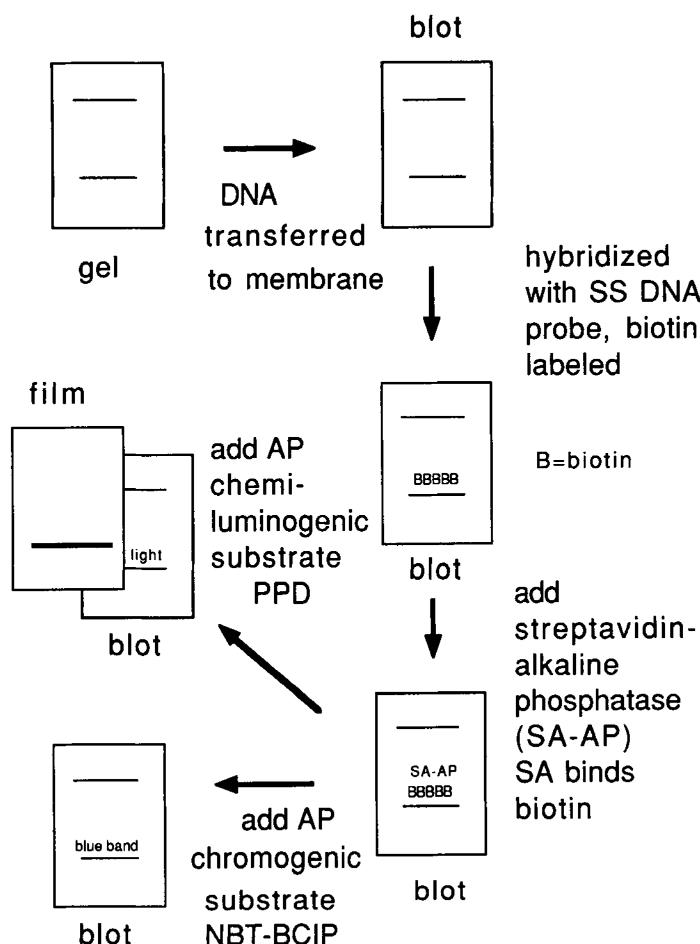


Figure 3.1 Overview of biotin-based nonradioactive DNA detection systems. DNA from a gel is transferred to a membrane and hybridized with a specific biotin-labeled single-stranded DNA probe. The biotin-labeled DNA is detected by the addition of streptavidin-alkaline phosphatase (SA-AP). Streptavidin binds tightly to biotin. A chromogenic or chemiluminogenic substrate for alkaline phosphatase is then added either to produce a blue precipitate or to give off light.

Horseradish Peroxidase and Enhanced Chemiluminescence

In the horseradish peroxidase nonradioactive system, a chemical reaction is used to label the DNA to be used as a probe (Stone and Durrant, 1991). Horseradish peroxidase (HRP) is covalently linked to polyethyleneimine. In the labeling reaction, denatured DNA is mixed with HRP–polyethyleneimine plus 1% glutaraldehyde at room temperature. Glutaraldehyde is a bifunctional crosslinking reagent that covalently crosslinks the

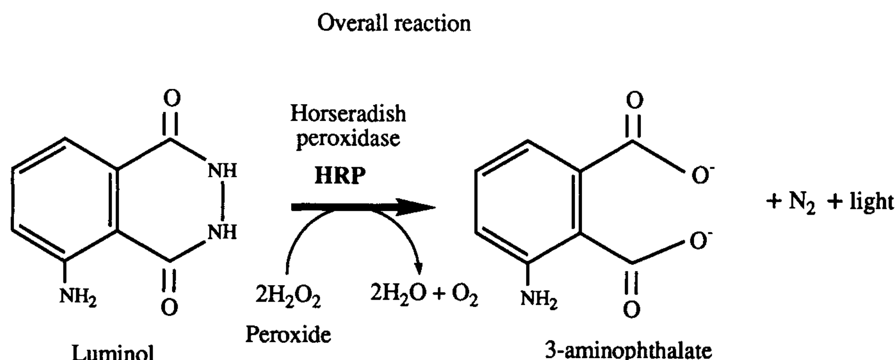


Figure 3.2 Enhanced chemiluminescence. The detection of horseradish peroxidase-labeled DNA by the addition of luminol. Light is given off.

marker enzyme HRP to the DNA probe. The crosslinking reaction takes an hour. Further reactions with the HRP-labeled probe DNA are carried out under conditions that keep the HRP in an active form. Urea (6 *M*) is used to lower the T_m so that the hybridization temperature is 42°C. Either chromogenic or chemiluminogenic substrates for horseradish peroxidase can be used. In the presence of peroxide and peroxidase, chloronaphthol, a chromogenic substrate for horseradish peroxidase, forms a purple insoluble product. HRP also catalyzes the oxidation of luminol, a chemiluminogenic substrate for HRP. The oxidized luminol enters an excited state that may emit light at a wavelength of 428 nm as it decays to the ground state. Figure 3.2 shows the overall reaction of enhanced chemiluminescence with HRP. Enhancers such as p-iodophenol are added to the reaction to increase the intensity of the light produced. About 1 pg of DNA can be detected in less than 1 hr (Stone and Durrant, 1991). The chemiluminescent reaction with luminol and enhancers reaches a maximum output very rapidly, within 1 to 5 min. The production of light also decays relatively rapidly and lasts about 3 hr. This rapid decay time can be a problem with HRP chemiluminescent detection, because all the exposures of film must be obtained in a relatively short time. However, if a blot is to be reprobed, this relatively rapid decay of light production is an advantage. A blot probed with a HRP-labeled probe detected with enhanced chemiluminescence can be rehybridized with a different probe in a very short time.

Digoxigenin Nonradioactive Labeling System

The digoxigenin–anti-digoxigenin labeling system uses digoxigenin (DIG), a cardenolide steroid isolated from *Digitalis* plants (Martin *et al.*,

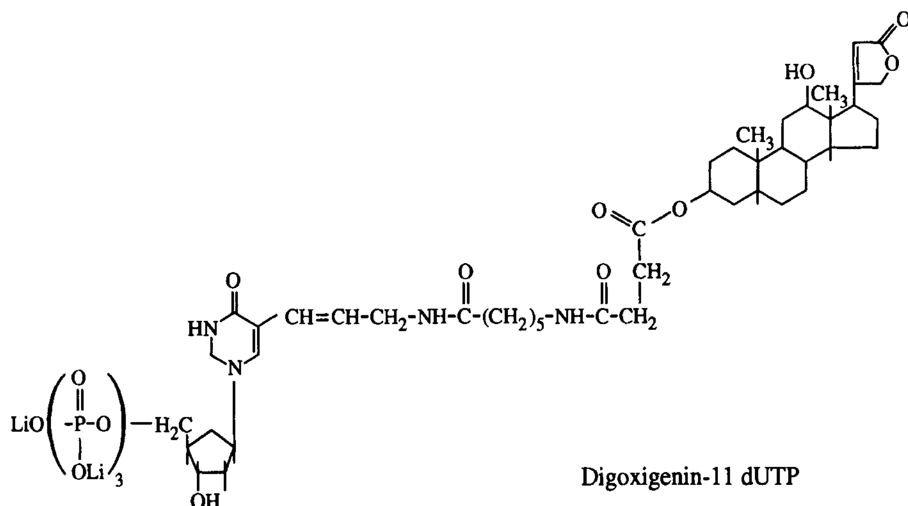


Figure 3.3 Structure of digoxigenin-11-dUTP. This nucleotide analog is incorporated into a DNA by nick translation or by oligo labeling. The digoxigenin-labeled DNA is detected by the binding of a digoxigenin-specific antibody conjugated to alkaline phosphatase and the addition of a chromogenic or chemiluminogenic substrate for alkaline phosphatase.

1990). A nucleotide triphosphate analog containing the digoxigenin moiety (Figure 3.3) is incorporated into DNA by nick translation or random primer labeling. The DIG-labeled probe is detected by an enzyme-linked immunoassay using an antibody to digoxigenin (anti-DIG) to which alkaline phosphatase has been conjugated. A chromogenic or chemiluminogenic substrate for alkaline phosphatase can then be used to detect the DIG-labeled probe. There is high specificity between DIG and anti-DIG, resulting in a detection system with low background.

Biotin–Streptavidin Labeling System

The interaction of biotin (Figure 3.4) and avidin has been widely used in immunology. Avidin, a basic glycoprotein of 68,000 Da isolated from egg white, binds strongly and noncovalently to biotin, a vitamin, with a K_d of 10^{-15} M^{-1}. Each avidin molecule binds four biotin molecules. Avidin–biotin binding reactions can have high backgrounds because the basic isoelectric point of avidin favors stronger electrostatic interactions and the carbohydrate moiety of avidin has a tendency to bind to lectin-like proteins. Problems with high backgrounds can be avoided by using a biotin–streptavidin system. Streptavidin is an extracellular protein from *Streptomyces avidinii* that is very similar to avidin. Streptavidin has a molecular weight of 60,000 and has four identical subunits, each of which can bind a biotin molecule. Streptavidin, like avidin, has strong biotin-

binding properties but has fewer nonspecific background binding problems than avidin.

A biotin-containing nucleotide analog is incorporated into DNA by nick translation or by random primer labeling (Figure 3.4) (Gebeyehu *et al.*, 1987; Mackey and Rashtchian, 1992; Mackey *et al.*, 1992b). After hybridization, the biotin-labeled DNA is detected by the tight and specific binding of streptavidin that has been conjugated to alkaline phosphatase. A chromogenic or a chemiluminogenic substrate for alkaline phosphatase is used (Klevan and Gebeyehu, 1990; Langer *et al.*, 1981; Leary *et al.*, 1983). Gibco/BRL reports the ability to detect less than 100 fg of bacteriophage DNA in a Southern blot using a biotinylated probe (Mackey *et al.*, 1993).

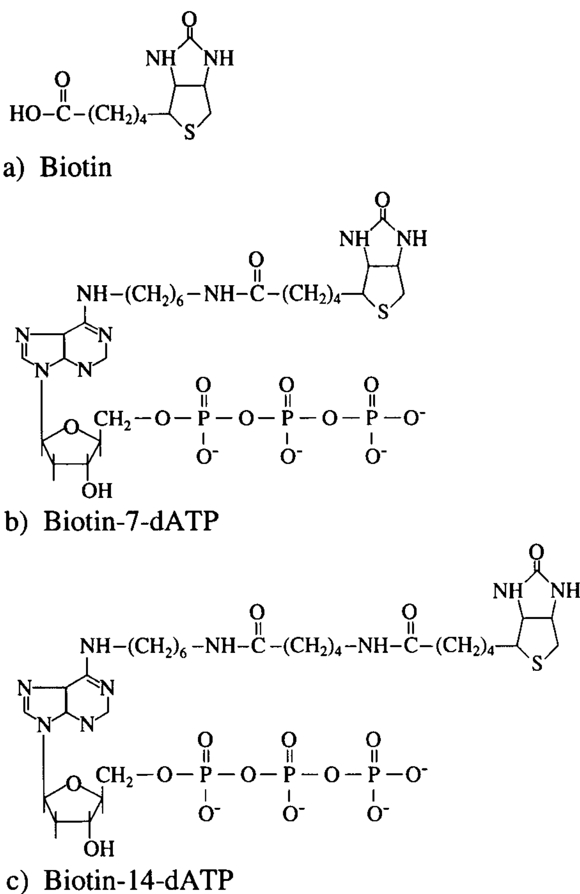


Figure 3.4 Structures of biotin and two biotin nucleotide analogs, biotin-7-dATP and biotin-14-dATP.

Chromogenic Substrate for Alkaline Phosphatase

A commonly used chromogenic substrate system for alkaline phosphatase is 5-bromo-4-chloro-3-indolyl phosphate (BCIP) with nitroblue tetrazolium chloride (NBT). After the phosphate group of the soluble, colorless BCIP is removed enzymatically by alkaline phosphatase, the indolyl product formed is oxidized to produce an indigoid dye that is an insoluble dimer. The indigo color produced is amplified by the addition of NBT to the system. NBT acts as an oxidant for the indolyl and is itself reduced to produce the intense blue dye NBT-formazan. NBT is a soluble salt that, when reduced to NBT formazan, is insoluble (Leary *et al.*, 1983; Guder, 1992) (Figures 3.5A and 3.5B).

Chemiluminogenic Substrate for Alkaline Phosphatase

Dioxetane chemiluminogenic substrates are used with digoxigenin or biotin–streptavidin systems. There are a number of stable 1,2-dioxetane derivatives that emit light when activated by enzymes (Beck and Koster, 1990; Bronstein and McGrath, 1989; Bronstein *et al.*, 1989). A 1,2-dioxetane that is commonly used has a phosphate group attached and can be enzymatically activated by alkaline phosphatase. The alkaline phosphatase can be directly linked to the DNA used as a probe (Mackey *et al.*, 1992a) or it can be covalently linked to streptavidin and then bound to a biotin-labeled probe. Alternatively, alkaline phosphatase can be covalently linked to an antibody directed against a hapten, such as digoxigenin. Chemiluminogenic substrates for alkaline phosphatase include PPD (Figure 3.6) and CSPD. PPD, also called AMPPD, is 4-methoxy-4-(3-phosphatephenyl) spiro [1,2-dioxetane-3,2′-adamantane] (Lumigen, Detroit, MI). CSPD is disodium 3-(4-methoxy-spiro[1,2-dioxetane-3,2′-(5′-chloro)-tricyclo [3.3.1.$1^{3,7}$]decan]-4-yl)phenyl phosphate (Tropix, Bedford, MA).

The emission of light in this reaction is a two-step process. First, enzymatic dephosphorylation occurs. The anion produced subsequently decomposes and gives off light. In the presence of excess substrate, the light intensity produced depends upon the concentration of alkaline phosphatase. This chemiluminescent reaction has a high quantum yield because of a rapid turnover of the enzyme. In addition, other components present in the reaction can enhance the signal intensity. The presence of macromolecules such as bovine serum albumin (BSA) or the formation of aqueous micelles with molecules such as cetyltrimethyl-ammonium bromide (CTAB) enhances the signal intensity and shifts the wavelength of light emitted. In solution, the light emission maximum is at 470 nm. In the presence of a micelle-forming material or a nylon membrane, the

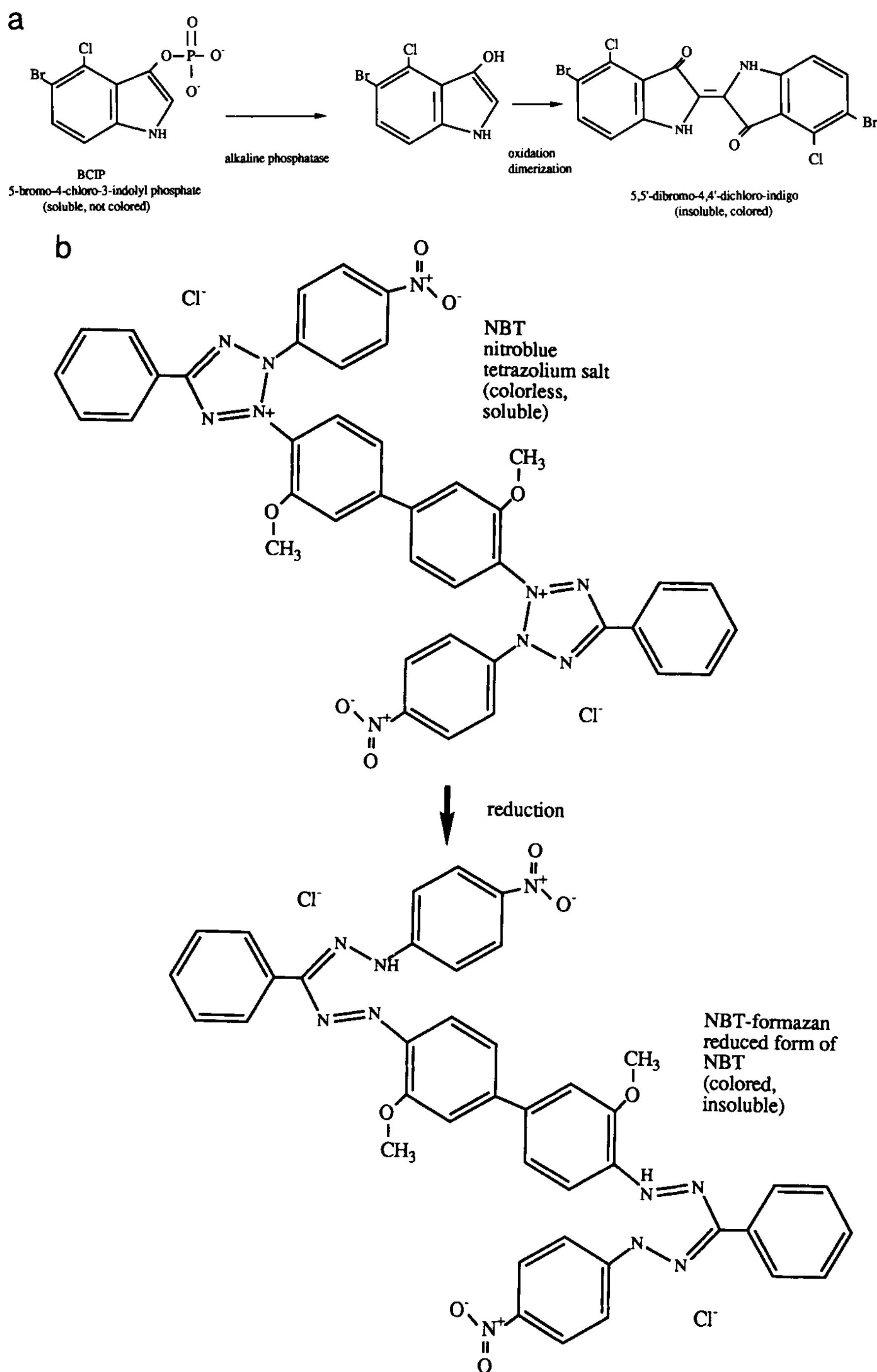


Figure 3.5 A chromogenic (color producing) substrate for alkaline phosphatase. (a) When alkaline phosphate removes the phosphate group from BCIP, 5-bromo-4-chloro-3-indolyl phosphate, the resulting molecule dimerizes under oxidating conditions to give the molecule 5,5′-dibromo-4,4′-dichloro-indigo, which is a blue precipitate. (b) Also during the reaction with BCIP, NBT, nitroblue tetrazolium, which is also present, is reduced to its colored form to give an enhanced color reaction.

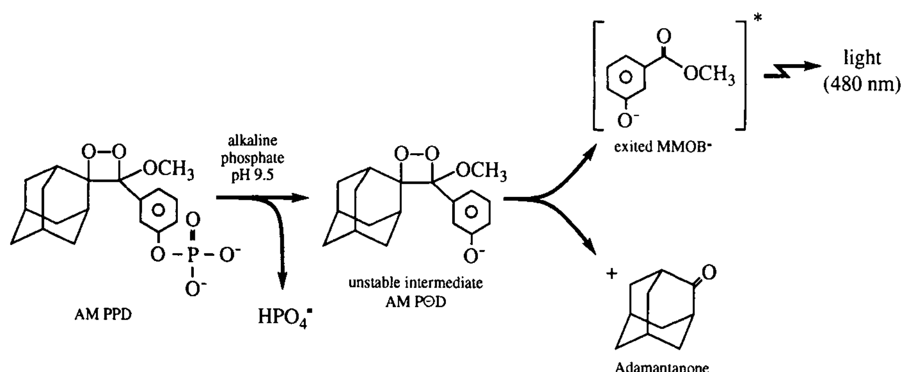


Figure 3.6 A chemiluminogenic substrate for alkaline phosphatase. When alkaline phosphatase removes a phosphate group from AMPPD (4-methoxy-4-(3-phosphatephenyl) spiro [1,2-dioxetane-3,2'-adamantane]), the unstable intermediate breaks down and emits light at 480 nm. The presence of other components in the reaction can shift the wavelength of the emitted light.

light emission maximum is about 460 nm. Other enhancers added to the reaction may also change the wavelength of light emitted. In addition, the excited molecule decays relatively slowly so the light signal lasts for many hours or days. This allows ample time to obtain optimum film exposure.

Autoradiography: Overview

The film used to detect the ^{32}P-labeled probe has a radiation-sensitive emulsion of minute crystals of silver halides such as bromides, chlorides, and/or iodides, suspended in gelatin (Hahn, 1983). The gelatin provides a stable matrix through which the processing solutions can readily pass. This emulsion in gelatin is coated onto a flexible film support. Often double-coated film, with emulsion on both sides, is used.

To produce a "developable" silver halide grain the exposing energy is absorbed by silver halide grains, causing electrons to be released. A mobile electron can then reduce Ag^+ to an Ag atom. These events are repeated, resulting in the production of more reduced Ag. In the most sensitive films, 3 to 6 Ag atoms are needed to form a stable center that can be detected when the film is developed. (Other references say between 6 and 30 Ag atoms are needed.) This stable center is called a *latent image center. Latent* means "present but invisible, lying hidden and undeveloped." The latent image centers in the emulsion are not very stable; they can decay to smaller than a stable size as Ag^+ is formed again. The rate of decay of these centers increases in the presence of oxygen and moisture. Thermal agitation of the crystal lattice at room temperature can also destroy the centers.

More than one photon of light is needed to produce these three to six Ag atoms, but for ionizing radiation such as β particles, X rays, or γ rays, one interaction can produce a latent image center of three to six Ag atoms. Note that only a very small amount of the silver halide grain or crystal is in the Ag form. Most is still Ag^+X^-.

Photographic processing makes the latent image visible. The developer solution reduces the rest of the silver halide grain to metallic silver Ag atoms in those silver grains that have Ag atoms in them, that is, have a latent image center (or have been exposed). Silver grains that do not contain Ag atoms (were not exposed) are not affected by the developer. The developing process amplifies the latent image about a billion times! The stop bath neutralizes the developer. Then, a fixer solution is used to remove all the silver halide that was not affected by the developer.

Sambrook *et al.* (1989) state that 1000–5000 cpm of ^{32}P in a 1-cm-wide band can be detected in a 12- to 16-hr exposure. ^{32}P has a half-life of 14.3 days; when it decays, a β^- particle is emitted. To increase the sensitivity of detection of the film, the film and blot are placed at −70°C. This temperature reduction delays the decay of the latent image caused by thermal agitation. Also, the X-ray film can be "preflashed"—exposed to a brief period of light (about a millisecond!). Longer flashes of light increase background on the film without increasing sensitivity. This flash of light sensitizes the film by activating the silver halide crystals. If more areas of the crystals already contain Ag atoms, then it is more likely that the next single event will produce an image that will be recorded when the film is developed.

Intensification screens are also used. These screens contain inorganic phosphor crystals such as calcium tungstate. When an X ray hits the screen, inner shell electrons of the phosphor are expelled, emitting another X ray. In addition, the electrons excite the outer shell electrons of the phosphor, producing fluorescence. The net result is that for every X ray that collides with the screen, several thousand photons are produced that can then be recorded on the film. The mechanism of interaction with intensification screens for the β^- particle produced by ^{32}P is unknown but is probably similar to that of X rays. Sambrook *et al.* (1989) report that the use of two calcium tungstate phosphor screens can increase the sensitivity of detection of ^{32}P 8- to 10-fold.

One concern is the linearity of response, important for comparing the strength of different signals. The response of the film at low light intensities is nonlinear. The use of very low temperature also prolongs the period of fluorescence, thereby increasing the chance that two events will occur close enough in time to be detected on the film. Another concern is resolution—the ability to detect two closely spaced bands as separate bands. The use of intensification screens increases the sensitivity but decreases the resolution of the film because the photons produced by

fluorescence may disperse—spread out a bit—before they are recorded on the film.

For detection of a nonradioactive probe: When a chemiluminescent substrate is used for a nonradioactive probe, X-ray film or other film is used. Preflashing the film can make it more sensitive, but preflashing the film is not helpful for exposure times of less than 24 hr. The chemiluminescent signal is so strong that generally exposure times are less than 24 hr. The chemiluminescent reaction must be at a temperature that allows activity of the enzyme used in the detection reaction, such as alkaline phosphatase. Typically, this assay is performed at room temperature. United States Biochemical (USB) suggests incubation at 37°C be used for the chemiluminescent assay for biotin-labeled probes.

Isolation of Nucleic Acid Fragments from Gels

Frequently, it is useful to isolate individual bands of a particular size of nucleic acid from gels. This technique is useful for isolation of individual framents to be used for subcloning a specific fragment of DNA. DNA isolated from bands in gels can also be digested with additional restriction endonucleases to generate restriction endonuclease site maps. In addition, individual bands of DNA can be labeled and used as specific probes in hybridization experiments.

Over the years numerous methods have been employed to isolate individual bands of DNA from gels. A general trend reported in all of these procedures is that is is easier to recover smaller DNA fragments quantitatively. An ideal procedure would have at least a 90% yield for a wide range of DNA fragment sizes. One of the early methods was to electroelute the specific DNA into a trough cut in the gel. Following restriction endonuclease digestion and agarose gel electrophoresis to separate DNA fragments on the basis of size, the agarose gel is stained with ethidium bromide and bands are visualized by UV illumination. Using a clean razor blade, a rectangular piece of agarose is cut out of the gel in front (toward the direction the DNA migrates—that is, toward the positive pole) of the DNA fragment to be isolated. Removal of this rectangle of agarose creates a trough in the gel. The trough can then be filled in several ways. The leading edge of the trough can be lined with a piece of dialysis tubing and the trough filled with electrophoresis running buffer. Electrophoresis is resumed and the DNA fragment of interest migrates toward the positive pole and into the buffer in the trough. The DNA cannot migrate through the dialysis tubing and so is trapped in the trough. The buffer and DNA solution are then removed. Usually, the DNA sample would be precipitated, by adding salt and alcohol, to concentrate the sample. Alternatively,

the trough could be filled with a matrix such as hydroxyapatite or diethylaminoethyl (DEAE)-cellulose. As the DNA migrates into the trough during electrophoresis, the DNA binds to the matrix material in the trough and cannot migrate farther. The matrix material is removed from the trough and the DNA is eluted from the matrix with buffers of the appropriate salt concentration. Note that if electrophoresis is continued for too long, DNA bands larger than the band of interest can also migrate into the trough.

In many other methods of isolating DNA from a gel, the specific band containing the DNA of interest is first cut from the gel and then one of a number of methods is employed to remove the DNA from the gel slice. One such method of removing DNA from a gel slice is the "freeze and squeeze" method. The agarose slice with DNA is frozen to −20°C and then, using a gloved hand, the gel is squeezed. With this procedure as much as 70% of the gel weight can be squeezed out as a clear liquid. The liquid, containing the DNA, is centrifuged for 5 min at 8000g in a microfuge to pellet the gel. After centrifugation, the DNA sample in the supernatant solution can be used as is (Thuring *et al.*, 1975).

Another means of removing DNA from a slice of an agarose gel is to dissolve or disrupt the agarose gel. A chaiotrophic agent such as NaI disrupts the agarose gel. Glass beads to which DNA will bind are then added. The glass beads with DNA bound are pelleted by centifugation to separate the DNA from the agarose. Then, under high salt conditions, the DNA is washed from the glass beads (Vogelstein and Gillespie, 1979).

Agarase has been used to solubilize the agarose gel and thereby release the DNA from the gel slice. Commercially available agarase may, however, be contaminated with nucleases.

Low-melting-point agarose has also been used to facilitate separation of agarose from DNA. Low-melting-point agarose melts at a lower temperature than standard agarose. The lower melting temperature does not denature double-stranded DNA. Once the agarose has been melted, a phenol extraction of the melted agarose is typically done. Some procedures use a quaternary ammonium salt such as hexadecyltrimethylammonium bromide to partition the DNA away from the melted agarose. The cost of special low-melting-point agarose is approximately two and one-half times greater than that of standard agarose.

Several physical procedures have been used to separate DNA from an agarose gel slice (Weichenhan, 1991; Zhu *et al.*, 1985). In some protocols, centrifugation through siliconized glass wool, through chromatography paper, or through a filter membrane such as GeneScreen is used. Through centrifugal force, the DNA is eluted from the agarose. The agarose is retained by the glass wool, membrane, or paper while the DNA solution passes through. A related procedure uses ultracentrifugation of a gel slice to cause the collapse of the agarose into a pellet. After such an ultracentrifugation, the DNA is found in the supernatant.

Electroelution is a method that has been used quite successfully to separate the DNA from an agarose gel slice. The gel slice and electrophoresis buffer are placed within a piece of dialysis tubing. During electrophoresis, the DNA migrates out of the gel slice into the buffer in the dialysis bag. The DNA cannot migrate through the tubing because of the small pore size of the dialysis tubing. The buffer and DNA are then removed from the dialysis bag and ethanol is added to precipitate the DNA. Recently, several modifications of electroelution have been used in which the dialysis bag is replaced by a small chamber made in a microfuge tube. These modifications have the advantage of keeping the electroelution volumes smaller.

Labeling Methods

Nick Translation

Nick translation is one method of labeling DNA to be used as a hybridization probe. This method uses the enzymes pancreatic DNase I and *Escherichia coli* DNA polymerase I. Under the nick translation reaction conditions that use Mg^{2+}, DNase I randomly nicks double-stranded DNA to leave 5′ phosphate termini. A nick is a break in a phosphodiester bond in one strand of a double-helix. In the presence of Mn^{2+}, DNase I behaves differently and generates cuts in both strands of the DNA at approximately the same site.

Escherichia coli DNA polymerase I has three activities: (1) a 5′ to 3′ polymerase activity that requires a single-stranded template and a primer with a 3′ hydroxyl group to synthesize a new nucleotide chain complementary to the template; (2) a 5′ to 3′ exonuclease activity that degrades double-stranded DNA from a free 5′ end; and (3) a 3′ to 5′ exonuclease activity that degrades double- or single-stranded DNA from a free 3′ hydroxyl end. This latter activity is a proofreading or editing function. On double-stranded DNA, the 3′ to 5′ exonuclease activity is blocked by the 5′ to 3′ polymerase activity. Overall the nick translation reaction results from the process by which *E. coli* DNA polymerase I adds nucleotides to the 3′-OH created by the nicking activity of DNase I while the 5′ to 3′ exonuclease activity simultaneously removes nucleotides from the 5′ side of the nick. The result of these activities is that nucleotides are eliminated from the 5′ side of the nick while nucleotides are added to the 3′ side of the nick. This results in the movement—or translation—of the nick along the DNA. If labeled precursor nucleotides are present in the reaction, the preexisting nucleotides are replaced with labeled nucleotides. For radioactive labeling of DNA, the precursor nucleotide is an [α-^{32}P]dNTP. The phosphate group that is incorporated in the growing nucleic acid chain is the α-phosphate (see Figure 3.7).

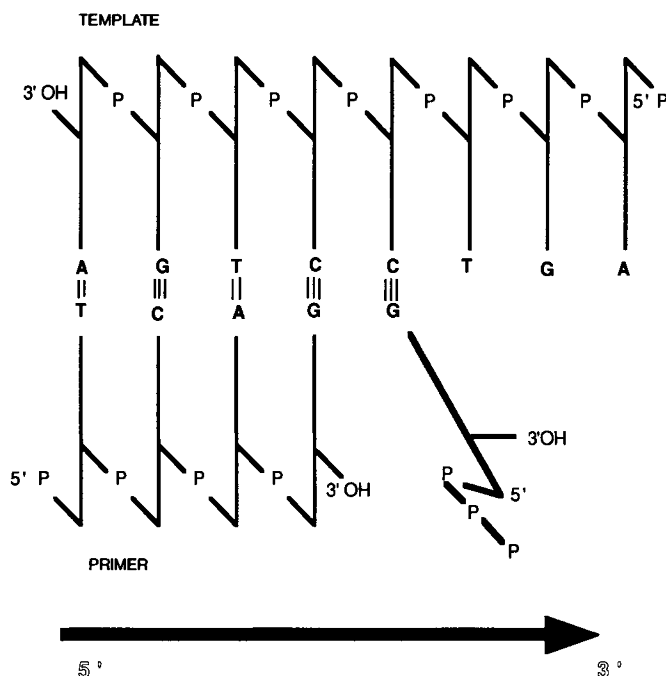


Figure 3.7 The action of a DNA polymerase showing how a nucleotide triphosphate is added to the primer.

For nonradioactive labeling procedures, a biotin or digoxigenin moiety attached to a dNTP analog is used (see Figures 3.3 and 3.4).

The nick translation labeling reaction works with a concentration of dNTPs as low as 2 μM, but the labeling is more efficient if higher concentrations of dNTPs are used. For example, a nick translation reaction might use a concentration of 2 μM labeled nucleotide and 20 μM unlabeled nucleotides. To increase the amount of labeled nucleotide incorporated, and thus increase the specific activity of the probe, more than one labeled dNTP can be used in the reaction. Alternatively, the ratio of labeled to unlabeled dNTPs can be increased to incorporate more labeled nucleotides. In addition, the amount of DNase I in the reaction can be increased to increase the number of nicks and then the extent of replacement of the template. The size of the labeled DNA resulting from nick translation will depend on the amount of DNase I in the reaction and is typically from 400 to 800 bases. In some cases if a longer probe were desired, the probe could be ligated after the labeling reaction. A smaller probe may be useful for *in situ* labeling detection. Kits for nick translation labeling reactions are commercially available. As little as 20 ng of DNA can be used in a nick translation labeling reaction, but a typical amount of template to be

labeled is 1 μg of template in a 50-μl reaction. The nick translation reaction is performed at 16°C for 60 to 90 min. At higher temperatures, the polymerase can copy the newly synthesized strand, thus producing DNA molecules that are self-complementary and called "snap-back" DNA. To stop the nick translation reaction, EDTA can be added. For a radioactive labeling reaction, phenol can be added to extract enzymes. For a biotin labeling reaction, phenol extraction is not recommended because the biotin moiety may partition into the phenol phase and the labeled DNA would be lost.

After the labeling reaction, the unincorporated labeled nucleotide is separated from the labeled DNA. The presence of unincorporated labeled nucleotides in a hybridization reaction can give a high level of background resulting from the random binding of the nucleotides to the membrane.

To separate the incorporated from unincorporated labeled nucleotides, the sample can be ethanol precipitated. The labeled DNA precipitates while the unincopoporated nucleotides do not precipitate. Typically, in an ethanol precipitation of DNA, polynucleotides longer than 15 to 20 nucleotides will precipitate while smaller oligonucleotides will remain in solution. The size of the polynucleotide that can be ethanol precipitated will also vary with the concentration of the polynucleotide or the amount of extra DNA, called carrier DNA, added.

Another way of separating incorporated from unincorporated nucleotides is by gel exclusion chromatography. A small column made from a Pasteur pipet is packed with a matrix material such as Sephadex G-100. The G-100 matrix consists of many small beads with small pores or channels in the beads. To make the column, the G-100 beads are packed into the pipet. The nick translation reaction is combined with a mixture of two dyes: blue dextran and orange G. The nick translation reaction is loaded onto the column and fractions are eluted. The blue dextran is a large molecule; the orange G is a very small molecule. The labeled DNA and blue dextran are too large to enter the small channels in the G-100 beads so these molecules elute from the column first as part of the excluded volume of the column. Small molecules such as the labeled unincorporated nucleotides and the orange G dye can enter the channels of the G-100 beads and therefore have a longer pathway through the column. The smaller molecules elute from the column later than the larger molecules. This is the basis of size exclusion column chromatography. The size of the poly- or oligonucleotide that is excluded varies with the matrix material. Pharmacia says that its Sephadex G-100 matrix excludes oligonucleotides that are longer than 25 nucleotides. The presence of the two dyes makes it easy to track the progress of the molecules to be separated through the column. The blue dye comigrates with the labeled DNA; the orange dye comigrates with the unincorporated labeled nucleotide. Thus the blue fraction from the G-100 column is collected.

Oligo Labeling

Another way to label DNA is oligo labeling, also called random primer or primer extension labeling. In this labeling procedure, double-stranded DNA is denatured to the single-stranded form. Short oligonucleotides of random sequence that hybridize randomly to the single-stranded DNA are added. The hybridized oligonucleotides serve as primers for the initiation of DNA synthesis by a DNA polymerase. The polymerase used is the Klenow fragment of *E. coli* DNA polymerase I. Intact *E. coli* DNA polymerase is 109,000 Da. When the enzyme is cleaved with subtilisin, a large fragment of 76,000 Da, the Klenow fragment, is generated. It has the 5′ to 3′ polymerase activity and the 3′ to 5′ exonuclease activity, but not the 5′ to 3′ exonuclease activity. The use of the Klenow fragment ensures that DNA synthesis occurs only by primer extension and that the hybridized primers will not be degraded because there is no 5′ to 3′ exonuclease activity. If the primers are not degraded, the labeling is more random. The random primers can be obtained by a digestion of calf thymus or salmon sperm DNA with DNase I in the presence of Mn^{2+} to cut the DNA. The DNA is then denatured to yield single-stranded oligonucleotides 6 to 12 nucleotides long. Alternatively, the primers can be synthesized by an automated DNA synthesizer to generate octamers that have any of the four bases at each position. The synthesized primers are of equal length and without sequence bias. Kits with all the components for oligo labeling are commercially available. If the primers used in the oligo labeling reaction are random, hybrids will form at many positions along the single-stranded DNA template. All parts of the template DNA will be copied at equal frequency except perhaps the extreme 5′ end of the template. Since the polymerase activity is 5′ to 3′ from a primer, the extreme 5′ end cannot be copied unless a primer has hybridized at the very end of the template.

The oligo labeling reaction is performed at room temperature or at 37°C for 2 to 3 hr, although the reaction can be allowed to go for up to 16 hr. Frequently the reaction results in greater than 90% of the labeled precursor incorporated into a probe. In such cases it may not be necessary to separate the unincorporated labeled nucleotide from the labeled probe. In an oligo labeling reaction, a molar excess of primers is used. The lower the concentration of template in the reaction, the greater the specific activity achieved in the labeling. For radioactive labeling, specific activities in the range of 5×10^8 to 4×10^9 cpm/μg DNA can be obtained. It is interesting to note that ^{32}P-labeled probes with very high specific activities should be used immediately because the probes will degrade rapidly due to radiochemical decay.

The average size of the labeled probe is inversely proportional to the concentration of primers. At low primer concentrations, the probes produced may be up to 50% the length of the entire template molecule. At high primer concentrations, probes between 400 and 600 bp are obtained. It

is possible to label small amounts of DNA, as little as 10 ng. Reactions with low concentrations of template DNA will have slower rates and may take at least 5 hr to complete. There is a net synthesis of DNA during the labeling reaction.

High hybridization backgrounds with oligo-labeled probes can occur for several reasons, including the presence of a large amount of unincorporated label or the presence of too much template DNA in the labeling reaction. When there is a large excess of template DNA, shorter probes are generated because the reaction runs out of precursor dNTPs and only short chains are synthesized. Shorter probes may hybridize randomly. See Table 3.1 for a comparison of nick translation and oligo labeling.

Table 3.1
Comparison of Nick Translation and Oligo Labeling of DNA

	Nick translation	Oligo labeling or random primer labeling
Enzymes	Pancreatic DNase I and *E. coli* DNA polymerase I 5′ to 3′ polymerase 3′ to 5′ exonuclease 5′ to 3′ exonuclease	Klenow fragment of *E. coli* DNA Polymerase I 5′ to 3′ polymerase 3′ to 5′ exonuclease Lacks 5′ to 3′ exonuclease
DNA to be labeled	Double-stranded DNA, can be circular DNA	Single-stranded DNA, usually best if linear
Concentration of DNA to label	0.5 μg in 25-μl reaction; can use as little as 20 ng BRL kit recommends 1 μg	10–25 ng
To increase specific activity	Increase number of nicks by increasing concentration of DNase I; add another dNTP with label	Decrease concentration of probe template to be labeled
Sequence bias	None	May not label extreme 5′ end of probe
Size of labeled DNA	400–800 bases, varies with the concentration of DNase I used	If 25 ng of probe used in reaction: greater than 50% the length of the probe If 200 ng of probe used in reaction: 400–600 bases
Temperature and time to label	16°C for 60–90 min	Room temperature or 37°C for 2–3 hr up to 16 hr
Labeling electroeluted fragment	DNase I is sometimes inhibited by contaminating agarose *E. coli* DNA polymerase I is not affected by agarose	Klenow fragment is not affected by agarose

With a radioactive labeling system, oligo labeling is typically the method of choice because a small amount of template can be readily amplified and labeled to give a high-specific-activity probe. When biotin is used in a labeling system, either nick translation or oligo labeling can be used. The method of choice would depend on the amount of DNA available to use in the labeling reaction. When very little template DNA is available, an oligo labeling is more useful because it requires less template DNA in the reaction. With a biotin labeling system, the sensitivity of the detection of biotin-labeled DNA probes does not increase very much when the degree of nucleotide incorporation of biotin increases beyond the value of 10 to 30 nucleotides per kilobase of template DNA. There must be other factors contributing to the sensitivity, such as the interaction of biotin with streptavidin.

Biotin-labeled probes should not be subjected to phenol extraction because the biotin may partition into the phenol phase. Biotin-labeled probes should be heat denatured, not denatured by high pH because alkaline pH may cause the biotin moiety to be cleaved from the dNTP. The kinetics of incorporation of a biotin-labeled nucleotide are the same as the kinetics for [^{32}P]dNTP incorporation. However, with a biotin-labeled probe, a small reduction in the T_m of hybrids is observed, presumably the result of steric hindrance of base pairing in the helix by the biotin moiety.

Photobiotin

An alternative, nonenzymatic way to label single-stranded or double-stranded nucleic acids with biotin is to use photobiotin. Photobiotin, *N*-(4-azido-2-nitrophenyl)-*N*′-(*N*-*d*-biotinyl-3-aminopropyl)-*N*′-methyl-1,3-propanediamine, a photo-activatable analog of biotin (see Figure 3.8). A photoreactive aryl azide group is attached to biotin through a charged linker arm. When photobiotin is illuminated with strong visible light in the presence of nucleic acids, the biotin moiety is covalently linked to

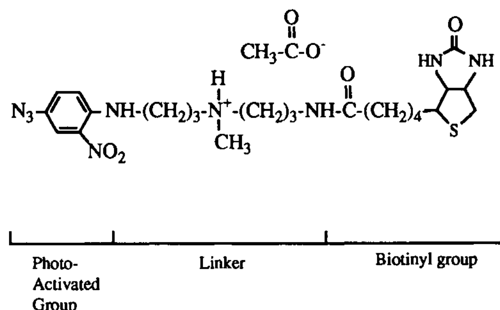


Figure 3.8 Structure of photobiotin acetate. Under bright visible light, the photoactivated group will interact with nucleic acids to label them with biotin.

the nucleic acid. The biotin-labeled nucleic acid is separated from photobiotin by 2-butanol extraction and ethanol precipitation. Forster *et al.* (1985) report labeling single-stranded M13 DNA with photobiotin to give one biotin per 100–400 nucleotides. Using this as a probe for dot blots (where nucleic acid is spotted on a membrane), they were able to detect as little as 0.5 pg of sequences complementary to the probe with a chromogenic biotin detection system. Others report that a photobiotin-labeled probe is not as sensitive as an enzymaticly labeled probe. Photobiotin labeling is a chemical reaction, not an enzymatic one. The materials for photobiotin labeling are more stable than the enzymes needed in nick translation or oligo labeling and are less expensive. Photobiotin may be the labeling method of choice when large quantities of probe are needed and when very high sensitivities are not needed.

Hybridization to Membranes

Methods for immobilizing DNA on nitrocellulose began with the work of Gillespie and Spiegelman (1965). Southern's pioneering work describing the transfer of DNA from an agarose gel to a nitrocellulose membrane—what is now called a Southern blot—was published in 1975. In this method, called a mixed-phase hybridization, one nucleic acid is tethered or attached to a membrane and the other is a labeled nucleic acid used as a probe. This mixed-phase hybridization had limited applications initially. It was not until the development of molecular cloning, which made available a myriad of gene-specific probes for hybridization, that this method became so important.

As Meinkoth and Wahl (1984) state in their review of this area, hybridization of nucleic acids immobilized on solid supports is "the cornerstone of the gene (and gene product) detection methods which have revolutionized our understanding of gene structure, genomic organization, and control of gene expression." The technique of Southern blotting is relatively rapid and inexpensive and requires a minimal amount of materials. The method is very sensitive. Detection of complementary sequence is routinely in the picogram range. The minimal amount of a complementary sequence that can be detected is reported to be in the femtogram range. This method has been essential for basic research, for the diagnosis of genetic diseases, and for the detection of microbial and viral pathogens.

The kinetics of the hybridization reaction between DNA or RNA probes and DNA tethered to a membrane are very similar to the kinetics of hybridization for nucleic acids free in solution. The rate of hybrid formation for single-stranded probes in mixed-phase hybridizations follows first-order kinetics because the concentration of probe is much greater than the concentration of target sequences. The kinetics of complementary,

i.e., nick-translated, probes is a little more complex because the probe can reanneal with itself. The reaction can be described by the equation

$$t_{1/2} = \ln 2/kc,$$

where $t_{1/2}$ is the time for half of the probe to anneal with the tethered DNA, k is the first-order rate constant, and c is the concentration of the probe.

k, the hybridization rate constant, depends on the following parameters:

The probe stand length
The molecular complexity (that is, the total number of base pairs of nonrepeating sequence)
The temperature
The ionic strength of the solution. At greater than 0.4 *M* the effects of ionic strength are small.
The viscosity of the solution
The pH of the solution. When the pH is between 5.0 and 9.0, the effects of pH on the rate constant are small.

For probes that are longer than 150 nucleotides, an empirical observation is that the maximum rate of hybridization for nucleic acids either free in solution or in mixed phase occurs at a temperature 25°C below the temperature of melting. The temperature of melting, or T_m, is the temperature at which half of the nucleotides in a double helix are dissociated. If the probe length is less than 150 nucleotides, in mixed-phase hybridization, the maximum rate of hybridization occurs at a lower temperature. It is possible to increase the rate of probe reannealing in mixed-phase hybridizations by adding dextran sulfate or other polyanions to the solution. The addition of the polyanion dextran sulfate increases the "effective concentration" of the probe by an "excluded volume effect." The large polyanion dextran sulfate and the negatively charged nucleic acid probe cannot occupy the same space in the solution. The volume of the solution that the probe can occupy is reduced, thus effectively increasing the concentration of the probe. Recall in the equation for $t_{1/2}$ discussed earlier that as c increases, $t_{1/2}$ decreases. Thus the presence of the dextran sulfate decreases the time needed for hybridization.

The stability of a hybrid formed between two single-stranded DNA molecules is reflected in the temperature of melting, T_m. The T_m depends on the ionic strength of the solution, the percentage GC of the DNA sequence, the length of the shortest strand in the duplex, and the concentration of any helix-destabilizing agents. For example, formamide is a chemical that destabilizes the duplex DNA. Hybridizations are typically

performed at T_m −25°C (25°C below the T_m) or at approximately 65°C. If 50% formamide were present, the temperature for hybridization would be lowered to 42°C.

An empirically derived equation can be used to estimate the T_m if the GC content of a DNA is known,

$$T_m = 81.5°C - 16.6(\log_{10}[Na^+]) + 0.41(\%\ G+C) - 0.63(\%\ \text{formamide}) - (600/l),$$

where l is the length of the hybrid in base pairs.

Other empirical equations have been derived for DNA:RNA hybrids, etc. See Sambrook *et al.* (1989, pp. 9.50–9.51) for more information about such equations.

The stability of duplexes containing mismatched bases is decreased. For hybrids that are larger than 150 bp, the T_m decreases about 1°C for every 1% base pair mismatch. For very small pieces of DNA, with hybrids smaller than 20 bp, the T_m decreases 5°C for every base pair mismatch.

Britten *et al.* (1974) defined the term stringency as the difference between the T_m of a perfect duplex and the temperature of incubation, T_i. High stringency means a T_i very close to T_m. High-stringency conditions would allow only sequences that are perfectly base paired or with very little mismatch to remain as duplex molecules. Low-stringency conditions would allow more mismatch, and imperfectly paired molecules could still remain as duplex molecules.

Typically, hybridizations of a probe to target DNA are performed under low-stringency conditions and subsequent washes are done under high-stringency conditions. The temperature and salt concentration of washes can be changed to adjust the stringency.

Blot of a Dry Gel

Hybridization of probes to nucleic acids can be done in a dried gel without transferring the nucleic acid to a membrane or blot. This dried gel has been called an "unblot." Lueders and Fewell (1994) used either single-stranded oligonucleotide probes or double-stranded DNA fragments of 0.5 to 1.4 kb prepared by nick translation and labeled with ^{32}P. They found the dried gels had a higher sensitivity than conventional nylon or nitrocellulose blots. Hybridization of dried gels alleviates the problem of inefficient transfer of very large DNA from gels to blots. No prehybridization step is needed with dried gels. Using dried gels instead of blots is also less expensive because membranes are not needed.

One possible explanation for the difference in sensitivities is that the usual acid depurination step used to fragment large DNAs for more

efficient transfer out of the gel disrupts the target sequence for the probe hybridization.

The Attachment of Nucleic Acids to a Membrane

The exact mechanism of the binding of nucleic acids to a nitrocellulose membrane is not completely understood, but the binding is thought to involve hydrophobic interactions, hydrogen bonding, and salt bridges (Schleicher and Schuell, 1992). Although the bonds involved are not covalent, the nucleic acid is essentially permanently bound to the membrane. DNA in a gel is first denatured because single-stranded, but not double-stranded, DNA binds the nitrocellulose membrane. The gel is then neutralized before the Southern transfer is set up (Figure 3.9). This is necessary when nitrocellulose is the membrane to be used because nitrocellulose is destroyed by alkaline pH. There is a rapid transfer–alkaline blotting procedure that can be used with nylon membranes that can withstand the alkaline pH. When the nitrocellulose is dried, the nucleic acid is irreversibly attached to the membrane. The nitrocellulose is dried by baking at 80°C in a vacuum oven for 2 hr. The vacuum is necessary because the flash point of nitrocellulose is 200°C. Although 2 hr is the typical time suggested for baking the nitrocellulose membrane, as little as 20 min or until the nitrocellulose is dry is an adequate amount of time. If nitrocellulose is baked for an excessive length of time, it may become brittle.

Both single-stranded and double-stranded DNA can bind to nylon or positively charged nylon (Brown, 1991, p. 283). To fix nucleic acids to a nylon membrane, the membrane may be baked at 65–80°C for 30 min. For a nylon membrane, it is not necessary to bake the membrane in a vacuum. Alternatively, DNA may be covalently bound to the nylon mem-

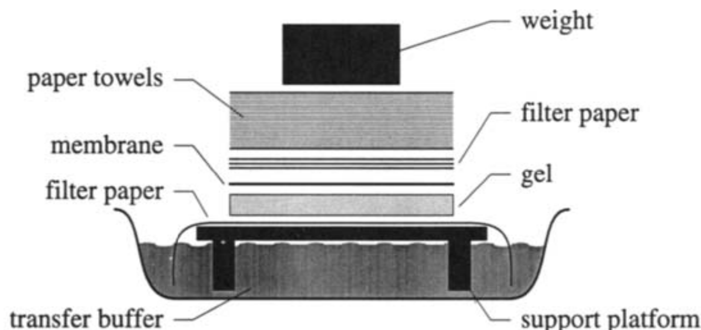


Figure 3.9 The setup of a Southern blot.

brane by UV crosslinking (Church and Gilbert, 1984). Using a UV light of 254 nm, thymine groups (and to a lesser degree other nucleotides) in nucleic acids are activated and react with amine groups on the nylon surface to link the nucleic acid covalently to the nylon membrane. The binding of the nucleic acid to the nylon is dependent on the UV dose. An excess of UV light can result in an increase in crosslinking of the nucleic acid to the membrane and reduced ability of the nucleic acid to hybridize to a probe sequence. The time of UV exposure for crosslinking must be empirically determined for a particular UV source. Some sources suggest doing the UV crosslinking with a wet membrane; others suggest allowing the membrane to dry before crosslinking (Beck, 1992).

The procedure for a standard Southern blot and variations of the standard blot are given in protocols 3.1a through 3.1d.

PROTOCOL 3.1a: Southern Blot (Southern, 1975)

Materials

- Denaturation solution: 0.5 *M* NaOH, 1.0 *M* NaCl
- Neutralization solution: 0.5 *M* Tris and 1.5 *M* NaCl, pH 7.0. Dissolve the needed amount of Tris and NaCl in a volume of sterile-distilled water that is smaller than the final volume. Add concentrated HCl to bring the pH to 7.0. Adjust to the final volume.

CAUTION: Wear gloves, goggles, a face mask, and a laboratory coat when handling concentrated HCl.

- 20× standard sodium citrate (SSC): 3 *M* NaCl and 0.3 *M* Na_3 citrate, pH 7.0. Use concentrated HCl to adjust the solution to pH 7.0, and then adjust the solution to the final volume.

Procedure

1. Cut the DNA samples to be studied with the appropriate restriction endonucleases. Separate the DNA samples by agarose gel electrophoresis. Include in the agarose gel a size standard such as λ DNA cut with *Hin*dIII. If a biotin-labeled DNA probe will be used to hybridize to the final Southern blot, a size standard of biotinylated λ DNA cut with *Hin*dIII (Gibco/BRL) can be used in the gel. Stain the gel with ethidium bromide (1 μg/ml) and photograph the gel on a ultraviolet transilluminator.

CAUTION: Remember to wear gloves and UV protective goggles when working with ethidium bromide and UV light.

2. Denature the DNA in the gel. Place the gel in a plastic box or a glass tray. Add 1 liter denaturation solution to the box. Gently rock the gel in the denaturing solution on a moving platform for 1 hr.
3. Wear gloves when handling the gel. Rinse the gel and tray with about 1 liter of sterile distilled water.

CAUTION: Be careful. A gel that has been soaked in denaturation solution is very slippery!

4. Gently rock the gel in 1 liter of neutralization solution for 1 hr.

CAUTION: Handle the membrane only at the edges with forceps or a gloved hand. Do not apply a great deal of pressure to the membrane. Incorrect handling of the membrane can result in a spotty background.

5. Cut the membrane to be used to the exact size of the gel. Wet the membrane in sterile distilled water. Soak the membrane in 10× SSC (for a nylon or Photogene membrane) for 15 min before using the membrane for DNA transfer. Use 20× SSC for nitrocellulose.
6. Set up the gel for blotting as shown in Figure 3.9. Wrap a piece of Whatman No. 3 chromatography paper around a plastic plate or tray that will serve as a platform. Place the plate with paper in a shallow glass or plastic dish. Make a platform by placing the tray with paper on bottle lids or other means to elevate it about 1–2 cm off the bottom of the glass dish.
7. Pour 10× SSC over the chromatography paper to wet it and to fill the glass dish partially. Place the gel on the chromatography paper-covered plate. A horizontal gel should be placed with the bottom of the gel up. The bottom of the gel should have a smoother surface because of the way the gel was cast. There will be better contact between the gel and membrane if the surface is smooth.
8. Carefully position the wet membrane on top of the gel; make sure there are no air bubbles between the gel and the membrane. Once the membrane is in position, it should not be moved because some transfer and binding of DNA to the membrane can occur rapidly.
9. Wet three or four pieces of Whatman paper the same size as the membrane in 10× SSC. Carefully place the pieces of Whatman paper on top of the membrane.
10. Place strips of plastic wrap beside the gel to cover the paper on the plate. Place a stack of paper towels, 1 to 2 in. thick, on top of the Whatman papers on the gel. Place a glass or plastic tray on top of the paper towels. Put a weight on top of the glass tray.
11. Let the DNA transfer progress overnight. During this time, DNA moves out of the gel by capillary action and binds to the membrane. The

length of time needed to obtain complete transfer of DNA from the gel varies. Mark a corner of the blot to identify the orientation of the blot. Disassemble the transfer setup and soak the membrane in 10× SSC for 5 min. Place the membrane on a clean piece of chromatography paper and allow the membrane to air-dry completely. (Use 2× SSC for nitrocellulose.)

12. Bake the membrane in a vacuum oven at 80°C for 2 hr to fix the DNA to the membrane. For nitrocellulose, such baking must be done in a vacuum because the flash point of nitrocellulose is about 200°C. If nitrocellulose is baked for an extensive time, it may become very brittle. It will also become brittle and shatter if exposed to alkaline pH. Other membranes do not require baking in a vacuum. Many procedures suggest baking the membrane only until it is completely dry.

NOTES

1. For many sizes of DNA, the process of photographing the gel using UV light in the presence of ethidium bromide nicks the DNA sufficiently that the DNA elutes from the gel readily during the blotting process.
2. Alternatively, the gel can be soaked in 0.25 *N* HCl for 8 to 10 min before it is denatured. The acid treatment depurinates DNA; during the subsequent base treatment, the DNA is cleaved at the depurinated sites. This treatment ensures that all sizes of DNA bands on the gel will transfer out of the gel readily. However, excessive acid treatment can result in very small DNA fragments that do not bind well to the membrane (Meinkoth and Wahl, 1984).
3. The volume of denaturant and length of time the gel is soaked in denaturant can vary with the size and percentage agarose of the gel. A smaller volume and shorter time can be used for smaller gels.
4. The selection of membrane to be used in blotting depends on the detection method. For chromogenic (colorimetric) detection of biotin using streptavidin–alkaline phosphatase conjugate, nitrocellulose membranes work well. For chemiluminescent detection of digoxigenin or biotin, nylon membranes are recommended. Nitrocellulose membranes are not recommended because nitrocellulose may quench light and therefore decrease the signal. Neutral nylon membranes may give less background than charge-modified nylon membranes. BRL/Gibco provides a specific nylon membrane, Photogene, for use with their chemiluminescent detection system, PhotoGene.
5. The length of time for transfer from the gel to the membrane can vary widely and still give acceptable results. Some researchers indicate that the majority of the transfer of DNA occurs rapidly, and further time for transfer may result in a decrease in the signal.

6. Several papers suggest that a transfer setup that does not compact the gel is better for complete transfer of the nucleic acids (Chomczynski, 1992; Koetsier *et al.*, 1993; Khandjian, 1987; Lichtenstein *et al.*, 1990). For routine work, the traditional setup of a Southern blot as described here works very well. If an experiment requires the detection of extremely small amounts of complementary sequence on a membrane, the experimenter should consider the possible effects of variations in the transfer conditions, gel setup, and type of membrane.
7. Neuhaus-Url and Neuhaus (1993) report equally good results with nucleic acids attached to membranes by UV crosslinking and those attached by baking. However, Beck (1992) claims that the moisture content of a membrane can greatly affect UV crosslinking and that too much or too little crosslinking can reduce the signal observed. Beck recommends that a membrane be dried completely by baking at 80°C for 15 min before UV crosslinking; such treatment may improve the signal-to-noise ratio.

Modifications of Standard Blotting Procedures

In the standard Southern blotting protocol for a very large 1% agarose gel, about 3 hr (1.5 hr to denature and 1.5 hr to neutralize) is required to prepare a gel so that the nucleic acids in it can be transferred to a nitrocellulose filter, followed by about 16 hr of blotting for the transfer to occur. If a small, low-percentage agarose gel is being used, the denaturing and neutralizing times can be reduced to 30–45 min each, and the transfer time can be reduced to 2–3 hr.

Mini-Southern Blotting

This procedure is suitable for a small gel of a low-percentage agarose. There is no large reservoir of transfer buffer used. The procedures described here are for a nitrocellulose membrane, but the general setup can be used for any membrane.

1. Ethidium bromide stain and photograph a mini-gel. Soak the gel in 250 ml of 1 *M* NaCl and 0.5 *M* NaOH for 30 to 45 min. The solution can be replaced with fresh solution during that time.
2. Wash the gel four times in 250 ml of water.

CAUTION: A gel soaked in NaOH is slippery.

3. Soak the gel in 250 ml of 1.5 *M* NaCl, 0.5 *M* Tris, pH 7.0, for 30 to 45 min. The solution can be replaced with fresh solution during that time.
4. Wet four Whatman No. 3 filter papers cut to the exact size of the gel in 20× SSC and place the filter papers over a glass plate.

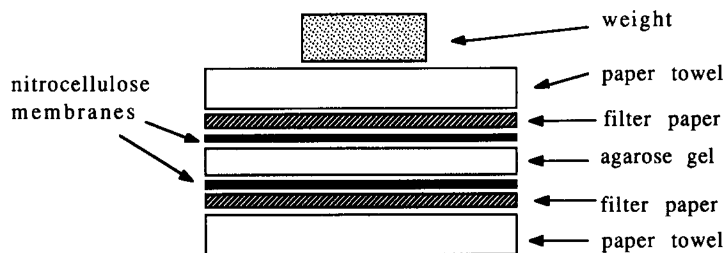


Figure 3.10 The setup of a bidirectional Southern blot in which two blots are produced from one gel.

5. Place the gel on the wet filter papers such that the bottom side (smooth surface) of the gel faces up.
6. Wet a piece of nitrocellulose cut to the exact size of the gel in water. Soak the nitrocellulose in 20× SSC. Place the nitrocellulose on the gel. Do not trap any air bubbles between the membrane and the gel.
7. Wet three Whatman No. 3 filter papers the same size as the gel in 20× SSC. Place the filter papers over the nitrocellulose.
8. Stack about 1 in. of paper towels cut to the same size on top. Place a glass plate or other weight on top of the paper towels.
9. Remove the blot after 4 to 6 hr. Mark a corner of the blot. Wash the blot briefly for 30 sec in 2× SSC. Dry the blot. Bake the blot for 2 hr at 80°C under vacuum.

PROTOCOL 3.1b: Bidirectional Blotting—A Sandwich Blot

In this modification of the standard Southern blotting protocol, two identical blots are made from one gel. Pieces of membrane are placed above and below the gel to be blotted. There is no reservoir of blotting buffer; all the buffer is supplied by the liquid in the gel (Figure 3.10).

PROTOCOL 3.1c: Alkaline Blotting

This is an alternative, rapid blotting procedure that can be used with a nylon membrane, such as Nytran or Gene Screen Plus, but not with nitrocellulose. A gel is soaked in an alkaline NaOH solution and blotted directly without neutralization. Nitrocellulose is not suitable for this protocol because it is degraded by high pH.

1. Run the gel. Stain and photograph the gel.
2. Soak the gel in wick buffer for 10 to 30 min. Use 10 min for a small gel; use 30 min for a large gel.
   Wick buffer: 0.4 N NaOH; 1.5 N NaCl. Add 16 g NaOH and 87.6 g NaCl per liter of H_2O.
3. Cut 3 pieces of thick Whatman filter paper (3-mm chromatography paper) and the nylon membrane to fit the gel.
4. Soak the nylon membrane in H_2O to wet it and then soak it in wick buffer until ready to use.
5. Set up a blotting tray, wick, wick buffer, and gel as for the standard Southern blotting.
6. Place a weight on top of the stack of paper towels and blot for 4 to 6 hr.
7. Take off the nylon membrane and wash it for 5 min in 5× SSC. Bake the membrane at 60–80°C in a vacuum oven for 60 min.

NOTES

1. Although this method is more rapid, it can result in poor transfer of some DNA fragments compared to the standard Southern blotting protocol. Prolonged transfer times with alkaline blotting can reduce the DNA bound to the membrane.
2. Recommended prehybridization and hybridization solutions for Nytran or Gene Screen Plus differ slightly from those for nitrocellulose.

PROTOCOL 3.1d: Colony Hybridization

This procedure allows DNA from individual bacterial colonies to be transferred to a membrane. The membrane can then be probed with a specific DNA to determine if the bacteria contains that sequence. Colony hybridization is useful to screen many recombinant DNA colonies to determine which colonies contain a specific DNA sequence.

Perform the following steps in advance:

1. Streak bacterial colonies onto the appropriate selective medium. Incubate plates at the appropriate temperature overnight.
2. Use circular nitrocellulose filters that just fit inside the petri plates. Place the nitrocellulose filters between layers of filter paper. Wrap the filters in aluminum foil and autoclave them on slow exhaust to sterilize.

3. Just before the transfer process, prepare the following:
   A. 20 ml of lysozyme (1.5 mg/ml) dissolved in 25% sucrose, 50 m*M* Tris, and 10 m*M* EDTA, pH 8.0
   B. 20 ml of 0.5 *N* NaOH, 0.1% SDS
   C. 20 ml of 0.5 *N* NaOH
   D. 20 ml of 1 *M* Tris, pH 7.5
   E. 20 ml of 0.15 *M* NaCl and 0.1 *M* Tris, pH 7.5
4. Prepare five petri plates with Whatman filter papers: Place three circular Whatman filter papers (that just fit the petri plates used) inside a petri plate lid. Label each petri plate lid and fill each plate with about 5 ml of one solution (A–E above). The filter papers should be completely wet but not have much extra solution above the papers.
5. Place the petri plate lid with solution A, the lysozyme in sucrose, Tris, and EDTA on ice.
6. Keep the other petri plates lids at room temperature.

To do colony transfers or colony lifts, perform the following steps:

1. Use forceps that are flamed to surface sterilize them to handle nitrocellulose filters.
2. Press a nitrocellulose filter onto the surface of a plate with colonies grown on it. Leave the filter in place momentarily. Gently rub the surface of the filter with a gloved hand to ensure uniform transfer of colonies from the plate to the filter.
3. Remove the nitrocellulose filter from the petri plate and place the filter, colony side up, in the petri plate containing solution A on ice. Be sure to keep the plate horizontal. Colonies will smear into each other if the plate is tipped. Incubate for 1 min.
4. Using forceps, pick up the nitrocellulose filter and drag the filter across the edge of the petri plate lid to remove the excess solution from the bottom of the filter. Place the filter colony side up in the petri plate with solution B. Incubate for 1 min.
5. Again using forceps, remove the filter from solution B and place the filter in solution C for 1 min.
6. Remove the filter from solution C and place the filter in solution D for 1 min.
7. Remove the filter from solution D and place the filter in solution E for 1 min.
8. Remove the filter from solution E and place on a piece of filter paper. Allow the nitrocellulose filter to air-dry completely. Place nitrocellulose filters between pieces of filter paper. If not completely dried before baking, filters may stick to the paper.

9. Bake the filter under vacuum at 80°C for 2 to 4 hr.
10. Hybridize the filter per the usual Southern blot hybridization conditions.

NOTES

1. This protocol is based on modifications of Thayer (1979). For additional discussion of the use of colony hybridization, see Grunstein and Wallis (1979) and Grunstein and Hogness (1975).
2. Plates can be streaked with colonies, grown overnight at the appropriate temperature, and then wrapped with Parafilm and stored at 4°C. Plates with colonies may be kept at least 4 days before the colony transfers are done.
3. Occasionally, some of the bacterial cell debris still on the filters may come off in the prehybridization solution. Such a solution will be a yellowish color. If there is a lot of debris from the filters, change the prehybridization solution before adding the probe.
4. If many filters are to be hybridized with the same DNA probe, those filters can be stacked on top of each other in the same hybridization bag and hybridized together.

PROTOCOL 3.2: Isolation of DNA Fragments by Electroelution

Overview

This method uses agarose gel electrophoresis for the purification of DNA fragments. The DNA obtained can readily be used in nick translation (to label DNA to use as a probe) or ligation (to clone a piece of DNA) reactions or be cut with additional restriction endonucleases to map restriction sites within the DNA fragment.

The method of electroelution allows the isolation of a particular band of DNA from an agarose gel. Often a highly pure grade of agarose, such as Pharmacia NA agarose, is used for electroelution. The more highly purified grades of agarose have lower amounts of sulfated polysaccharides. Polysulfonates can copurify with DNA and interfere with subsequent enzymatic reactions. Alternatively, if a high grade of agarose is not used, after electroelution, it may be necessary to phenol extract the DNA sample before use.

Time required: 1 hr for restriction endonuclease digestion, 1–1.5 hr for gel electrophoresis and staining of gel, 1–2 hr for electroelution.

Materials

- High-quality agarose, such as NA agarose from Pharmacia or Gibco/BRL Ultrapure agarose
- Plasmid DNA, digested with appropriate restriction endonuclease
- Apparatus for gel electrophoresis
- Gel box
- Power supply
- Ultraviolet transilluminator
- Dialysis tubing
- Isopropanol or ethanol
- 1× TBE (Tris–borate gel buffer)
- 0.5× TBE (half-strength 1× TBE solution)
- 10× TBE buffer: 0.89 *M* Tris (Trizma base), 0.89 *M* boric acid, 0.02 *M* EDTA
- Gel loading dye: gel loading dye, stop mix, or stop buffer: 50% glycerol; 0.7% sodium dodecyl sulfate (SDS, a detergent); 0.1% bromophenol blue (BPB, a dye)

Procedure

1. Digest the plasmid DNA with the appropriate restriction enzymes in a final volume of 100–200 μl.
2. Using Pharmacia NA agarose or BRL Ultrapure agarose, make an 0.8% gel in 1× TBE gel running buffer. For a mini-gel apparatus, use 30–50 ml of agarose solution. Set up the gel with a wide slot comb to make a wide well.
3. If there is any question about complete restriction digestion of the DNA, check a small aliquot of the large restriction digestion on a different mini-gel to be sure there has been complete cutting with the restriction enzymes. If the cutting was not complete, another aliquot of restriction enzyme and an extended digestion time can be used. When it is clear that the restriction digestion is complete, add gel loading buffer to the sample. Load the rest of the sample into the broad slot of the mini-gel, and subject the sample to electrophoresis.

CAUTION: Ethidium bromide is toxic and is potentially a mutagen. Wear gloves and a laboratory coat when handling ethidium bromide. Dispose of ethidium bromide-containing materials properly.

CAUTION: The UV transilluminator is a strong source of UV light. Protect eyes and skin from UV light. Wear UV protective eye glasses or goggles and a UV protective face shield. Wear gloves and a laboratory coat to protect skin from UV light.

4. After electrophoresis, stain the gel for 10 to 15 min in an ethidium bromide solution (0.5–1.0 μg/ml final concentration). Rinse the gel with water. Examine the gel on a UV transilluminator. Place a piece of plastic wrap underneath the gel to cover the surface of the transilluminator. Using a new, clean razor blade, cut out a strip of the gel that contains the fragment of interest.

CAUTION: Be sure to place the gel on plastic wrap so that the surface of the transilluminator is not scratched by the razor blade. Remember to protect eyes and skin from UV light.

5. Cut a piece of prepared dialysis tubing long enough to enclose the gel strip and have room to tie or clamp the tubing closed. Rinse the inside and outside of the dialysis tubing thoroughly with sterile H_2O and then with 0.5× TBE gel buffer. Tie off one end of the tubing, fill the tubing with buffer, and then slide the excised gel fragment into the tubing. Pour out the excess buffer, and knot the end of the bag, making sure that there are no air bubbles trapped inside. The volume of buffer left inside the dialysis bag should be very small (just enough to cover the agarose gel fragment).
6. Fill a mini-gel apparatus with 0.5× TBE gel buffer, and immerse the tubing with the gel fragment in the buffer, arranged perpendicular to the direction of electrophoresis.
7. Electroelute the fragment at 100 V for 1 hr.
8. At the end of the electroelution, reverse the positions of the leads to the power supply. Turn on the power supply for 1–3 sec. This reversal of the current will help remove DNA that is against the dialysis tubing.
9. Remove the dialysis bag from the electrophoresis chamber. Wipe the outside of the dialysis tubing with a tissue. Squeeze the tubing to disperse the electroeluted DNA in the buffer that is in the sack. Inspect the slice under UV light to make sure that all the DNA has migrated out of the agarose. All these operations can be monitored by looking at the ethidium bromide fluorescence under UV light.
10. Cut open one end of the bag. Use a micropipettor to collect all the liquid from the inside of the dialysis bag into a microfuge tube. Add 50–100 μl of 0.5× TBE to rinse out the inside of the dialysis bag. Collect this wash solution with the other liquid. Typically, at this point, the sample volume will be 200–500 μl.
11. If a phenol extraction is to be done, add an equal volume of phenol: chloroform to the DNA sample. Mix well by shaking the closed microfuge tube vigorously. Centrifuge the sample for 5 min to separate the phases. Carefully remove the aqueous (top) phase.

12. Measure the sample volume, and add 1/10 volume of 3 *M* NaOAc. Precipitate the DNA by adding either 1 volume of isopropanol or 2 volumes of ethanol. Store the sample at −20°C overnight. Pellet the DNA by centrifugating the sample in a microfuge for 10 min. Decant the alcohol. Rinse the pellet with 50 μl of 70% ethanol. Centrifuge the sample for 5 min. Decant the 70% ethanol and dry the pellet completely. Resuspend the pellet in approximately 10–30 μl of 1 m*M* Tris and 0.1 m*M* EDTA, pH 7.0.
13. Label the sample. Store the DNA at 4°C.
14. Estimate the DNA concentration of the sample by gel electrophoresis of an aliquot of this DNA and an aliquot of a DNA of known concentration. Compare the intensity of ethidium bromide-stained DNA fragments to estimate the concentration of DNA.

NOTES

1. The DNA can now be used for restriction endonuclease digestions, labeling reactions, or ligations for cloning.
2. Yields should be 90% or greater, although yields may decrease for larger DNA fragments.
3. There may be some agarose left in the sample. If a high-quality agarose were used, this would not interfere with subsequent enzymatic reactions.
4. An alternative electroelution procedure that uses far less dialysis tubing is illustrated in Figure 3.11 (Peloquin and Platzer, 1991).

Preparation of Dialysis Tubing

1. *Always wear gloves when handling dialysis tubing.* Using a clean razor blade or scissors, cut off the desired length of tubing.
2. Boil the tubing for 10 min in a large volume of 2% sodium bicarbonate and 1 m*M* EDTA. To make this solution, dissolve 40 g of sodium bicarbonate in about 1600 ml of distilled water. Add 20 ml of 0.5 *M* EDTA, pH 7.0. Bring the volume to 2000 ml with distilled water.
3. Rinse the dialysis tubing thoroughly with distilled water.
4. Boil the tubing for an additional 10 min in a large volume (about 2000 ml) of distilled water.
5. Let the dialysis tubing cool in the distilled water. When it is cool, transfer the tubing to a closed container of 50% ethanol in water. Store in the refrigerator at 4°C.

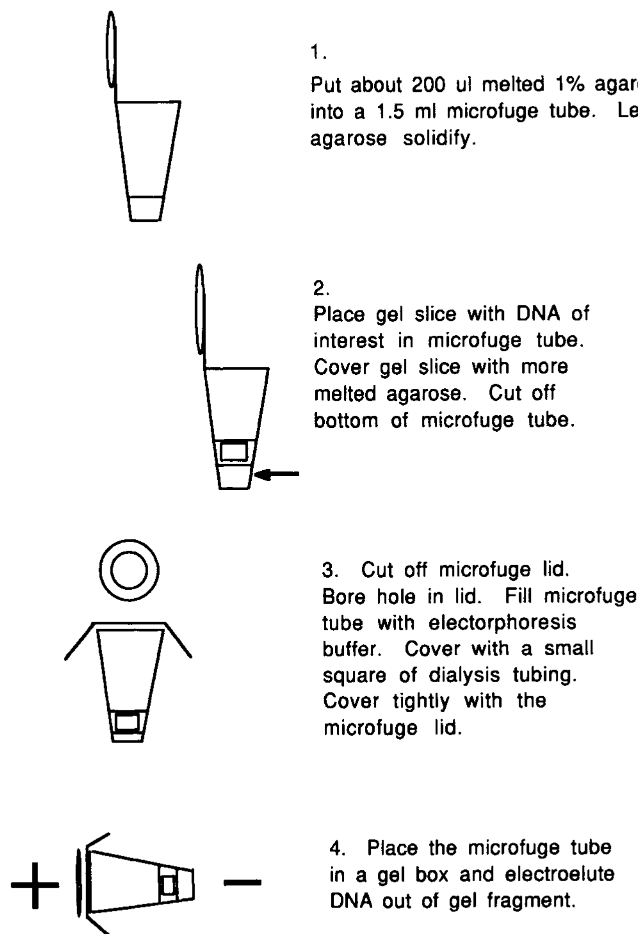


Figure 3.11 An apparatus made from a microfuge tube for electroelution of DNA from an agarose gel.

6. When the dialysis tubing is needed, rinse the tubing carefully inside and out with sterile distilled water and then rinse the tubing with electrophoresis running buffer.

NOTES

1. Once the dialysis tubing is wet, do not allow the tubing to dry out again. If the tubing is allowed to dry, holes may form in the tubing.
2. An example of the type of dialysis tubing used for electroelution of DNA from gel fragments is Spectra/por Molecular porous membrane

tubing with a molecular weight cut off of 12,000–14,000 from VWR Scientific Co. (Catalog No. 25225-226; 25-mm × 100-ft roll, with a diameter of 15.9 mm).

Labeling DNA to Be Used as Probes

For labeling DNA probes with radioactivity, random primer extension labeling rather than nick translation is often used because the random primer method has several advantages. These include the ability to label smaller amounts of DNA (in the 25-ng range for the random primer method; in the 1-μg range for nick translation) and the ability to produce probes with higher specific activity (Mackey *et al.*, 1992a). Interestingly, BRL has found no difference in sensitivity between random primer extension and nick translation labeling for biotin-labeled probes that are detected with streptavidin conjugated to alkaline phosphatase (Mackey and Rashtchian, 1992; Mackey *et al.*, 1992b, 1993). Using Southern blots, BRL reports the ability to detect less than 100 fg of bacteriophage DNA and less than 400 fg of a 5.5-kb β-globin fragment of human genomic DNA by biotin probes labeled by either method. Even with nonradioactive probes, random primer labeling can be more useful if the amount of DNA available to label is small because the random primer method uses only nanogram amounts of DNA (Mackey *et al.*, 1993).

PROTOCOL 3.3a: Labeling of Probe with Biotin Using Nick Translation

Components

10× dNTP mix: 0.2 m*M* each dCTP, dGTP, dTTP; 0.1 m*M* dATP; 0.1 m*M* biotin-14-dATP; 500 m*M* Tris–HCl, pH 7.8; 100 m*M* β-mercaptoethanol; 100 μg/ml nuclease-free BSA; available as part of Gibco/BRL's BioNick labeling kit.

10× enzyme mix: 0.5 unit/μl DNA polymerase I; 0.0075 unit/μl DNase I; 50 m*M* Tris–HCl, pH 7.5; 5 m*M* magnesium acetate; 1 m*M* β-mercaptoethanol; 0.1 m*M* phenylmethylsulfonyl fluoride; 50% (v/v) glycerol; 100 μg/ml nuclease-free BSA; available as part of Gibco/BRL's BioNick labeling kit.

100 μl nick translation dye mixture: 0.5 *M* EDTA, pH 8.0; 2 mg/ml blue dextran (Sigma; average molecular weight of 2×10^6); 1 mg/ml orange G (Sigma; 7-hydroxy-8-phenylazo-1,3-naphthalenedisulfonic acid). Prepare a stock of this in advance.

Procedure

1. Place a microfuge tube on ice. To the tube add the following components in the order listed:
   - 10 μl 10× dNTP mix
   - *X* μl DNA; enough to give 1 μg double-stranded, either linear or circular DNA
   - *Y* μl sterile distilled water; add the correct amount of water to bring final volume of the reaction, including the enzyme mix, to 50 μl
   - 5 μl 10× enzyme mix
2. Mix components gently using a micropipettor tip to stir the components.
3. Incubate the labeling reaction mixture at 15°C for 60 min.
4. Stop the labeling reaction by adding 100 μl nick translation dye mixture.
5. Add 2 μl of 5% SDS. The presence of the detergent reduces the chances of biotin sticking to materials such as glass.
6. Mix thoroughly.

Separation of the Biotin-Labeled DNA from the Unincorporated Biotin-14-dATP by Exclusion Chromatography Using a Sephadex G-100 Column

Components

G-100 column buffer: 0.1 *M* NaCl; 10 m*M* Tris, pH 7.5; 2 m*M* EDTA; 0.1% SDS.

Sephadex G-100 (Pharmacia): Add 5 g of G-100 beads to 150 ml of G-100 column buffer. Autoclave the solution for 20 min on slow exhaust. Store the solution at 4°C. Before using, warm the solution to room temperature to redissolve the SDS.

Procedure

1. Prepare a Sephadex G-100 column in a sterile Pasteur pipet:
   a. Insert a small piece of glass wool into a sterile Pasteur pipet. Push the glass wool into the bottom of the Pasteur pipet using a small disposable plastic 1-ml pipet or other suitable object that will fit inside the Pasteur pipet. The piece of glass wool should be about 1/2 cm long when packed into the bottom of the Pasteur pipet. There should be enough glass wool in the pipet to hold the Sephadex beads in place. If too much glass wool is tamped into the pipet, the flow rate of the column will be very slow.

b. Invert the bottle of Sephadex G-100 beads in G-100 column buffer several times to resuspend the beads that have settled to the bottom of the bottle. Add the Sephadex G-100 beads in G-100 column buffer to the Pasteur pipet. Let the beads in column buffer run down along the inside of the Pasteur pipet. Do not trap air bubbles in the pipet when packing the column. Do not let the column run dry. Collect and then discard the column buffer that runs off the column as the G-100 beads settle or pack into place. Continue to add G-100 beads until the Sephadex column is packed to a height of about 8 cm (to the constricted point on a Pasteur pipet). Add about 300 μl of G-100 column buffer to the top of the packed column. Lay the packed Pasteur pipet column horizontally until needed.

2. Assemble all the materials needed. Clamp the Pasteur pipet column into a vertical position on a stand. Position a test tube rack with microfuge tubes under the column. Position a microfuge tube under the column to collect the buffer running through the column.
3. When the meniscus of the column buffer just enters the column, load the nick translation labeling reaction mixed with the nick translation dyes and SDS into the column. When all the labeling reaction has run into the column, add column buffer to the top of the column.
3. Elute the sample with G-100 column buffer. Continue to add column buffer to the top of the column as needed.
4. Collect the blue fraction (blue dextran and biotin-labeled DNA) that elutes first off the column. Discard the orange fraction (orange G and free nucleotides).
5. Store the biotin-labeled DNA at 4°C or at −20°C. The biotin-labeled DNA may be stored in this manner for at least a year.
6. To monitor the incorporation of biotin into DNA, spot dilutions of the biotin-labeled DNA and known amounts of biotinylated DNA onto a membrane. Detect the biotin-labeled DNA with either a chromogenic or chemiluminescent substrate as described under Detection Procedures.

PROTOCOL 3.3b: Oligo Labeling of a Probe

Materials

- USB Images Nucleotide Mixture:
  0.167 m*M* each of dGTP, dATP, and dTTP
  0.125 m*M* biotin-14-dCTP
  0.042 m*M* dCTP

- USB Images Reaction Mixture:
  $10A_{260}$ units of random hexanucleotides in 2 M Hepes, pH 6.6
  2 mM Tris, pH 7.0
  0.1 mM EDTA
  4 mg/ml bovine serum albumin

Procedure

1. Mix in a microfuge tube the following:
   - 25–100 ng of DNA to be labeled. The volume should not be more than 14 μl.
   - 2 μl of Images Reaction Mixture (random hexanucleotide primers).
   - X μl of sterile, distilled H_2O, enough to bring the total volume to 16 μl.
2. Place the microfuge tube in a boiling H_2O bath for 10 min to denature the DNA to be labeled. Remove the microfuge tube from the boiling H_2O bath and immediately put the tube on ice. Let the tube sit on ice for 10 min.
3. Keep the tube on ice. Add these additional components to the tube:
   - 3 μl Images Nucleotide Mixture
   - 1 μl Klenow enzyme (10 units).

   The final reaction volume is 20 μl.
4. Mix thoroughly. Centrifuge the tube for a few seconds in a microfuge to bring droplets on the side of the microfuge tube down to the bottom.
5. Incubate the reaction at 37°C for 2 hr to overnight.
6. At the end of the incubation time, stop the labeling reaction by adding 100 μl nick translation dye mixture. (See Labeling of Probe with Biotin Using Nick Translation.)
7. Add 2 μl of 5% SDS. The presence of the detergent reduces the chances of biotin sticking to materials such as glass.
8. Mix thoroughly.
9. To separate the labeled DNA from the unincorporated biotin nucleotide, follow the protocol for Separation of the Biotin-Labeled DNA from the Unincorporated Biotin-14-dATP by Exclusion Chromatography Using a Sephadex G-100 Column described above.

NOTES

1. The oligo or random primer labeling reaction may be done at 37°C or at room temperature.

2. It may not be necessary to separate the incorporated biotin-labeled probe from the unincorporated biotin nucleotide because, typically, the extent of incorporation is very high (greater than 90%).
3. This procedure is based on the Random Primed Images Biotin Labeling Kit of USB.

PROTOCOL 3.3c: Photobiotin Labeling of a Probe

Materials

- Photobiotin acetate, concentration 1 $\mu g/\mu l$, dissolved in water.
- DNA to be labeled, at a concentration of 1 $\mu g/\mu l$ in water or in 0.1 mM EDTA, pH 7.0.
- Strong visible light source, such as a sunlamp (Philips Ultraphil MLU 300 W or Philips HPL-N 400 W).

Procedure

1. Work in very subdued light. Mix from 5 to 25 μl of photobiotin acetate with the same volume of the DNA to be labeled.
2. Seal the solution inside a siliconized glass capillary tube.
3. Place the capillary tube in an ice water bath approximately 10 cm below a sunlamp.
4. Irradiate the sample with the sunlamp for 15 min.
5. Open the capillary tube. Add the solution to 50 μl of 0.1 M Tris, pH 9.0. Add H_2O to the sample to bring the final sample volume to 100 μl.
6. Add 100 μl of 2-butanol. Mix. Remove and keep the upper aqueous phase.
7. Add an additional 100 μl of 2-butanol to the sample. Mix. Remove and keep the upper aqueous phase.
8. Add H_2O to the aqueous phase to bring the total volume to 45 μl. Add 5 μl of 3 M NaOAc (sodium acetate). Add 125 μl of cold ethanol. Mix.
9. Store the sample at −20°C overnight or place the sample in a dry ice–ethanol bath for 15 min.
10. Centrifuge the sample in a microfuge for 15 min.
11. Wash the pellet with 50 μl of cold 70% (v/v) ethanol. Centrifuge the sample for 1 min.
12. Decant the 70% ethanol. Dry the pellet completely using a Speed-vac.
13. Resuspend the pellet in a small volume (10 to 50 μl) of 1 mM Tris and 0.1 mM EDTA, pH 7.0.

The procedure described here is based on the protocol of Forster *et al.* (1985). The same procedure is used to label single- or double-stranded nucleic acids.

PROTOCOL 3.4a: Hybridization and Detection of Labeled Probe—A Biotin-Labeled Nonradioactive Probe and Chromogenic Substrate

This protocol uses materials from the Blugene kit from Gibco/BRL.

Hybridization for a Chromogenic Nonradioactive Detection System

1. Prepare a Southern blot as described in the Southern blot protocols. Membranes that work well for a chromogenic detection system include nitrocellulose. Always wear gloves when handling the blot. Touch the blot only at the edges. Do not apply too much pressure to the blot. Excess pressure at a point on the membrane may result in a background spot at that location when the probe is detected. The prehybridization mix (PM) and calf thymus DNA are added to prehybridization and hybridization solutions to block or inhibit the nonspecific binding of the probe to the membrane.
2. Prepare 10 ml of prehybridization solution:

Final concentration of component	Concentration of stock component	Amount of stock component to add
50% formamide	Formamide, deionized or molecular biology grade	5 ml
5× SSC	20× SSC	2.5 ml
5× PM or Denhardt's solution	100× PM	0.5 ml
25 m*M* sodium phosphate, pH 6.5	1 *M* sodium phosphate, pH 6.5	0.25 ml
0.5 mg/ml freshly denatured sheared calf thymus DNA	2 mg/ml freshly denatured sheared calf thymus DNA	2.5 ml
	H_2O	To a total volume of 10 ml

20× SSC is 3 *M* NaCl, 0.3 *M* Na_3 citrate, pH 7.0. 100× PM or Denhardt's is 2% (w/v) Ficoll, 2% (w/v) polyvinylpyrrolidone, 2% bovine serum albumin (BSA).

Do not autoclave this solution. Use sterile water to make up the solution.

3. Place membrane in a small plastic box just large enough to allow the membrane to lie flat or in a Seal-a-Meal bag. Add 20–100 μl prehybridization solution/cm² surface area of the membrane. Incubate the membrane in the prehybridization solution at 42°C for 2 to 4 hr.
4. Prepare 10 ml of hybridization solution:

Final concentration of component	Concentration of stock component	Amount of stock component to add
45% formamide	Formamide, deionized or molecular biology grade	4.5 ml
5× SSC	20× SSC	2.5 ml
1× PM or Denhardt's solution	100× PM	0.1 ml
20 m*M* sodium phosphate, pH 6.5	1 *M* sodium phosphate, pH 6.5	0.2 ml
0.2 mg/ml sheared, denatured calf thymus DNA	2 mg/ml sheared, denatured calf thymus DNA	1 ml
	H_2O	To a total volume of 10 ml

5. Immediately before use, heat denature the biotin-labeled DNA to be used as a probe. Use the amount of probe that will give a final concentration in the hybridization solution of 0.1 to 0.5 μg/ml. Place a tube with the probe DNA in a boiling H_2O bath for 10 min. After denaturation, place the tube with the probe on ice for 10 min. The probe must be single-stranded to be able to hybridize with its complementary sequence.
6. Add the denatured probe to the hybridization solution. Mix thoroughly. Remove the prehybridization solution from the membrane. Add the hybridization solution.
7. Place the membrane in hybridization solution at 42°C for 14 to 16 hr.
8. Prepare the wash solutions needed in steps 9 to 16. Prewarm the wash solution used in steps 14 and 15 to 50°C.

For each wash step, place the wash solution in a plastic box, beaker, or dish large enough to allow the membrane to lie flat. Using forceps, place the membrane in the wash solution. The wash solution should completely cover the membrane. The washes remove probe that has not correctly hybridized to its complementary sequence.

9. Wash the membrane after hybridization to remove the probe that has not hybridized to its complementary sequences.

10. Wash the membrane in 250 ml of 2× SSC/0.1% SDS for 3 min at room temperature.
11. Again, wash the membrane in 250 ml of 2× SSC/0.1% SDS for 3 min at room temperature.
12. Wash the membrane in 250 ml of 0.25× SSC/0.1% SDS for 3 min at room temperature.
13. Again, wash the membrane in 250 ml of 0.25× SSC/0.1% SDS for 3 min at room temperature.
14. Wash the membrane in 250 ml of 0.16× SSC/0.1% SDS for 15 min at 50°C.
15. Again, wash the membrane in 250 ml of 0.16× SSC/0.1% SDS for 15 min at 50°C.
16. Rinse the membrane in 2× SSC at room temperature for 1 min.
17. Place the membrane on a piece of chromatography paper. Let the membrane air-dry or continue with the membrane blocking protocol below.

Detection of a Biotin-Labeled Probe for a Chromogenic Nonradioactive Detection System

The membrane blocking eliminates the nonspecific binding of streptavidin–alkaline phosphatase (SA-AP) to the membrane. Streptavidin binds tightly and specifically to the biotin of the labeled probe. A substrate for alkaline phosphatase is added to detect the biotin-labeled probe.

Membrane Blocking

1. Prepare and filter the buffers.
   - Buffer 1: 0.1 *M* Tris–HCl, 0.15 *M* NaCl, pH 7.5
   - Buffer 2: 3% (w/v) BSA in Buffer 1. Use 3 g BSA/100 ml Buffer 1. The BSA used should be fraction V. Other preparations of BSA may contain alkaline phosphatase activity and should be checked carefully before use.
   - Buffer 3: 0.1 *M* Tris, 0.1 *M* NaCl, 50 m*M* $MgCl_2$, pH 9.5

   Filter all the buffers through 0.45-μm filters to reduce the background. Before use, prewarm the buffer needed in step 2 to 65°C.

2. Working at room temperature, soak the hybridized membrane for 1 min in Buffer 1 in a plastic box large enough to allow the membrane to lie flat. Use enough volume of Buffer 1 to cover the membrane completely. If the membrane has been dried before this blocking step, soak the membrane in Buffer 1 for 5 to 10 min to rehydrate the membrane completely.

3. Use forceps to transfer the membrane from the plastic box containing Buffer 1 into a plastic box containing prewarmed Buffer 2. Incubate the membrane for 1 hr at 65°C in Buffer 2.
4. Remove the membrane from Buffer 2. Continue with the application of the detection system. Alternatively, the membrane may be dried at this point in a vacuum oven at 80°C for 10 to 20 min. Dried membranes may be stored for months before the detection procedure is continued.

Application of Detection System

5. If the membrane is dry, thoroughly rehydrate it by soaking in Buffer 2 for 10 min at room temperature.
6. In a polypropylene tube, immediately before use, add 7 μl of streptavidin–alkaline phosphatase conjugate (at 1 mg/ml) to 7.0 ml of Buffer 1. Place the diluted SA-AP in a plastic box just large enough to allow the membrane to lie flat.
7. Incubate the membrane in the diluted SA-AP for 10 min at room temperature with gentle agitation. Occasionally during the 10-min incubation, pipet the SA-AP solution over the membrane.
8. Use forceps to move the membrane from the SA-AP into a plastic box or a dish containing 250 ml of Buffer 1. Gently agitate for 15 min.
9. Decant Buffer 1. Add 250 ml of fresh Buffer 1. Gently agitate for 15 min.
10. Transfer the membrane into 250 ml of Buffer 3. Incubate for at least 10 min at room temperature.

Visualization

Prepare dyes just prior to use.

CAUTION: The dye solutions contain dimethylformamide, which is harmful if inhaled, swallowed, or absorbed through the skin. Wear gloves when handling the dye solutions and wash hands thoroughly after use.

11. In a polypropylene tube, add 33 μl of nitroblue tetrazolium (NBT) solution to 7.5 ml of Buffer 3. Mix gently by inverting the tube. Add 25 μl of 5-bromo-4-chloro-3-indolylphosphate (BCIP) solution. Mix gently. Pour the dye solution into a small plastic box just large enough for the membrane to lie flat. The NBT solution is 75 mg/ml NBT in 70% dimethylformamide. The BCIP solution is 50 mg/ml BCIP in dimethylformamide.
12. Place the membrane in the dye solution. Incubate at room temperature in the dark or in low light. Periodically examine the membrane for the development of purple bands on the membrane. Color development may require from 30 min to 3 hr. Incubations of longer than

3 hr may result in increased background. DNA bands will be most evident on only one side of the membrane.

13. When the bands are visible, stop the color development reaction. Wash the membrane in 20 m*M* Tris and 0.5 m*M* Na_2 EDTA, pH 7.5.
14. Allow the membrane to air-dry. Often the purple color will appear less intense when the membrane is dry. To examine faint bands, place the membrane back in 20 m*M* Tris and 0.5 m*M* Na_2 EDTA, pH 7.5. When the membrane is wet, the color intensity increases. After observing bands, allow the membrane to air-dry again. Store the membrane away from strong light to prevent fading of the color.

NBT and BCIP form an insoluble precipitate that makes it difficult to reprobe a membrane. NBT and BCIP can be solublized in dimethylformamide. Nitrocellulose is destroyed by dimethylformamide. However, if a nylon membrane was used in the blot, the dyes can be stripped from the membrane by soaking the membrane in dimethylformamide.

PROTOCOL 3.4b: Hybridization and Detection of Labeled Probe—A Biotin-Labeled Nonradioactive Probe and Chemiluminogenic Substrate

This protocol uses materials from the Photogene Detection System from Gibco/BRL.

The chemiluminescent substrate for alkaline phosphatase used here is PPD or 4-methoxy-4-(3-phosphatephenyl)spiro [1,2-dioxetane-3,2′-adamantane]. Enhancers added (that form fluorescent micelles) are cetyltrimethyl ammonium bromide and 5-(N-tetradecanoyl)-aminofluorescein in 0.75 *M* 2-amino-2-methyl-1-propanol at pH 9.6.

This dioxetane system is able to detect less than 1 pg of target DNA. It appears to be more sensitive than the chromogenic NBT/BCIP substrates.

For optimal results, use Photogene nylon membrane for which BRL has optimized conditions to minimize background and maximize sensitivity.

Southern Blot

1. Prepare a Southern blot per standard protocol with one modification to accommodate the use of the Photogene membrane. When using a Photogene membrane, wet the membrane initially in distilled water, then soak the membrane in 10× SSC for 15 min before it is put on top of the agarose gel for the Southern transfer. Also use 10× SSC for the transfer solution. (Nitrocellulose membranes use 20× SSC for transfers.)

2. Bake the membrane to attach the DNA to it.

Hybridization

1. Prepare the prehybridization solution to make 100 ml of solution:

Component	Final concentration	Amount
NaCl	0.9 *M*	5.26 g
$NaH_2PO_4 \cdot H_2O$	0.06 *M*	0.83 g
$Na_2EDTA \cdot H_2O$	0.006 *M*	0.22 g
Ficoll	0.1% (w/v)	0.1 g
Polyvinylpyrrolidone	0.1% (w/v)	0.1 g
Bovine serum albumin	0.1% (w/v)	0.1 g
Sodium dodecyl sulfate	1.0% (w/v)	1.0 g; 5 ml of 20% (w/v) stock
Sheared, denatured salmon sperm DNA	200 μ/ml	20 mg; 2 ml of a 10 mg/ml stock
Formamide	50% (v/v)	50 ml

Add the components (except the DNA and formamide) in the order indicated above to less than 38 ml of sterile distilled water. Be sure each solid component is completely dissolved before adding the next component. Adjust the pH to 7.4 with 4 *M* NaOH. Add the DNA. Adjust the volume to 50 ml with distilled water. Add 50 ml formamide. Mix thoroughly. Store excess solution at −20°C. If desired, a stock of an EDTA solution and a stock containing Ficoll, PVP, and BSA can also be used.

2. Place the membrane in a plastic Seal-a-Meal bag or a plastic box large enough to allow the membrane to lie flat. Add prehybridization solution. Use 250 μl prehybridization solution for each square centimeter of membrane surface area. For example, a 10 by 10-cm membrane requires 25 ml of prehybridization solution. Seal the plastic bag. Incubate the membrane 2 to 4 hr at 42°C.
3. Prepare the hybridization solution.

Hybridization Solution

a. To make 50 ml of 20% dextran sulfate solution, add 10 g dextran sulfate to about 40 ml of formamide. Dextran sulfate will dissolve very slowly. Dissolve the dextran sulfate by gentle rocking or stirring overnight at room temperature. When all the dextran sulfate has dissolved, adjust the final volume to 50 ml with formamide and mix thoroughly. Store the solution at 4°C. This solution is very viscous. Warm the solution in a 42°C bath before using it.

b. To make 50 ml of 2× hybridization solution, use the following:

Component	Final concentration	Amount
NaCl	1.8 *M*	5.26 g
$NaH_2PO_4 \cdot H_2O$	0.12 M	0.83 g
$Na_2EDTA \cdot H_2O$	0.012 *M*	0.22 g
Ficoll	0.2% (w/v)	0.1 g
Polyvinylpyrrolidone	0.2% (w/v)	0.1 g
Bovine serum albumin	0.2% (w/v)	0.1 g
Sodium dodecyl sulfate	2.0% (w/v)	1 g; 5 ml of 20% (w/v) stock
Sheared, denatured salmon sperm DNA	400 μg/ml	20 mg; 2 ml of a 10 mg/ml stock

Add the components (except the DNA) in the order indicated above to less than 38 ml of sterile distilled water. Be sure that each solid component is completely dissolved before adding the next component. Adjust the pH to 7.4 with 4 *M* NaOH. Add the DNA. Adjust the volume to 50 ml with distilled water. Store solution at −20°C. Note that the dextran sulfate is not added to the 2× hybridization solution at this time.

4. Determine the amount of probe to use. Use this factor: ng probe = area of membrane (cm^2) × 50 ng probe/ml × 0.1 ml/cm^2 membrane. For example, a 10 by 10-cm membrane requires 0.5 μg of probe DNA.
5. Ethanol precipitate the probe. Place the correct volume of probe DNA needed in a 30-ml Corex centrifuge tube. Add 0.1 volume of 3 *M* NaOAc (sodium acetate). Add 2 volumes of 100% ethanol. Mix thoroughly. Place at −20°C for at least 2 hr. Centrifuge the probe sample in a microfuge for 10 min. Decant the ethanol. Dry the DNA pellet completely. Either let the pellet air-dry or use a vacuum to dry it.
6. Dissolve the probe DNA in 2× hybridization solution. Use 50 μl of 2× hybridization solution per square centimeter surface area of membrane. For a 10 by 10-cm membrane, 5 ml of 2× hybridization solution is added. Be sure the pellet is completely dissolved. Add an equal volume of 20% dextran sulfate in formamide. For a 10 by 10-cm membrane, add 5 ml of 20% dextran sulfate in formamide.
7. Denature the probe just before the probe is added to the membrane. Place the centrifuge tube with the redissolved probe in a boiling water bath for 10 min. Then place the tube on ice for 10 min.
8. Cut open a corner of the plastic bag. Pour off the prehybridization solution. Add the hybridization solution and reseal the plastic bag.
9. Incubate the membrane with the probe overnight at 42°C.

Washes

These washes are used after hybridization to remove any probe that is not correctly base paired to its homologous sequences.

1. Make all wash solutions needed in advance. For each of the wash solutions, use 2 ml of wash solution/cm^2 surface area of the membrane. For a 10 by 10-cm membrane, use 200 ml of each wash solution. Prewarm the wash solutions to the temperatures at which they will be used.
2. Remove the membrane from the 42°C bath. Cut open the plastic bag. The hybridization solution with the probe can be saved and reused to probe another membrane. Store used hybridization solution with probe at −20°C. Using forceps, move the membrane into a plastic box containing the first wash solution.
3. Wash the membrane in 5× SSC, 0.5% (w/v) SDS at 65°C for 5 min.
4. Again, wash the membrane in 5× SSC, 0.5% (w/v) SDS at 65°C for 5 min.
5. Wash the membrane in 0.1× SSC, 1% (w/v) SDS at 50°C for 30 min. To vary the level of hybridization stringency, the temperature may be varied.
6. Wash the membrane in 2× SSC for 5 min at room temperature.

NOTES

As an alternative to the prehybridization and hybridization solutions used above, Gibco/BRL Tech-Line 800-828-6686 reports that the following modification has been used successfully by some of their research scientists. Instead of the prehybridization and hybridization solutions described above, use 5× SSC, 1% SDS, 0.5% BSA; a probe concentration of 50 ng/ml; and hybridization temperatures of 65°C. This solution is simpler to prepare and uses less-expensive components.

Detection of Biotin-Labeled DNA: Binding the Streptavidin–Alkaline Phosphatase Conjugate to the Biotin-Labeled DNA

During these steps, be sure solutions flow freely around the membranes. Make sure the membranes do not stick to the container or to each other. Use forceps to move the membrane when necessary.

1. Make the TBS–Tween 20 and blocking solutions in advance:

Component	Final concentration	Amount
Tris base	100 m*M*	21.1 g
NaCl	150 m*M*	8.77 g
Tween 20	0.05% (v/w)	0.5 ml

Adjust the pH to 7.5 with 4 *M* HCl. Filter sterilize using a 0.2-μm filter. Store the sterile solution at 4°C. Prewarm TBS–Tween 20 to room temperature before use.

Blocking Solution (100 ml)

Dissolve 3 g of bovine serum albumin (fraction V BSA) in 100 ml TBS–Tween 20. Adjust the pH to 7.5. Filter sterilize the solution using a 0.45-μm filter. Store the solution at 4°C. Other preparations of BSA may contain alkaline phosphatase activity and should be checked carefully before use. Prewarm the blocking solution to 65°C before use.

2. Wet the hybridized and washed membrane thoroughly in TBS–Tween 20.
3. Incubate the membrane in blocking solution for 1 hr at 65°C in a covered plastic box. Use 0.75 ml blocking solution/cm^2 surface area of the membrane. Agitate the membrane gently during blocking. If working with more than one membrane in a box, be sure membranes do not stick to each other or to the box.
4. Spin the tube of streptavidin–alkaline phosphatase conjugate in a microfuge for 4 min at room temperature. The solution of SA-AP conjugate is 1.0 mg/ml SA-AP in 3 *M* NaCl, 1 m*M* $MgCl_2$, 0.1 m*M* $ZnCl_2$, 30 m*M* triethanolamine, pH 7.6. A precipitate may or may not be present in the bottom of the microfuge tube. Remove the needed amount of SA-AP from the supernatant solution. Do not pipet any of the precipitate in the bottom of the tube. Use 7 μl of SA-AP for each 100 cm^2 surface area of the membrane.
5. Dilute the supernatant SA-AP 1:1000 in TBS–Tween 20. For a 10 by 10-cm membrane, add 7 μl of SA-AP to 7 ml of TBS–Tween 20.
6. Incubate the membrane in the diluted SA-AP for 10 min at room temperature with gentle agitation. Do not let membranes stick to each other or to the plastic box.
7. Use forceps to remove the membrane from the diluted SA-AP and place the membrane in a clean plastic box containing TBS–Tween 20. Use 1 ml TBS–Tween 20/cm^2 surface area of the membrane. Wash the membrane in TBS–Tween 20 for 15 min at room temperature. Gently agitate or rock the membrane during this time.
8. Use forceps to remove the membrane from the TBS–Tween 20 and place the membrane in a clean plastic box containing fresh TBS–Tween 20. Again, wash the membrane in TBS–Tween 20 for 15 min at room temperature. Gently agitate or rock the membrane.
9. Dilute the 10× final wash buffer 1:10 with distilled water. Use 1 ml 1× final wash buffer/cm^2 surface area of the membrane. Wash the membrane in final wash buffer for at least 60 min at room temperature. Agitate gently. 1× final wash buffer: 0.65 *M* 2-amino-2-methyl-1-propanol, pH 9.6, 0.88 *M* $MgCl_2$.

10. Use forceps to remove the membrane from the final wash solution. Touch the membrane to a piece of chromatography paper to remove excess buffer. Place the membrane in between two clear acetate sheets or in plastic wrap.

Addition of the Chemiluminogenic Substrate for Alkaline Phosphatase

The chemiluminogenic substrate (PPD) should not be exposed to bright lights. Work in subdued light when adding the substrate.

CAUTION: The chemiluminogenic substrate may be a skin irritant. The substrate solution is flammable. Wear gloves when handling the chemiluminogenic substrate. Wash hands thoroughly when finished working with the substrate.

1. Use 0.01 ml PPD/cm^2 surface area of the membrane. For a 10 by 10-cm membrane, 1 ml of substrate is used. Remove the top acetate sheet covering the membrane. Pipet the needed amount of chemiluminogenic substrate over the top of the membrane. Immediately cover the membrane with the acetate sheet or plastic wrap. Gently roll a 10-ml pipet over the plastic sheet to spread the detection reagent evenly over the membrane and to remove any air bubbles that are over the membrane. Do not apply too much pressure.
2. Seal the edges of the acetate sheets or plastic wrap with tape.
3. Place the membranes in folders in the dark at room temperature, 23–25°C.
4. In the dark or under a photographic red safety light, place a piece of X-ray film over the acetate sheet above the membrane. Place the film and membrane in acetate sheet inside a light-tight film holder. Expose the film for the desired length of time. Develop the film. During the course of this light-generating reaction, the light emission increases during the first 3 to 5 hr after addition of the substrate (Carlson *et al.*, 1990). The intensity of light emission remains relatively constant for the next 24 hr. Often light can still be detected 48 hr or more later. This allows time for several different exposures to be obtained.

NOTES

1. The chemiluminogenic substrate system used here is based on the Gibco/BRL Photogene system.
2. A film can be exposed by the membrane as soon as the substrate has been added, but waiting 3 to 5 hr when the rate of light emission is relatively constant will make it easier to plan the length of exposure times. Initially, try a 15-min exposure, then adjust exposure times as

needed. Typical exposure times for the detection of a plasmid DNA would be minutes and for the detection of a signal copy sequence in a complex eukaryotic genome would be more than an hour. The emitted light can also be detected on Polaroid film, by the dark-adapted eye, or by some phosphoimagizing systems.

3. If a very strong signal is detected, there may be a local depletion of substrate. If a once strong signal has disappeared in only 24 hr, an additional aliquot of the chemiluminogenic substrate will restore the signal (Karcher and Goodner, 1990).
4. To increase the final chemiluminogenic signal, the amount of SA-AP stock used can be increased or the length of time allowed for the SA-AP to bind to biotin can be increased.
5. If nonspecific background is observed, the following suggestions may reduce the background.
   a. Increase the length of time of the washes to remove the unbound SA-AP.
   b. Increase the time of incubation in the final wash to greater than 1 hr. Longer incubations in the final wash solution generally result in backgrounds. The membrane can be incubated in final wash solution for up to 24 hr at room temperature without adverse consequences.
   c. Be sure to filter sterilize the solutions using a 0.45-μm filter. The removal of dust by the filtration of solutions frequently helps to minimize background spots.
   d. Use powder-free disposable gloves while working with the chemiluminogenic substrate because powder residues from disposable gloves may also contribute to background problems.

Reprobing Blots

A Photogene membrane may be reprobed many times with little or no loss of signal. To remove the hybridized probe, place the blot in a large container; cover the blot with 0.1× SSC, 0.1% SDS; and heat the blot to 100°C for 15 min.

Additional Notes about Nonradioactive DNA Detection Systems

1. The following are comments about selecting the membrane to use.
   a. Nitrocellulose membranes do not work well with a chemiluminogenic substrate because nitrocellulose quenches the signal from chemiluminogenic substrates such as Lumi-Phos 530 or Lumigen PPD. For a chromogenic substrate, nitrocellulose or nylon membranes can be used. The color can be removed from a nylon mem-

brane by soaking the membrane in N,N-dimethylformamide (DMF). However, the color cannot be removed from nitrocellulose membranes because DMF dissolves nitrocellulose.

b. The membrane used with a digoxigenin labeling system can be a positively charged nylon membrane. However, membranes with a high positive charge density can cause unacceptably high backgrounds that cannot be avoided even with increased blocking and washing steps. Uncharged membranes give low backgrounds but may be more apt to leach off nucleic acid during the hybridization and prehybridization steps. Suppliers of dioxigenin labeling systems, such as the Genius Kit from Boehringer-Mannheim, recommend a nylon membrane with a moderate amount of positive charge (Boehringer-Mannheim, 1994). Often the supplier of the kit also sells a nylon membrane that has been optimized for its labeling kits. Other membranes must be tested to determine if the background obtained will be acceptably low.

2. Lakhotia (1993) described the use of gelatin as a blocking agent. After transferring DNA to a membrane and crosslinking to bind the DNA to the membrane, he dipped the membrane into a 0.1% gelatin solution for approximately 5 sec. The membrane was then air-dried. Lakhotia says that for Southern blots, after a gelatin treatment, no prehybridization was necessary. In addition, blocking agents such as Denhardt's solution or single-stranded calf thymus DNA were not needed. He used the procedure successfully for a ^{32}P-labeled probe and for a digoxigenin-labeled probe detected with a chromogenic or a chemiluminogenic substrate.
3. Other chemiluminogenic substrates are Lumi-Phos 480 dioxetane, Lumi-Phos, Lumigen-PPD (all Trademarks of Lumigen, Inc., Detroit, MI) and AMPPD (Trademark of Tropix, Bedford, MA). Lumi-Phos 530 is PPD in a solution containing enhancers; Lumigen-PPD contains concentrated PPD without enhancers and is diluted before use. Lumi-Phos 530 is used in the Gibco/BRL Photogene system. These different substrates vary in the enhancers that are added to the substituted dioxetane and vary in the wavelength of maximum emission.
4. The following are alternative methods for adding the chemiluminogenic substrate.

a. In the USB "Gene Images" kit, the substrate is sprayed onto the surface of the membrane as a mist from a spray bottle.

CAUTION: If using such a spray to distribute the substrate, avoid inhalation of the sprayed mist.

b. Soak the blot briefly in a larger volume of the substrate solution and then remove the blot from the substrate solution. For example,

Mackey and Rashtchian (1992) soak the blot in the chemiluminogenic substrate for 60 sec and then remove the blot from the substrate solution. Neuhaus-Url and Neuhaus (1993) add to the DNA side of a membrane 2.5 to 5 ml of chemiluminogenic substrate for a 100-cm^2 membrane. The substrate they use is AMPPD from Tropix or Boehringer-Mannheim diluted to 100 μg/ml in 100 m*M* Tris–HCl, 100 m*M* NaCl, 50 m*M* $MgCl_2$, pH 9.5. After 5 min, they remove the diluted AMPPD and reuse it for up to three more membranes over the next week. Figure 3.12 shows an example of the detection of a chemiluminogenic probe.

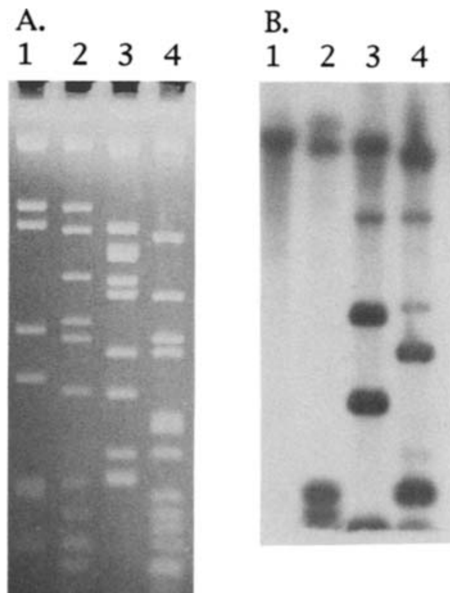


Figure 3.12 An example of a Southern blot used to map a restriction fragment from a cosmid. (A) An ethidium bromide-stained gel. In each lane is 0.5 μg of cosmid DNA cut with different restriction endonucleases. (B) The hybridization of a specific probe only to certain fragments of the cosmid. The probe was a cloned fragment of the cosmid and was labeled with biotin and detected with streptavidin conjugated to alkaline phosphatase using a chemiluminogenic substrate for alkaline phosphatase. This information is analyzed to generate a map of restriction endonuclease sites in the cosmid. These data are from Biology 542 classes at Purdue University.

PROTOCOL 3.5: Standard Southern Blot Hybridization with ^{32}P-Labeled Probe

Materials

Prehybridization Solution

Final concentration of component	Concentration of stock component	Amount of stock component to add
6 × SSC	20 × SSC	90 ml
10 × PM	100 × PM	30 ml
20 m*M* Tris, pH 7.0	2 *M* Tris, pH 7.0	3 ml
	H_2O	To a total volume of 300 ml

Do not autoclave this solution. Use sterile water to make up the solution.

Southern Hybridization Solution

Final concentration of component	Concentration of stock component	Amount of stock component to add
3 × SSC	20 × SSC	15 ml
5 × PM	100 × PM	5 ml
20 m*M* Tris, pH 7.0	2 *M* Tris, pH 7.0	1 ml
2 m*M* EDTA	0.5 *M* EDTA, pH 7.0	0.4 ml
100 μg/ml sheared, denatured calf thymus DNA	2 mg/ml sheared, denatured calf thymus DNA	5 ml
0.5% SDS	20% (w/v) SDS	2.5 ml
	H_2O	To a total volume of 100 ml

Do not autoclave this solution. Use sterile water to make up the solution. SDS should be added last because SDS will precipitate in high salt.

Washes for Southern Blot

Final concentration of component	Concentration of stock component	Amount of stock component to add
0.3 × SSC	20 × SSC	15 ml
5 m*M* EDTA	0.5 *M* EDTA	10 ml
0.1% SDS	20% SDS	5 ml
	H_2O	To a total volume of 1 liter

NOTES

1. Use molecular biology grade formamide or deionize the formamide before using. To deionize formamide, add approximately 1 g of Dowex mixed resin beads to approximately 100 ml of formamide. Mix thoroughly. Store in the dark or wrap the bottle with aluminum foil. Let the formamide and beads stand for several hours before being used.
2. SSC is standard sodium citrate. 1× SSC is 8.8 g NaCl, 4.4 g Na_3 citrate/liter, pH 7.0 (0.15 *M* NaCl, 0.015 *M* Na_3 citrate).
3. PB is phosphate buffer. 1 *M* PB is pH 6.8. Make 1 *M* PB by mixing equal volumes of 1 *M* mono-basic sodium phosphate and di-basic sodium phosphate.
4. PM is prehybridization mix or Denhardt's solution.

100× Denhardt's solution:

2% (w/v) BSA (bovine serum albumin)

2% (w/v) PVP (polyvinylpyrrolidone, molecular weight (MW) 4×10^4)

2% (w/v) Ficoll (MW 4×10^5, a nonionic synthetic polymer of sucrose)

These three components are "nonspecific blockers." They help decrease "background" of nonspecific binding of probe to nitrocellulose membrane.

5. SDS is sodium dodecyl sulfate.

CAUTION: Before working with radioactive ^{32}P, review the radiation safety rules and disposal protocols for your school.

Procedure

1. Seal the baked nitrocellulose blot in a Seal-a-Meal plastic bag containing 25 ml of Southern prehybridization solution.
2. Incubate the blot overnight at 65°C.
3. Replace prehybridization solution with Southern hybridization solution.
4. Incubate at 65°C for a minimum of 4–6 hr.
5. Add denatured probe.
6. Allow hybridization to take place. For a plasmid DNA, overnight hybridization is sufficient. Hybridize over two nights for a eukaryotic genomic Southern.
7. Wash blot to remove unbound probe. Wash 1× with 3XSSC, 0.1% SDS, 5 m*M* EDTA. Wash 3× with 0.3XSSC, 0.1% SDS, 5 m*M* EDTA. Each wash is at 65–68°C for at least 30 min. Rinse blot in 2× SSC.

8. Wrap the blot in plastic wrap and mount it on intensification screens for autoradiography.
9. Expose X-ray films.

References

Beck, S. (1992). Nonradioactive detection of DNA using dioxetane chemiluminescence. *In* "Methods in Enzymology" (R. Wu, ed.), Vol. 216, pp. 143–153. Academic Press, San Diego.

Beck, S., and Koster, H. (1990). Applications of dioxetane chemiluminescent probes to molecular biology. *Anal. Chem.* **62,** 2258–2270.

Boehringer-Mannheim (1994). Technical tips: Selecting membranes to use with the Genius system. *Biochemica* **11,** 10.

Britten, R., Graham, D., and Neufeld, B. (1974). Analysis of repeating DNA sequences by reassociation. *In* "Methods in Enzymology" (L. Grossman and K. Moldave, eds.) Vol. 29, pp. 363–418. Academic Press, San Diego.

Bronstein, I., and McGrath, P. (1989). Chemiluminescence lights up. *Nature (London)* **338,** 599–600.

Bronstein, I., Edwards, B., and Voyta, I. C. (1989). 1,2-Dioxetanes: Novel chemiluminescent enzyme substrates: Applications to immunoassays. *J. Biolumin. Chemilumin.* **4,** 99–111.

Burmeister, M., and Lehrach, H. (1987). Isolation of large DNA fragments from agarose gels using agarase. *Trends Genet.* **5,** 41.

Carlson, D. P., Superko, C., Mackey, J., Gaskill, M. E., and Hansen, P. (1990). Chemiluminescent detection of nucleic acid hybridization. *Focus* **12**(1), 9–12.

Chomczynski, P. (1992). One-hour downward alkaline capillary transfer for blotting of DNA and RNA. *Anal. Biochem.* **201,** 134–139.

Church, G. M., and Gilbert, W. (1984). Genomic sequencing. *Proc. Natl. Acad. Sci. U.S.A.* **81,** 1991–1995.

Dretzen, G. M., Bellard, M., Sassone-Corsi, P., and Chambon, P. (1981). A reliable method for the recovery of DNA fragments from agarose and acrylamide gels. *Anal. Biochem.* **112,** 295–298. [Use of strips of DEAE-cellulose paper]

Danna, K. J. (1980). Determination of fragment order through partial digests and multiple enzyme digests. In "Methods in Enzymology" (L. Grossman and K. Moldave, eds.). Vol. 65, 449–467. Academic Press, San Diego.

Ericson, M. L. (1990). Quick DNA recovery from agarose gels by ultracentrifuge run. Trends *Genet.* **6,** 278.

Feinberg, A. P., and Vogelstein, B. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **132,** 6–13.

Feinberg, A. P., and Vogelstein, B. (1984). Addendum: A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **137,** 266–269.

Forster, A. C., McInnes, J. L., Skingle, D. C., and Symons, R. H. (1985). Non-radioactive hybridization probes prepared by the chemical labeling of DNA and RNA with a novel reagent, photobiotin. *Nucleic Acids Res.* **13,** 745–761.

Gebeyehu, G., Pao, P. Y., Simms, D. A., and Klevan, L. (1987). Novel biotinylated nucleotide-analogs for labeling and colorimetric detection of DNA. *Nucleic Acids Res.* **15,** 4513–4534.

Gillespie, D., and Spiegelman, S. (1965). A quantitative assay for DNA–RNA hybrids with DNA immobilized on a membrane. *J. Mol. Biol.* **12,** 829–842.

Grunstein, M., and Hogness, D. S. (1975). Colony hybridization: A method for the isolation of cloned DNAs that contain a specific gene. *Proc. Natl. Acad. Sci. U.S.A.* **72,** 5417–5421.

Grunstein, M., and Wallis, J. (1979). Colony hybridization. *In* "Methods in Enzymology." (R. Wu, ed.) Vol. 68, pp. 379–389. Academic Press, San Diego.

Guder, H.-J. (1992). Colorimetric systems—indigo/tetrazolium dyes. *In* "Nonradioactive Labeling and Detection of Biomolecules" (C. Kessler, ed.), pp. 159–161. Springer-Verlag, Berlin.

Hahn, E. J. (1983). Autoradiography: A review of basic principles. *Am. Lab.* **15,** 64–71.

Hanahan, D., and Meselson, M. (1980). Plasmid screening at high colony density. *Gene* **10,** 63–67.

Heery, D. M., Gannon, F., and Powell, R. (1990). A simple method for subcloning DNA fragments from gel slices. *Trends Genet.* **6,** 173. [Centrifugation through glass wool]

Jacob, A. T. (1991). "The Chemistry of Black and White Photography." Institute for Chemical Education, Univ. Wisconsin, Madison, WI.

Karcher, S. J., and Goodner, B. W. (1990). Chemiluminescent detection of multiple copy genes. *Focus* **12,** 112–113.

Kessler, C. (1992). "Nonradioactive Labeling and Detection of Biomolecules." Springer-Verlag, Berlin.

Khandjian, E. W. (1987). Optimized hybridization of DNA blotted and fixed to nitrocellulose and nylon membranes. *Biotechnology* **5,** 165–167.

Klevan, L., and Gebeyehu, G. (1990). Biotinylated nucleotides for labeling and detecting DNA. *In* "Methods in Enzymology: Avidin-Biotin Technology" (M. Wilchek and E. A. Bayer, eds.), Vol. 184, pp. 561–577. Academic Press, San Diego.

Koenen, M. (1989). Recovery of DNA from agarose gels using liquid nitrogen. *Trends Genet.* **5,** 137.

Koetsier, P. A., Schorr, J., and Doerfler, W. (1993). A rapid optimized protocol for downward alkaline Southern blotting of DNA. *Biotechniques* **15,** 260–262.

Kricka, L. J. (1992). "Nonisotopic DNA Probe Techniques." Academic Press, San Diego.

Lakhotia, S. C. (1993). Gelatin as a blocking agent in Southern blot and chromosomel *in situ* hybridizations. *Trends Genet.* **9,** 261.

Langer, P. R., Waldrop, A. A., and Ward, D. C. (1981). Enzymatic synthesis of biotin-labeled polynucleotides: Novel nucleic acid affinity probes. *Proc. Natl. Acad. Sci. U.S.A.* **78,** 6633–6637.

Langridge, J., Langridge, P. J., and Bergquist, P. L. (1980). Extraction of nucleic acids from agarose gels. *Anal. Biochem.* **103,** 246–271. [Use of quaternary ammonium compound to complex with nucleic acids]

Leary, J. L., Brigate, D. J., and Ward, D. C. (1983). Rapid and sensitive colorimetric method for visualizing biotin-labeled DNA or RNA immobilized on nitrocellulose Bio-blots. *Proc. Natl. Acad. Sci. U.S.A.* **80,** 4045–4049.

Lerman, L. S. (1986). "DNA Probes: Applications in Genetics and Infectious Diseases." Cold Spring Harbor Press, Cold Spring Harbor, NY.

Lichtenstein, A. V., Moiseev, V. L., and Zaboikin, M. M. (1990). A procedure for DNA and RNA transfer to membrane filters avoiding weight-induced gel flattening. *Anal. Biochem.* **191,** 187–191.

Lueders, K. K., and Fewell, J. W. (1994). Hybridization of DNA in dried gels provides increased sensitivity compared with hybridization to blots. *Biotechniques* **16,** 66–67.

Mackey, J., and Rashtchian, A. (1992). A method for simultaneous nonradioactive labeling and amplification of DNA using random primers. *Focus* **14,** 21–23.

Mackey, J., Guan, N., and Rashtchian, A. (1992a). Direct enzyme coupling to oligonucleotide probes for increased sensitivity and simplified nonradioactive detection. *Focus* **14,** 112–116.

Mackey, J., Guan, N., and Rashtchian, A. (1992b). Rapid labeling of DNA probes using random primers. *Focus* **14,** 117–119.

Mackey, J., Darfler, M., Nisson, P., and Rashtchian, A. (1993). Use of random primer extension for concurrent amplification and nonradioactive labeling of nucleic acids. *Anal. Biochem.* **212,** 428–435.

Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982). "Molecular Cloning: A Laboratory Manual," p. 471. Cold Spring Harbor Press, Cold Spring Harbor, NY.

Martin, R., Hoover, C., Grimme, S., Grogan, C., Holtke, J., and Kessler, C. (1990). A highly sensitive, nonradioactive DNA labeling and detection system. *Biotechniques* **9**, 762–768.

Meinkoth, J., and Wahl, G. (1984). Hybridization of nucleic acids immobilized on solid supports. *Anal. Biochem.* **138**, 267–284.

Neuhaus-Url, G., and Neuhaus, G. (1993). The use of the nonradioactive digoxigenin chemiluminescent technology for plant genomic Southern blot hybridization: A comparison with radioactivity. *Transgen. Res.* **2**, 115–120.

Neufeld, P. J., and Colman, N. (1990). When science takes the witness stand. *Sci. Am.* **262**, 46–53.

Peloquin, J. J., and Platzer, E. G. (1991). A simple inexpensive electroelution device for the recovery of nucleic acid fragments from agarose gels. *Biotechniques* **10**, 159–160. [Electroelution using a microfuge tube instead of dialysis bag]

Pollard-Knight, D. (1990). Current methods in nonradioactive nucleic acid labeling and detection. *Technique* **2**, 113–132.

Reed, K. C., and Mann, D. A. (1985). Rapid transfer of DNA from agarose gels to nylon membranes. *Nucleic Acids Res.* **13**, 7207–7221.

Rackwitz, H. R., Zehetner, G., Frischauf, A.-M., and Lehrach, H. (1984). Rapid restriction mapping of DNA cloned in lambda phage vectors. *Gene* **30**, 195–200.

Rigaud, G., Grange, T., and Pictet, R. (1987). The use of NaOH as transfer solution of DNA onto nylon membrane decreases the hybridization efficiency. *Nucleic Acids Res.* **15**, 857.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). "Molecular Cloning: A Laboratory Manual," 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schleicher and Schuell (1992). "Product Guide and Methods," Keene, NH.

Smith, G. E., and Summers, M. D. (1980). The bidirectional transfer of DNA and RNA to nitrocellulose or diazobenzyloxymethyl-paper. *Anal. Biochem.* **109**, 123–129.

Smith, H. O., and Birnstiel, M. L. (1976). A simple method for DNA restriction mapping. *Nucleic Acids Res.* **3**, 2387–2398.

Southern E. (1975). Detection of specific sequences among DNA fragments separated by electrophoresis. *J. Mol. Biol.* **98**, 503–517.

Stone, T., and Durrant, I. (1991). Enhanced chemiluminescence for the detection of membrane-bound nucleic acid sequences: Advantages of the Amersham system. *Genet. Anal. Techniques Appl.* **8**, 230–237.

Stoye, J. P., Frankel, W. N., and Coffin, J. M. (1991). DNA hybridization in dried gels with fragmented probes: An improvement over blotting techniques. *Technique* **3**, 123–128.

Thayer, R. (1979). An improved method for detecting foreign DNA in plasmids of *Escherichia coli*. *Anal. Biochem.* **98**, 60–63.

Thuring, R. W. J., Sanders, J. P. M., and Borst, P. (1975). A freeze-squeeze method for recovering long DNA from agarose gels. *Anal. Biochem.* **66**, 213–220.

Twoney, T. A., and Krawetz, S. A. (1990). Parameters affecting hybridization of nucleic acids blotted onto nylon or nitrocellulose membranes. *Biotechniques* **8**, 478–481.

Vogelstein, B., and Gillespie, D. (1979). Preparative and analytical purification of DNA from agarose. *Proc. Nat. Acad. Sci. U.S.A.* **76**, 615–619. [Use of chaiotropic salt NaI]

Weichenhan, D. (1991). Fast recovery of DNA from agarose gels by centrifugation through blotting paper. *Trends Genet.* **7**, 109.

Wiley, A. M. (1988). "Nucleic Acid Probes in Diagnosis of Human Genetic Diseases." A. R. Liss, New York.

Zhu, J., Kempenaers, D., Van der Straeten, D., Contreras, R., and Fiers, W. (1985). A method for fast and pure DNA elution from agarose gels by centrifugal filtration. *Biotechnology* **3**, 1014–1016.

Suggested Reading

Recovery of DNA from Gels

Dretzen, G., Bellard, M., Sassone-Corsi, P., and Chambon, P. (1981). A reliable method for recovery of DNA fragments from agarose and acrylamide gels. *Anal. Biochem.* **112,** 295–298.

Smith, H. O. (1980). Recovery of DNA from gels. *In* "Methods in Enzymology" (L. Grossman and K. Moldave, eds.), Vol. 65, p. 371. Academic Press, San Diego.

Weislander L. (1979). A simple method to recover intact high molecular weight RNA and DNA after electrophoretic separation in low gelling temperature agarose gels. *Anal. Biochem.* **98,** 305–309.

Nucleic Acid Hybridization

Casey, J., and Davidson, N. (1977). Rates of formation and thermal stabilities of RNA : DNA and DNA : DNA duplexes at high concentration of formamide. *Nucleic Acids Res.* **4,** 1539–1552.

Chaconas, G., and van de Sande, J. H. (1980). '5-^{32}P labeling of RNA and DNA restriction fragments. *In* "Methods in Enzymology" (L. Grossman and K. Moldave, eds.), Vol. 65, p. 75. Academic Press, San Diego.

Dove, W. F., and Davidson, N. (1962). Cation effects on the denaturation of DNA. *J. Mol. Biol.* **5,** 467–478.

Dunn, A. R., and Sambrook, J. (1980). Mapping viral mRNAs by sandwich hybridization. *In* "Methods in Enzymology" (L. Grossman and K. Moldave, eds.), Vol. 65, p. 468. Academic Press, San Diego.

Grunstein, M., and Hogness, D. S. (1975). Colony hybridization: A method for the isolation of cloned DNAs that contain a specific gene. *Proc. Natl. Acad. Sci. U.S.A.* **72,** 3961.

Laskey, R. A. (1980). Use of intensification screens or organic scintillators for visualizing radioactive molecules resolved by gel electrophoresis. *In* "Methods in Enzymology" (L. Grossman and K. Moldave, eds.), Vol. 65, p. 363. Academic Press, San Diego.

Laskey, R. A., and Mills, A. D. (1977). Enhanced autoradiographic detection of ^{32}P and ^{125}I using intensifying screens and hypersensitized films. *FEBS Lett.* **82,** 314–316.

McConaughy, B. L., Laird, C. D., and McCarthy, B. J. (1969). Nucleic acid reassociation in formamide. *Biochemistry* **8,** 3289–3295.

Meinkoth, J., and Wahl, G. (1984). Hybridization of nucleic acids immobilized on solid supports. *Anal. Biochem.* **138,** 267–284.

Rigby, P. W. J., Diechmann, M., Rhodes, C., and Berg, P. (1977). Labeling DNA to high specific activity *in vitro* by nick translation with DNA polymerase I. *J. Mol. Biol.* **133,** 237–251.

Southern E. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98,** 503–517.

Swanstrom, R., and Shank, P. R. (1978). X-ray intensifying screens greatly enhance the detection by autoradiography of the radioactive isotypes ^{32}P and ^{125}I. *Anal. Biochem.* **86,** 184–192.

Thomas, P. (1980). Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc. Natl. Acad. Sci. U.S.A.* **77,** 5201–5205.

Autoradiography

Hahn, E. J. (1983). Autoradiography: A review of basic principles *Am. Lab.* **15**(7), 64–71.

Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982). "Molecular Cloning: A Laboratory Manual," pp. 470–471. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

4

PLANT GENOMIC SOUTHERN BLOTTING WITH PROBES FOR LOW- AND HIGH-COPY-NUMBER GENES

Overview of Experiment

Genomic Southern

In this experiment, plant genomic Southern blots are probed with a clone of a high-copy-number gene (for ribosomal RNA) and a clone of a low-copy-number gene (for the small subunit of ribulose bisphosphate carboxylase/oxygenase).

The ribosome is a complex cellular particle on which protein synthesis occurs. The subunits of the ribosome are made up of at least one ribosomal RNA (rRNA) molecule and a large number of ribosomal proteins. (See Russell (1992) and Grierson and Covey (1984) for a more detailed description.) The sizes of the ribosomal RNAs of higher plants are 18S, 5.8S, 25S, and 5S. (S is a "Svedberg," a unit of centrifugation rate determined by the size and shape of a molecule.) The genes for these ribosomal RNAs are called the ribosomal DNAs (rDNA). The 18S, 5.8S, and 25S rRNAs are transcribed from rDNA as a single pre-rRNA molecule, which is then processed to produce the mature rRNAs. There are many copies of the rDNA genes in the eukaryotic genome. The rDNA genes are arranged as tandem repeats with nontranscribed spacer regions that can be of variable length.

The rDNA clone to be used as a probe in this experiment is pBG35 (Goldsbrough and Cullis, 1981), an 8.7-kb fragment containing a single rDNA repeat from flax cloned into the vector pAT153. These rDNA genes are present at very high copy number, as high as 10^4 copies per cell, in the plant genome.

The rDNA clone will be used as a probe for a high-copy-number sequence. What predictions can be made about the hybridization pattern of a high-copy-number sequence that is present in tandem repeats?

Ribulose bisphosphate carboxylase/oxygenase (RUBISCO) is a 550-kDa enzyme, the most abundant protein on earth, that carries out the photosynthetic fixation of carbon dioxide in the chloroplast. In higher plants RUBISCO consists of eight 55-kDa large subunits and eight 12- to 16-kDa small subunits. The genes for the large subunit are encoded by the chloroplast; the genes for the small subunit are encoded by the nuclear genome. The small subunit (SS) of RUBISCO is made in the cytoplasm as a precursor polypeptide containing a transit sequence that directs the transport of SS to the chloroplast.

The gene for SSRUBISCO used in this experiment was cloned from tobacco based on homology to soybean and pea SSRUBISCO sequences (Mazur and Chui, 1985). In the tobacco genome, it has been estimated that 4 to 10 copies of the SS gene are present. Different members of the family of genes encoding SSRUBISCO are expressed at different developmental times or in different tissues. This cloned probe is part of one of the members of the SSRUBISCO gene family. Mazur and Chui noted that this probe hybridized to five *Eco*RI fragments in a Southern blot of the tobacco genome. It is expected that this probe for a low-copy-number gene will be more difficult to detect than the rRNA genes because there are fewer DNA sequences homologous to this probe in the complex tobacco genome.

In this experiment, students will do the following:

1. Isolate DNA from tobacco leaves using the mini-prep procedure (Dellaporta *et al.*, 1983). With this procedure, a CsCl gradient is not needed to prepare DNA that can readily be cut with restriction enzymes. (The term "mini-prep" refers to a simple and rapid procedure.)
2. After the DNA is isolated, digest an aliquot of DNA with restriction enzymes and run that aliquot of DNA on a gel to check that the DNA has been digested to completion. Include an uncut sample of DNA on the gel to use to estimate the DNA concentration.
3. Digest samples of tobacco DNA with *Eco*RI or with *Bam*HI. Separate the fragments by gel electrophoresis. In addition to a λ *Hin*dIII size standard, include in the gel reconstructions (known amounts of the probe DNA) to estimate the copy numbers of the probed sequences.
4. Prepare a Southern blot of the gel.
5. Probe the blot either with the cloned gene for the small subunit of RUBISCO (a low-copy-number gene) or with the cloned genes of the ribosomal DNA (rDNA) repeat unit (a very high-copy-number sequence).

The goals of this experiment are the following:

1. To teach a simple and rapid plant DNA extraction procedure.
2. To give students more experience with the Southern blotting procedure. (In this experiment, students use the procedures learned in Chapters 2 and 3 in a new project.)
3. To compare the ease of detection of a high-copy-number and a low-copy-number gene sequence.

PROTOCOL 4.1: Plant DNA Extraction Mini-Prep Procedure

Solutions Needed

Extraction buffer minus β-mercaptoethanol: 100 m*M* Tris, pH 8.0; 50 m*M* EDTA, pH 8.0; 500 m*M* NaCl.

NOTE

Add β-mercaptoethanol after autoclaving the solution. Extraction buffer is used after enough β-mercaptoethanol is added to make the buffer 10 m*M* β-mercaptoethanol. β-Mercaptoethanol can be added only to the aliquot of extraction buffer to be used.

Procedure

1. Weigh the tissue. Quick freeze the tissue in liquid nitrogen. Grind the tissue to a fine powder in a mortar and pestle.

CAUTION: Wear double layers of plastic gloves to protect your hands from the cold.

Transfer the slurry of powder with liquid nitrogen into a 30-ml Oakridge or other screw-capped centrifuge tube.

NOTE

It is crucial that the tissue not thaw once it is frozen until the plant extraction buffer is added. *Do not cap* tubes while nitrogen is evaporating.

2. Add 15 ml of plant extraction buffer up to 5 g of plant callus tissue. Before using the extraction buffer, add 10.5 μl of β-mercaptoethanol for every 15 ml of extraction buffer.

NOTE

Plant callus tissue contains more water than many other plant tissues. If using this procedure to extract DNA from plant leaf tissue, use 15 ml of extraction buffer for 0.5 to 1.0 g of leaf tissue.

3. Add 1 ml of 20% sodium dodecyl sulfate (SDS). Mix thoroughly by vigorous shaking. Incubate tubes at 65°C for 10 min.
4. Add 5.0 ml of 5 *M* potassium acetate. Shake the capped tubes vigorously and set the tubes on ice for 20 min.
5. Centrifuge tubes at 13,000 rpm for 20 min. Pour the supernatant solution through a Miracloth filter (Calbiochem) into a clean 30-ml Oakridge tube containing 10 ml isopropanol. Mix the solution well by inverting the capped centrifuge tube several times. Incubate the tube at −20°C for 30 min.
6. Pellet DNA by centrifugation at 12,000 rpm for 15 min. Gently pour off the supernatant fluid and drain pellets by inverting the tubes onto paper towels.
7. Redissolve the DNA pellet with 0.7 ml of 10 m*M* Tris and 1 m*M* EDTA, pH 8.0. Transfer the solution to a microfuge tube. Centrifuge the tubes in a microfuge for 10 min to remove insoluble debris.
8. Transfer the supernatant solution to a new microfuge tube. Add 75 μl of 3 *M* sodium acetate and 500 μl of isopropanol. Mix well and pellet the clot of DNA for 30 sec in a microfuge. Wash the DNA pellet with 75 μl of 70% ethanol; centrifuge the sample for 5 min. Pour off the 70% ethanol. Dry the DNA pellet. Redissolve the DNA in 100 μl of 1 m*M* Tris and 0.1 m*M* EDTA, pH 8.0.

NOTE

Precipitation from 0.3 *M* sodium acetate using relatively small amounts (~0.6 volume) of isopropanol separates high-molecular-weight DNA from polysaccharides. The sodium acetate also yields a tight fibrous precipitate that is easily washed and dried. The DNA will dissolve readily if allowed to rehydrate at 4°C for 1 hr followed by light vortexing.

"Reconstructions" for Gels

To estimate the copy number of sequences homologous to a probe in a Southern blot, known amounts of DNA homologous to the probe are loaded on the gel. For example, control lanes that contain the equivalent of 1 copy, 10 copies, etc., of a specific sequence of DNA per 5 μg of

the genome studied are subjected to electrophoresis through the gel. By comparing the intensity of the hybridization signal observed for the experimental sample and the "reconstructions," an estimate can be made of the number of copies of that fragment in the genome.

These calculations show how to determine the amount of DNA needed to make such "reconstructions." For example, the probe for the small subunit of the RUBISCO gene (Mazur and Chui, 1985) is pBM1, containing a 1.2-kb *Hin*dIII to *Sph*I fragment in a 12.2-kb vector.

Insert DNA 1.2 kb
Vector DNA 12.2 kb
Total 13.4 kb

The haploid genome size of tobacco is 6 pg = 6×10^6 kb.
Determine the ratio

$$\frac{\text{Size tobacco genome}}{\text{Size plasmid probe}} = \frac{6 \times 10^6 \text{kb}}{13.4 \text{ kb}} = 4.5 \times 10^5.$$

For 5 μg of plant DNA loaded on a gel, *one* copy of the probe would be contained within

$$\frac{5\ \mu\text{g}}{4.5 \times 10^5} = 1.11 \times 10^{-5}\ \mu\text{g of probe plasmid.}$$

This is an extremely small amount!

To obtain a readily measurable amount of the plasmid to be used as a probe, more copies of the sequence are considered.

$$1 \text{ copy} = 1.67 \times 10^{-5}\ \mu\text{g}; \qquad 100 \text{ copies} = 1.67 \times 10^{-3}\ \mu\text{g};$$
$$100 \text{ gel loadings of } 100 \text{ copies} = 1.67 \times 10^{-1}\ \mu\text{g!}$$

This is a measurable quantity. To prepare 1-copy, 10-copy, etc., reconstructions, dilute a stock of probe DNA of known concentration.

The probe for ribosomal DNA is pBG35 (Goldsbrough and Cullis, 1981) containing an 8.7-kb insert of a single rDNA (ribosomal DNA) repeat unit from flax, cloned into the *Bam*HI site of pAT153 (a pBR322 derivative).

8.7 kb insert
4.0 kb vector
12.7 kb Total plasmid size

Tobacco haploid genome = 6 pg = 6×10^6 kb.

$$\frac{\text{Ratio size tobacco genome}}{\text{size plasmid probe}} = 6 \text{ pg} = \frac{6 \times 10^6 \text{ kb}}{12.7 \text{ kb}} = 4.7 \times 10^5.$$

For 5 μg of tobacco DNA loaded on a gel lane, if there were one copy of the probe specific sequence, there would be

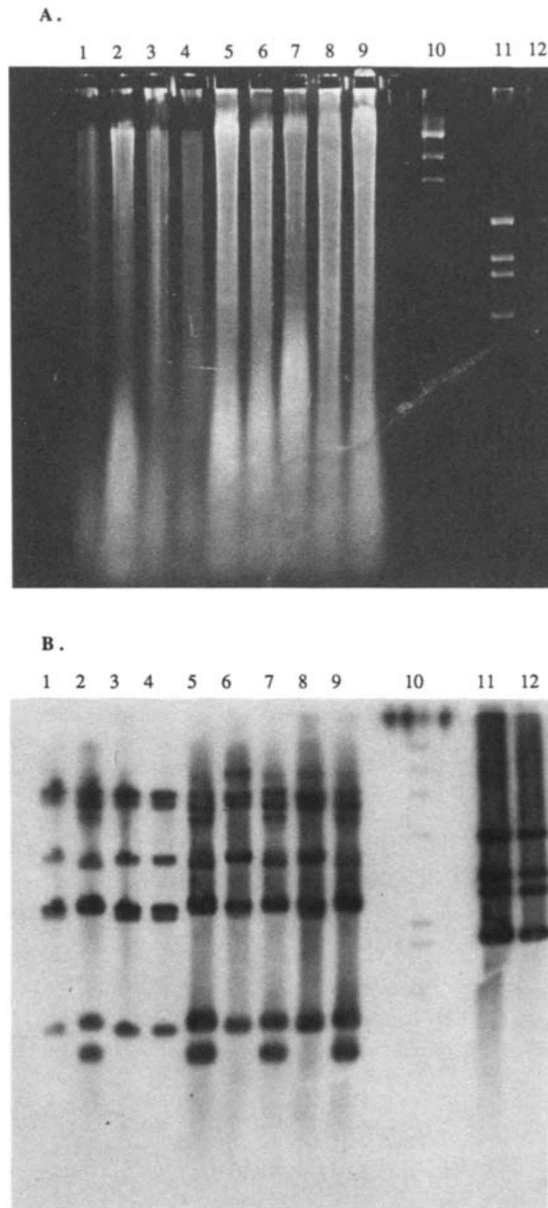


Figure 4.1 An example of a genomic Southern blot. (A) An ethidium bromide-stained agarose gel. Tobacco DNA (5 μg) cut with *Eco*RI or *Bam*HI is run in lanes 1–9. Lanes 11 and 12 are control lanes of the known amounts of the plasmid to be used as a probe. Lane 10 is biotin-labeled λ *Hin*dIII DNA used as a molecular weight standard. The gel in A was transferred to a membrane via Southern blotting methods. (B) The results of hybridization of a probe for ribosomal DNA using a chemiluminogenic substrate. These data are from Biology 542 classes at Purdue University.

$$\frac{5\ \mu g}{4.7 \times 10^5} = 1.06 \times 10^{-5}\ \mu g \text{ of probe plasmid.}$$

1 copy of rDNA probe/5 μg tobacco = 1.06×10^{-5} μg of probe plasmid; 10^4 copies = 1.06×10^{-1} μg.

This kind of calculation allows estimation of the copy number of probe-specific DNA sequences.

Figure 4.1 shows an example of a ribosomal DNA probe hybridized with tobacco DNA.

Details of the procedures needed in this experiment are presented in Chapter 3.

PROTOCOL 4.2: Steps of a Genomic Southern Blot

1. After isolating plant DNA using protocol 4.1, check that the DNA isolated can cut well. Digest a 1-μl aliquot of the plant DNA with 1 or 2 μl of the restriction endonuclease, such as *Eco*RI, *Bam*HI, or *Hin*dIII, that will be used for the Southern blot. Incubate the restriction digestions of the DNAs for 1 hr at 37°C. Examine the digestions and an uncut aliquot of DNA by gel electrophoresis to determine if cutting has occurred. The cut samples should show a smear of smaller size DNAs. The uncut sample should be a high-molecular-weight broad band that may have some smearing. If little or no cutting occurred, attempt to purify the DNA sample by phenol extraction. (Recall how to do a phenol extraction: *See* the DNA isolation procedures of Chapter 2.) After phenol extraction, recheck the cutting of the DNA sample.
2. Determine the DNA concentration of samples. The concentration can be estimated by comparing the intensity of ethidium bromide staining of an aliquot of the sample with DNAs of known concentration run on an 0.8% agarose gel. If the DNA sample has no contaminating RNA, the DNA concentration can be determined spectrophotometrically.
3. Digest the plant DNA for a Southern blot. Cut 5-μg aliquots of plant DNA with 50 units of a restriction endonuclease, such as *Eco*RI, *Bam*HI, or *Hin*dIII. Allow the digestion to proceed for 1 to 2 hr. This is an excess of restriction endonuclease to ensure complete cutting of the sample. For example, prepare a set of plant DNA samples cut with *Eco*RI and a set of samples cut with *Bam*HI.
4. Load DNA samples on an 0.8% agarose gel. Also include the following controls on the gel: If the gel will be probed with SSRUBISCO, include reconstructions of the SSRUBISCO clone that represent 0.1, 1, and 10 copies. If the gel will be probed with rDNA, include reconstruc-

tions of the rDNA clone that represent 10^2, 10^3, and 10^4 copies. Include a molecular weight standard, such as λ *Hin*dIII-cut DNA. If a nonradioactive, biotin-labeling detection system will be used, it is useful to use biotin-labeled λ *Hin*dIII-cut DNA so that the molecular weight marker can be detected when the hybridized probe is detected.

5. Run the gel; stain and photograph the gel. Prepare a Southern blot of the gel. Probe the gel with labeled SSRUBISCO or rDNA clones. The protocols for these procedures are described in Chapter 3.
6. Determine the size of the fragments that hybridize to the probes. Compare the intensity of the hybridization signal of the DNA sample lanes with the reconstruction lanes to estimate the copy number of the fragments. What differences are observed between the SSRUBISCO and the rDNA probes? What are the differences between the times needed to detect a hybridization signal for the two different probes? Examine the published restriction endonuclease maps for each probe to predict the sizes of fragments observed. Predict the hybridization results for tandem duplications of the rDNA probe. How might the protocol be modified to increase the signal strength for the probe that shows the less intense signal?

References

Dellaporta, S. L., Wood, J., and Hicks, J. B. (1983). A plant DNA minipreparation: version II. *Plant Mol. Biol. Rep.* **1,** 19–21.

Russell, P. J. (1992). "Genetics," 3 ed. Harper Collins, New York.

Suggested Reading

Ribosomal DNA (rDNA)

Agarwal, M. L., Aldrich, J., Agarwal, A., and Cullis, C. A. (1992). The flax ribosomal RNA-encoding genes are arranged in tandem at a single locus interspersed by 'non-rDNA' sequences. *Gene* **120,** 151–156.

Goldsbrough, P. B., and Cullis, C. A. (1981). Characterisation of the genes for ribosomal RNA in flax. *Nucleic Acids Res.* **9,** 1301–1309. [*Note: This is the source of the rDNA probe.*]

Grierson, D. (1982). RNA processing. *In* "Nucleic Acids and Proteins in Plants II Structure, Biochemistry and Physiology of Nucleic Acids" (Pathier and Boulter, eds.).

Grierson, D., and Covey, S. N. (1984). "Plant Molecular Biology." Blackie, Glasglow.

Gutell, R. R. (1993). Collection of small subunit (16S- and 16S-like) ribosomal RNA structures. *Nucleic Acids Res.* **21,** 3051–3054.

Hillis, D. M., and Dixon, M. T. (1991). Ribosomal DNA: Molecular evolution and phylogenetic inference. *Q. Rev. Biol.* **66,** 411–453.

Ingle, J., and Sinclair, J. (1972). Ribosomal RNA genes and plant development. *Nature (London)* **235,** 30–32.

Neefs, J.-M., Van de Peer, Y., De Rijk, P., Chapelle, S., and De Wachter, R. (1993). Compilation of small ribosomal subunit RNA structures. *Nucleic Acids Res.* **21,** 3025–3049.

Olsen, G. J., Overbeek, R., Larsen, N., Marsh, T. L., McCaughey, M. J., Maciukenas, M. A., Kuan, W.-M., Macke, T. J., Xing, Y., and Woese, C. R. (1991). The ribosomal database project. *Nucleic Acids Res.* **20**(Suppl) 2199–2200.

Rogers, S. O., and Bendich, A. J. (1987). Ribosomal RNA genes in plants: Variability in copy number and in the intergenic spacer. *Plant Mol. Biol.* **9,** 509–520.

Sollner-Webb, B., and Mougey, E. B. (1991). News from the nucleolus: rRNA gene expression. *Trends Biochem. Sci.* **16,** 58–62.

Steele, S. N., and Ingle, J. (1973). The genes for cytoplasmic ribosomal ribonucleic acid in higher plants. *Plant Phys.* **51,** 677–684.

RUBISCO (Ribulose Bisphosphate Carboxylase/Oxygenase)

Berry-Lowe, S. L., McKnight, T. D., Shah, D. M., and Meagher, R. B. (1982). The nucleotide sequence, expression, and evolution of one member of a multigene family encoding the small subunit of ribulose-1,5-bisphosphate carboxylase in soybean. *J. Mol. Appl. Genet.* **1,** 483–498.

Cashmore, A. R. (1979). Reiteration frequency of the gene coding for the small subunit of ribulose-1,5-bisphosphate carboxylase. *Cell* **17,** 383–388.

Kuhlemeier, C., Green, P. J., and Chua, N.-H. (1987). Regulation of gene expression in higher plants. *Annu. Rev. Plant Phys.* **38,** 221–257.

Mazur, B. J., and Chui, C.-F. (1985). Sequence of genomic DNA for small subunit of RUBISCO from tobacco. *Nucleic Acids Res.* **13,** 2373–2386. [*Note: This is the source of the SS RUBISCO probe.*]

Moses, P. B., and Chua, N. H. (1988). Light switches for plant genes. *Sci. Am.* **258,** 88–93.

Zhu, G., and Jensen, R. G. (1991). Fallover of ribulose 1,5-bisphosphate carboxylase/oxygenase activity. *Plant Physiol.* **97,** 1354–1358.

5

RNA PURIFICATION AND NORTHERN BLOT ANALYSIS

RNA Introduction: Overview of Experiment

The isolation and characterization of messenger RNA are important parts of the study of gene expression of an organism (Farrell, 1993). In this experiment, RNA is isolated from plants. The majority of RNA species isolated are ribosomal RNAs and tRNAs (Ausubel *et al.*, 1989). Poly(A)$^+$ RNA can also be isolated. The RNA isolated is separated on the basis of size using a denaturing formaldehyde agarose gel (Lehrach *et al.*, 1977; McMaster and Carmichael, 1977). The RNAs are then transferred from the gel to a membrane—producing a Northern blot (Alwine *et al.*, 1977, 1979; Thomas, 1980). The Northern blot is then hybridized with a cloned probe for a gene to determine the steady state levels of mRNA present in the cell that are complementary to the cloned probe.

The basic protocols needed are presented in this chapter. The student is to design the exact conditions for examining gene expression. For example, the student might use the cloned probe for the small subunit of ribulose bisphosphate carboxylase/oxygenase (SSRUBISCO) (see Chapter 4) to look for expression of SSRUBISCO in seedlings grown in the light and in the dark.

The isolation of RNA presents a challenge because RNases are ubiquitous. Endogenous RNases are quickly inactivated by the phenol/SDS extraction step of the RNA isolation procedure. Glassware and solutions are treated with agents such as diethyl pyrocarbonate to inactivate RNases.

The yield of RNA can vary depending on the source. Pea seedlings generally give a high yield, as much as 7 mg of total RNA from 15 g of

plant material. *Arabidopsis* plants may yield as little as 3 mg of total RNA per 15 g of plant tissue (Ausubel *et al.*, 1989).

PROTOCOL 5.1: RNA Extraction from Plant Leaves

Solutions

- TES: 50 m*M* Tris; 20 m*M* EDTA; 50 m*M* NaCl; pH 8.0
- TE: 50 m*M* Tris; 20 m*M* EDTA; pH 8.0
- 2 *M* LiCl
- 5 *M* LiCl
- 1:1 phenol:chloroform

The pheonol has been equilibrated with 3% NaCl and neutralized with Tris, and 0.1% 8-hydroxyquinoline has been added. The hydroxyquinoline is an antioxidant that reduces the formation of oxidation products of phenol such as quinones. The phenol is brought to a neutral pH because acidic phenol has a tendency to trap single-stranded nucleic acid at the interface between the phenol and the aqueous phases. Alternatively, if a molecular biology grade phenol is used, the hydroxyquinoline may be omitted.

- 3 *M* NaOAc (sodium acetate)
- 20% Sarkosyl

Preparation

RNases are ubiquitous. Great care should be taken to destroy RNases that might be present on glassware or in solutions. RNases are also present on the hands. Gloves should be worn during RNA handling steps.

1. Bake all glassware needed, except centrifuge tubes, overnight at 250°C to destroy RNases.
2. Soak glass and plastic centrifuge tubes and centrifuge bottles in sterile distilled water with 1% (v/v) diethyl pyrocarbonate (DEPC) for 10 to 15 min. Pour off the DEPC. Autoclave the tubes and bottles.

CAUTION: When handling diethyl pyrocarbonate, wear safety goggles, gloves, and a laboratory coat. Work in a fume hood.

3. Add DEPC to a final concentration of 0.1% (0.1 ml DEPC/100 ml solution) to all solutions, including H_2O. Shake the solutions. Let the solutions with the DEPC stand for 30 min. Autoclave the solutions.
4. Wear latex gloves at all times during the procedures to avoid contamina-

tion by RNases from the hands as well as to protect the hands from chemicals such as phenol.

5. Read and understand *all* instructions ahead of time. Calculate in advance the amounts of chemicals needed per 100 g of tissue. Work *quickly* and carefully.

Grinding Tissue

Have all necessary equipment and materials assembled beforehand. Proceed *rapidly* with work through the phenol blending (step 7) to minimize the length of time endogenous plant cell RNases can act on the RNAs.

1. Rinse a blender by blending about 100 ml absolute ethanol for a few seconds. Discard the ethanol. Blend 100–200 ml of sterile H_2O with 1% (v/v) DEPC for a few seconds. Discard the water.
2. Weigh out 1–20 g of plant leaf tissue. Put the tissue in the rinsed blender.
3. For each gram of tissue, add 1 ml of *cold* TES.
4. Add 20% Sarkosyl to give a final concentration of 1% Sarkosyl. Add β-mercaptoethanol to give a final concentration of 50 m*M* β-mercaptoethanol. Concentrated β-mercaptoethanol is 14 M. Add DEPC to give a final concentration of 1% (volume/volume) DEPC.

CAUTION: DO NOT pipet these compounds by mouth.

5. Cover the blender. Blend twice for 10 sec each at high speed.
6. For each gram of tissue used, add 1 ml of 1:1 phenol:chloroform. (This is the same as the volume of TES used.)
7. Blend twice for 10 sec at high speed. At this point RNases will be denatured by the phenol.
8. Pour the mixture into 250-ml centrifuge bottles. Centrifuge the solution at 9000 rpm for 10 min.
9. Carefully collect the upper phase. Avoid the white material, which is denatured proteins, at the interphase between the upper (aqueous) phase and the lower (phenolic) phase. Determine the volume of aqueous material collected.
10. Add 1/2 volume cold (−20°C) absolute ethanol to the aqueous solution. Mix well and let the solution sit on ice for approximately 15 min. Many polysaccharides are insoluble at this concentration of cold ethanol and will precipitate out of solution.
11. Centrifuge the solution at 10,000 rpm for 10 min. The polysaccharides will pellet. Decant and save the supernatant fluid. Discard the polysaccharide pellet. (The pellet will be clear and look like plastic.)

12. Add 2 volumes of cold absolute ethanol to the supernatant solution, cover, and mix well. Incubate the solution at −20°C for at least 2 hr. The sample may be left at −20°C overnight. A flocculant (fluffy), opaque white material, which is nucleic acids, primarily RNA, may precipitate as soon as the final 2 volumes of ethanol are added.

RNA Extraction

1. Centrifuge the nucleic acids precipitated in ethanol from step 12 above in a refrigerated centrifuge at 10,000 rpm for 10 min. Drain off the ethanol.
2. "Disperse" the pellet in cold 2 *M* LiCl. Use the minimum volume possible to resuspend the pellet. Any remaining polysaccharide, tRNAs, and some DNA are solubilized in the 2 *M* LiCl, but larger RNAs are insoluble in 2 *M* LiCl.
3. Centrifuge the solution at 10,000 rpm for 10 min. Keep the pellet. For convenience, switch to smaller centrifuge tubes when the volumes permit. This pellet will very likely be smaller in size than the preceding one because tRNAs, etc., have been solubilized.
4. Repeat the washing of the pellet in cold 2 *M* LiCl one to four times. Always discard the supernatant fluid and keep the pellet. Drain the pellet well. The number of 2 *M* LiCl washes used will be determined by the size and color of the pellet. The pellet should be opaque white (RNA), not clear (polysaccharide). After a few washes the pellet should remain approximately the same size after each 2 *M* LiCl wash.

   Optional step to purify RNA further:

5. Resuspend the pellet in a minimal volume of TE. Dissolve the entire pellet. Add cold 5 *M* LiCl to a final concentration of 2 *M*. Mix. Let the sample sit on ice overnight. Centrifuge the sample at 10,000 rpm for 10 min. Keep the pellet. Drain the pellet well.
6. Resuspend the pellet in TE. Add 3 *M* NaOAc to a final concentration of 0.3 *M*. Precipitate the sample with 2.5 volumes of cold absolute ethanol. Store the sample at −20°C more than 2 hr. The sample may be stored at −20°C overnight.
7. Centrifuge the sample at 10,000 rpm for 10 min. Drain and dry the pellet thoroughly. Resuspend the pellet in a minimal volume of sterile H_2O. The volume used will be about 1 ml. Determine the concentration of RNA by measuring the spectrum of a dilution of the RNA sample in a UV spectrophotometer. Typically a 1:40 dilution of the sample can be used. For RNAs and single-stranded DNAs, use an extinction coefficient of 1 A_{260} = 40 μg/ml. For double-stranded DNAs, 1 A_{260} = 50 μg/ml. This sample is total cellular RNA, excluding tRNAs.

PROTOCOL 5.2: Separating Poly(A)$^+$ RNA from Total Cellular RNA

1. Add 1/10 volume of 3 *M* NaOAc and 2.5 volumes of cold absolute ethanol to precipitate the sample of total RNA. Store the sample at −20°C more than 2 hr. Centrifuge the sample at 10,000 rpm for 10 min. Drain and dry the pellet thoroughly.
2. Resuspend the pellet in low-salt oligo(dT) buffer at room temperature. When the pellet is completely resuspended, add 5 *M* NaCl to give a final volume of 0.5 *M* NaCl. Centrifuge at 10,000 rpm for 10 min to pellet any debris. Keep the supernatant solution.

   Low-salt oligo(dT)-cellulose column buffer:
   - 10 m*M* Tris, pH 7.5
   - 5 m*M* EDTA
   - 0.1% Sarkosyl
3. Place the supernatant solution in a 65°C water bath for 10 min; put the solution on ice.
4. Prepare the oligo(dT)-cellulose for a column or for batch absorption.

Batch Elution

In a DEPC-treated 30-ml Corex centrifuge tube, add 5 ml **high-salt** oligo(dT) buffer (see below) to 0.5 g oligo(dT)-cellulose. Shake the cellulose and buffer well. Let the cellulose settle out at room temperature. Discard the top liquid. Rewash the cellulose in 10 ml of high-salt oligo(dT) buffer. This oligo(dT)-cellulose can be stored at 4°C and reused many times.

High-salt oligo(dT)-cellulose column buffer:
- Low-salt buffer *plus* 0.5 *M* NaCl

5. Make sure the oligo(dT)-cellulose has been equilibrated with **high-salt** buffer. Add RNA sample from step 3 to the oligo(dT)-cellulose. Mix. Let the mixture sit at room temperature for 1 hr. During the 1 hr, occasionally invert the sample to mix it.
6. After the final settling, remove the liquid and save it.
7. Rinse the pellet with 10 ml **high-salt** buffer. Let the cellulose settle. (After some settling, the procedure can be hastened by centrifuging at 4000 rpm for 3 min.) Save the supernatant solution and pool this sample with the supernatant solution from step 6. This is the poly(A)$^-$ component of the total RNA.
8. Do a second high-salt wash as in step 7. This time discard the supernatant fluid.
9. Add 2–3 ml of **low-salt** buffer. Mix and allow the cellulose to settle.

Save the supernatant fluid. Repeat the low-salt washes two to three times. Pool all the supernatant solutions from the low-salt washes. This is the RNA that bound to the oligo(dT)-cellulose—the poly(A)$^+$ fraction of RNA.

10. Ethanol precipitate the poly(A)$^-$ and poly(A)$^+$ fractions: For the poly(A)$^-$ RNA, add 2.5 volumes of cold ethanol. This RNA is in a high-salt buffer so no additional salt is needed for the precipitation. For the poly(A)$^+$ RNA, add 0.1 volume 3 *M* NaOAc and 2.5 volumes cold ethanol.
11. Resuspend each RNA in a minimal volume of H_2O. Typically, 0.5–1.0 ml of H_2O can be used. Centrifuge the sample to remove any debris. Determine the concentration of a dilution of the RNA. Aliquot the RNA. Store the RNAs frozen at −20°C.
12. Rinse the oligo(dT)-cellulose several times with H_2O. Store the cellulose in approximately 5–10 ml H_2O at 4°C.

PROTOCOL 5.3: RNA Gel—A Denaturing Formaldehyde Gel

CAUTION: Wear gloves when handling equipment.

1. Wash plates, gel boxes, and other equipment with detergent. Rinse the equipment thoroughly with H_2O. Rinse the equipment with 100% ethanol. Let the equipment air-dry.
2. Make a gel for RNA; i.e., for a large vertical apparatus, mix
   - 1.5% agarose 2.63 g BRL agarose
   - 17.5 ml 10 × Hepes buffer (see below)

   Bring the volume of the solution to 145 ml with sterile H_2O treated with 0.1% DEPC. Add 0.175 ml DEPC (this will be a final concentration of 0.1% DEPC). Mix.

   10× Hepes buffer
   - 200 m*M* Hepes
   - 10 m*M* EDTA

   Adjust the pH to 7.8 with KOH. Add 0.1% DEPC before autoclaving the solution.
3. Microwave the agarose suspension to dissolve the agarose.
4. Incubate the gel at 65°C for 10 to 15 min.

CAUTION: Wear gloves when working with formaldehyde. Use a fume hood. Set up and run the formaldehyde gel in a hood.

5. Add formaldehyde to give a final concentration of 6%. For this gel, add 29.2 ml of 36% formaldehyde (the concentration of the commercially available stock is 36%). Mix thoroughly, but be careful to avoid creating bubbles that are easily trapped in high-percentage gels.
6. Keep the agarose–formaldehyde gel at 65°C until the gel is poured. For gel samples, load 3 μg total RNA per 1-cm-wide slot in the gel.

If necessary, dry down the RNA sample using a Speed-vac and resuspend the sample in DEPC-treated H_2O. To prepare RNA samples for the formaldehyde gel:

1. Mix
   - 4.67 μl RNA sample
   - 2 μl 10× Hepes buffer
2. Vortex.
3. Add 10 μl deionized formamide and 3.3 μl formaldehyde.
4. Mix well.
5. Incubate the RNA samples at 65°C for 10 min. This heat treatment will disrupt the base pairing of any secondary structure of the RNAs.
6. Place the RNA samples on ice.
7. Add 4 μl RNA stop mix.
   - RNA stop mix or gel loading dye: 50% glycerol, 0.1% bromophenol blue.

Gels typically are run at 100 V with a current of about 25 mA for 8–10 hr using Hepes buffer as the gel running buffer. After gel electrophoresis, cut off any lanes to be stained from the gel and "blot" the rest of the gel as a Northern blot.

To stain a formaldehyde gel, soak the gel in an ethidium bromide solution (about 0.5 μg/ml) for about 20 min. Destain the gel for about 2 hr. Monitor the progress of the destaining. A gel may need to restained or destained longer.

To deionize formamide, add approximately 1 g of Dowex MR-3 mixed resin beads to about 100 ml of formamide. Mix well and let the sample incubate at room temperature for at least 30 min before the formamide is used. Store the deionized formamide tightly covered, in the dark, for up to 1 week. Alternatively, purchase molecular biology grade formamide which can be used as is. Store aliquots of high-quality formamide at −20°C.

PROTOCOL 5.4: A Northern Blot

For a Northern (reverse Southern) blot, RNAs are subjected to electrophoresis in a denaturing agarose gel, such as a formaldehyde gel, a glyoxal

gel, or a methyl mercury gel. When the RNAs have migrated far enough, the gel is ready to be blotted.

Procedure for Blotting a Gel

1. Obtain a glass baking dish, a glass plate large enough to hold the gel, and 4 bottle caps (supports for glass plates). Wear gloves when handling these. Wrap glass plate with a piece of Whatman (No. 1) chromatography paper cut to surround the glass plate completely. Put the glass plate on supports in the baking dish.
2. Pour 20× SSC (3 *M* NaCl, 0.3 *M* Na_3 citrate, pH 7) into the baking dish, wetting the filter paper. The level of 20× SSC should not quite reach the glass plate. The filter paper will act as a wick.
3. Place the gel on the wetted filter paper on glass plate. Mark a gel corner with a small notch to indicate the orientation of the gel. (A horizontal gel should be placed on the Whatman filter paper *upside down* so the nitrocellulose paper will contact the bottom of the gel.)
4. Cut a piece of nitrocellulose paper to fit the gel *exactly*. Gloves should be worn when handling the nitrocellulose paper. Cut the nitrocellulose paper with a very sharp razor blade; a dull blade will tear the nitrocellulose paper.
5. Wet the nitrocellulose paper with distilled H_2O: lay paper on top of a tray of H_2O and allow it to soak; do not submerge the paper. When one side is wetted, flip the paper over to wet the other side.
6. Once the nitrocellulose is totally wetted, soak paper in 20× SSC.
7. Place the nitrocellulose paper on top of the gel, taking care to position the paper precisely. Be especially careful to line up the top of the paper with the top of the gel (wells). Also take great care to avoid any air bubbles between the gel and the paper.
8. Cut 4 to 5 pieces of Whatman filter paper exactly to the size (or just slightly smaller) of the nitrocellulose paper. Wet 2 pieces of filter paper in 20× SSC and place on top of the nitrocellulose paper. Avoid air bubbles. Then place 2 to 3 pieces of dry filter paper on top. DO NOT allow paper to hang over edge so that it might act as a wick.
9. Cut a stack of paper towels ≥ 2 in. high just to fit or be slightly smaller than the nitrocellulose paper. Place a weight, such as another glass plate, on top of the paper towel stack.
10. Allow gel to set overnight, adding more 20× SSC as needed to keep the SSC level just at the bottom of the glass plate. Change paper towels as needed.
11. Blot the gel overnight.
12. Mark the top edge of the nitrocellulose paper with indelible ink.

Procedure for a Northern Blot

DO NOT WASH THE BLOT. If the blot is to be cut into strips, this should be done with a sharp razor while the nitrocellulose paper is still wet.

13. Pat the blot dry on paper towels.
14. Bake the blot in a vacuum oven at 80°C 2–4 hr. This fixes the nucleic acid to the nitrocellulose paper.

PROTOCOL 5.5: Standard Northern Blot Hybridization Conditions for ^{32}P-Labeled Probe

Materials

Northern Prehybridization Solution

Final concentration of component	Concentration of stock component	Amount of stock component to add
50% formamide	Formamide	5 ml
5× SSC	20× SSC	2.5 ml
50 m*M* PB	1*M* PB	0.5 ml
5× PM	100× PM	0.5 ml
100 μg/ml sheared, denatured calf thymus DNA	2 mg/ml sheared, denatured calf thymus DNA	0.5 ml
0.1% SDS	20% SDS	50 μl
	H_2O	0.95 ml—to a total volume of 10 ml

Northern Hybridization Solution

Final concentration of component	Concentration of stock component	Amount of stock component to add
50% formamide	Formamide	5 ml
5× SSC	20× SSC	2.5 ml
20 m*M* PB	1*M* PB	0.2 ml
1× PM	100× PM	0.1 ml
100 μg/ml sheared, denatured calf thymus DNA	2 mg/ml sheared, denatured calf thymus DNA	0.5 ml
5% dextran sulfate	50% dextran sulfate	1.0 ml
	H_2O	1.1 ml—to a total volume of 10 ml

Washes for Northern blot: 1× SSC, 0.1% SDS, 5 m*M* EDTA.

NOTES

1. Use molecular biology grade formamide or deionize the formamide before using. To deionize formamide, add approximately 1 g of Dowex mixed resin beads to approximately 100 ml of formamide. Mix thoroughly. Store in the dark or wrap the bottle with aluminum foil. Let the formamide and beads stand for several hours before using.
2. SSC is standard sodium citrate. 1× SSC is 8.8 g NaCl, 4.4 g Na citrate/liter, pH 7.0.
3. PB is phosphate buffer. 1 *M* PB is pH 6.8. Make 1 *M* PB by mixing equal volumes of 1 *M* monobasic sodium phosphate and dibasic sodium phosphate.

100× Denhardt's solution:

2% (w/v) BSA (bovine serum albumin)

2% (w/v) PVP (polyvinylpyrrolidone, molecular weight (MW) 4×10^4)

2% (w/v) Ficoll (MW 4×10^5, a nonionic synthetic polymer of sucrose)

These three components are "nonspecific blockers." They help decrease "background" of nonspecific binding of probe to nitrocellulose membrane.

4. PM is prehybridization mix or Denhardt's solution.
5. SDS is sodium dodecyl sulfate.
6. Dextran sulfate is a high-molecular-weight (MW 500,000) synthetic polyanion. It is added to increase the rate of hybridization of the probe via an excluded volume effect, effectively increasing the concentration of the probe in solution.

CAUTION: Before working with ^{32}P, review the radiation safety rules and disposal protocols for your school.

Procedure

1. Seal the baked nitrocellulose blot in a Seal-a-Meal plastic bag containing 10 ml of Northern prehybridization solution.
2. Incubate the blot in prehybridization solution for 10 to 15 hr or overnight at 42°C.
3. Open the plastic bag by cutting open a corner. Pour out the prehybridization solution. Add 10 ml Northern blot hybridization solution. Reseal the plastic bag.
4. Incubate the blot in hybridization solution for 4 to 6 hr.

CAUTION: When working with radioactivity, wear double layers of gloves, a laboratory coat, and goggles. Work in an area designated for

radioactive work. Use a radioactive safety shield. Monitor the work area with a Geiger counter.

5. Place the probe in a boiling water bath for 5 min. After denaturing the probe, place it on ice for 5 min. (The probe was labeled by one of the procedures in Chapter 3.)
6. Open the plastic bag. Add the denatured probe. When adding the probe, put the probe directly into the hybridization solution. Do not allow the concentrated probe solution to touch the blot.

CAUTION: Label the plastic bag with radioactive tape.

7. Incubate the blot overnight at 42°C to allow hybridization.

CAUTION: Use a water bath designated for work with radioactivity.

8. The next day, wash the blot to remove any probe that did not hybridize.

CAUTION: Before opening the plastic bag, prepare for working with radioactivity. Wear laboratory coat, goggles, and double layers of gloves. Work in an area designated for radioactive work. Cover the work area with spill paper. Have waste containers for temporary storage of liquid and solid radioactive waste nearby.

9. Place about 500 ml of prewarmed wash solution in a beaker or dish. Carefully cut open a corner of the plastic bag with scissors dedicated to radioactive work. Pour off the hybridization solution with radioactive probe into the liquid radioactive waste container. Cut open the plastic bag to allow removal of the blot. Using forceps, move the blot into the wash solution. Dispose of the plastic bag in the solid radioactive waste container. Replace the outer layer of gloves. Monitor the area with a Geiger counter.
10. Incubate the blot in wash solution for 1 hr at 65°C.
11. Discard the wash solution. Add more wash solution. Incubate the blot in wash solution for an additional 1 hr at 65°C.
12. Repeat the washes a total of four times.
13. Dry the blot and mount the blot between acetate sheets for autoradiography.

NOTE

A hybridization oven is an alternative to working with radioactivity in sealed plastic bags. With a hybridization oven the blot and solutions are placed in bottles, which are rotated in the oven. It is generally much easier to remove the radioactive hybridization solution without spills from the bottles of a hybridization oven than from a plastic bag. A hybridization oven costs approximately $2000.

PROTOCOL 5.6: Nonradioactive Biotin-Labeled Probes for Northern Blots

To probe a Northern blot with a nonradioactive probe using the Gibco/BRL Photogene nucleic acid detection system:

1. Prepare a denaturing formaldehyde gel and set up a Northern blot (as described earlier in this chapter). Set up the Northern blot transfer using a Photogene nylon membrane.
2. Prepare the biotin-labeled probe as described in Chapter 3. Use the hybridization, SA-AP (streptavidin-alkaline phosphatase) binding, and detection procedures for the Photogene Southern blotting system (described in Chapter 3).

References

Alwine, J. C., Kemp, D. J., and Stark, G. R. (1977). Method for detection of specific RNAs in agarose gels by transfer to diazobenzyloxymethyl-paper and hybridization with DNA probes. *Proc. Natl. Acad. Sci. U.S.A.* **74,** 5350–5354.

Alwine, J. C., Kemp, D. J., Parker, B. A., Reiser, J., Renart, J., Stark, G. R., and Wahl, G. M. (1979). Detection of specific RNAs or specific fragments of DNA by fractionation in gels and transfer to diazobenzyloxymethyl paper. In "Methods in Enzymology" (R. Wu, ed.) Vol. 68, pp. 220–242. Academic Press, San Diego.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. A., Smith, J. A., and Struhl, K. (1989). "Current Protocols in Molecular Biology." Green Publishing Association and Wiley-Interscience, New York. (Vol. 1, ch. 4).

Farrell, R. E., Jr. (1993). "RNA Methodologies: A Laboratory Guide for Isolation and Characterization." Academic Press, San Diego.

Lehrach, H., Diamond, D., Wozney, J., and Boedtker, H. (1977). RNA molecular weight determination by gel electrophoresis under denaturing conditions, a critical reexamination. *Biochemistry* **16,** 4743–4751.

McMaster, G. K., and Carmichael, G. G. (1977). Analysis of single- and double-stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. *Proc. Natl. Acad. Sci. U.S.A.* **74,** 4835–4838.

Thomas, P. (1980). Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc. Natl. Acad. Sci. U.S.A.* **77,** 5201–5205.

Suggested Reading

RNA Isolation

Berger, S. L. (1987). Preparation and characterization of RNA: Overview. In "Methods in Enzymology" (S. L. Berger and A. R. Kimmel, eds.), Vol. 152, pp. 215–219. Academic Press, San Diego.

Birnboim, H. C. (1992). Extraction of high molecular weight RNA and DNA from cultured

mammalian cells. *In* "Methods in Enzymology" (R. Wu, ed.), Vol. 216, pp. 154–160. Academic Press, San Diego.

Jacobson, A. (1987). Purification and fractionation of poly(A)$^+$ RNA. *In* "Methods in Enzymology" (S. L. Berger and A. R. Kimmel, eds.), Vol. 152, pp. 254–261. Academic Press, San Diego.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). "Molecular Cloning: A Laboratory Manual." 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Applications

Becker-Andre, M. (1993). Absolute levels of mRNA by polymerase chain reaction aided transcript titration assay. *In* "Methods in Enzymology" (R. Wu, ed.), Vol. 218, pp. 420–445. Academic Press, San Diego.

Jagus, R. (1987). Translation in cell-free systems. *In* "Methods in Enzymology" (S. L. Berger and A. R. Kimmel, eds.), Vol. 152, pp. 267–276. Academic Press, San Diego.

Kimmel, A. R., and Berger, S. L. (1987). Preparation of cDNA and the generation of cDNA libraries: Overview. *In* "Methods in Enzymology" (S. L. Berger and A. R. Kimmel, eds.), Vol. 152, pp. 307–316. Academic Press, San Diego.

Song, Y.-R., Ye, Z.-H., and Varner, J. E. (1993). Tissue-print hybridization on membranes for localization of mRNA in plant tissue. *In* "Methods in Enzymology" (R. Wu, ed.), Vol. 218, pp. 671–681. Academic Press, San Diego.

Splicing

Apirion, D., and Miczak, A. (1993). RNA processing in prokaryotic cells. *BioEssays* **15,** 113–120.

Balvay, L., Libri, D., and Fiszman, M. Y. (1993). Pre-mRNA secondary structure and the regulation of splicing. *BioEssays* **15,** 165–169.

Green, M. R. (1991). Biochemical mechanisms of constitutive and regulated pre-mRNA splicing. *Annu. Rev. Cell Biol.* **7,** 559–599.

Lamond, A. I. (1993). The spliceosome. *BioEssays* **15,** 595–603.

6

POLYMERASE CHAIN REACTION

Background

Polymerase chain reaction (PCR) is the *in vitro* amplification of specific sequences of nucleic acid. The processes of PCR and the enzyme DNA polymerase were named by *Science* magazine as the 1989 "Molecule of the Year" because they were likely to have the greatest influence on history (Guyer and Koshland, 1989). *Science* said that PCR was "revolutionizing the approaches researchers are taking to many problems in biology."

The basic steps of PCR (Figure 6.1) are (1) the denaturation by heating of a template DNA molecule to be copied, (2) the annealing of pairs of oligonucleotides of specific sequences (primers, typically 10–14 nt long) chosen to be homologous to sequences within the template DNA molecule, and (3) the extension by DNA polymerase from the primers to copy the template DNA molecule. dNTPs must be present in the reaction. These three steps are repeated many times (for many cycles) to amplify the template DNA. If in each cycle one copy is made of each of the strands of the template, the number of DNA molecules produced doubles each cycle. Because of this doubling, at the end of 20 cycles, more than one million copies of the template DNA are made. The first experiments in PCR used *Escherichia coli* DNA polymerase I inactivated by the heat treatment to denature DNA template molecules. The polymerase had to be added repeatedly to the reaction. What made this *in vitro* DNA amplification so efficient was the discovery of heat-stable DNA polymerases such as *Taq* DNA polymerase from *Thermus aquaticus*, a eubacterium that grows in the elevated temperatures of aquatic hot springs. With heat-stable DNA polymerases, all the components can be added at the start of the

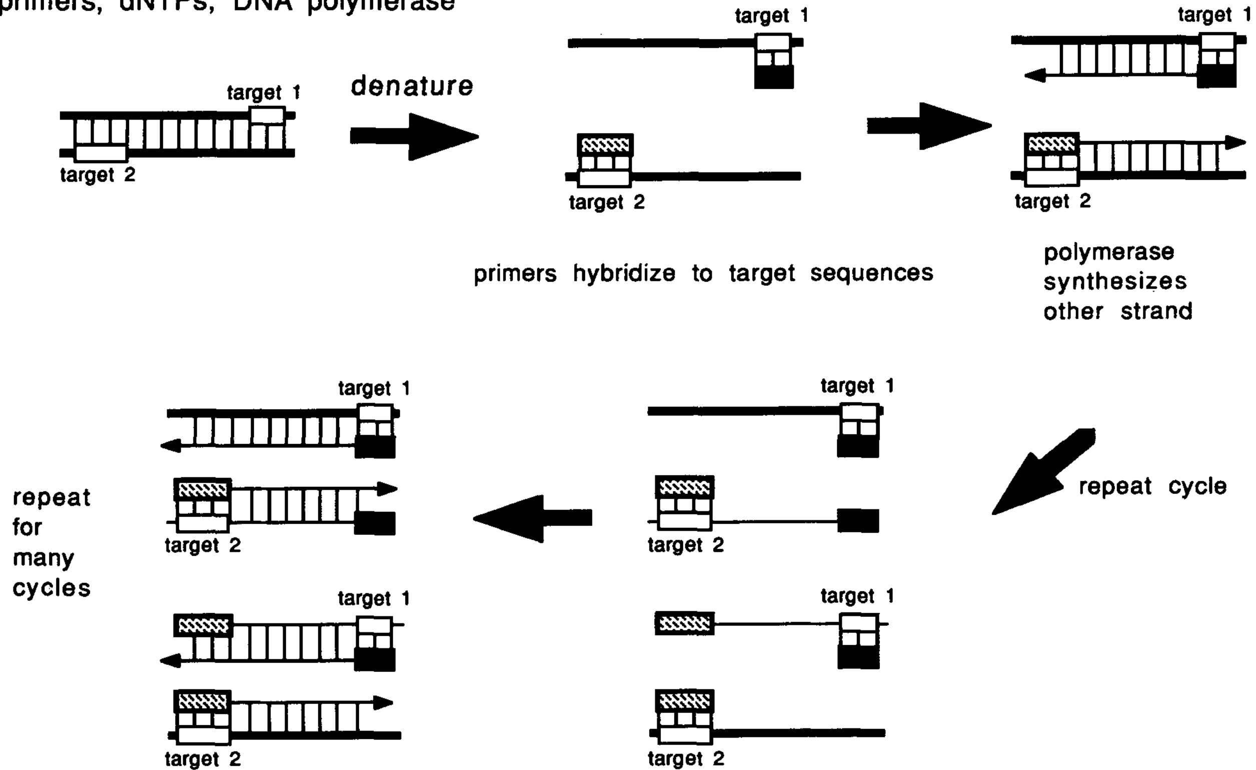


Figure 6.1 Polymerase chain reaction. (1) Double-stranded DNA to be amplified is denatured by high temperature (i.e., 95°C for 2 min). (2) The temperature is reduced to 55°C for 2 min to allow specific primers to hybridize to the target sequences. (3) The temperature is increased to 75°C for 2 min. The heat-stable DNA polymerase begins DNA synthesis at the primer and synthesizes the other DNA strand. This results in the duplication of the template DNA. Many cycles of steps 1–3 are repeated to amplify the template DNA many times.

reaction. The reaction is then cycled through the different temperatures that allow amplification to occur. The heat-stable DNA polymerases gradually lose activity over the course of the cycles. Although the temperature and time for each of the steps of 1 cycle described above will be varied according to the sequence of the primers used, a general example of the steps is

1. Denature template, 99°C, 2 min.
2. Anneal primers to template, 55°C, 2 min.
3. Extension of the primers by heat stable DNA polymerase, 72°C, 2 min.

Steps 1 through 3 are then repeated many times.

Using *Taq* DNA polymerase, the PCR amplification generally works best if primers hybridize to regions of the template not more than 2–4 kb apart. However, it is possible for *Taq* DNA polymerase to copy fragments up to 10 kb long. *Taq* DNA polymerase lacks a proofreading activity and can make a number of errors while copying the template. Because *Taq* DNA polymerase synthesis is error prone, care should be taken if the PCR amplification product is to be cloned. To clone a PCR product, several fragments from different PCR amplification reactions should be cloned and the sequences compared to be certain that sequence errors have not been cloned.

Other heat-stable DNA polymerases have been identified and characterized. For example, Vent DNA polymerase is isolated from the archaebacterium *Thermococcus litoralis*, which lives near thermal vents in the ocean floor and grows at temperatures of up to 98°C. Vent DNA polymerase (New England BioLabs) can produce PCR products up to 13 kb long and has a higher fidelity of DNA replication than *Taq* DNA polymerase. Another thermal-stable polymerase that has been characterized is Deep Vent DNA polymerase, isolated from *Pyrococcus* species GB-D that grow at ocean depths of 2,010 m where vent temperatures can be 104°C. Deep Vent DNA polymerase also has very high sequence fidelity of synthesis.

The polymerase chain reaction is very sensitive. It is possible to amplify a single or a very small number of molecules. This fact is simultaneously the great advantage of PCR and a problem with the technique. Because PCR is extremely sensitive, great care must be taken to avoid contamination of samples to be amplified by other DNA. Micropipettors with tips that contain a filter can be used so that the material being pipeted will not be contaminated from a previously contaminated pipet.

Some of the myriad of applications of the PCR technique include the following:

1. Disease diagnosis.
   a. Because a specific sequence can be amplified greatly, much less clinical material is needed to make a diagnosis.
   b. PCR can be used to detect pathogens that are difficult to culture, such as the causative agents for Lyme disease or for AIDS.
   c. PCR can be used for cancer diagnosis.
2. Forensics. DNA sequences from trace amounts of biological material such as semen, blood, and hair roots can be amplified. Probes for regions that show hypervariability in the population, and therefore make good markers to identify the source of the DNA, are available. PCR can be used to evaluate evidence at the scene of a crime, to help to identify missing persons, and in paternity cases.
3. Matching donor and recipient tissues for organ transplants.
4. Basic research.
   a. A comparison of sequence homology of conserved genes in different organisms can be made. It is even possible to study extinct organisms using samples of material from bones or museum specimens. The DNA used for PCR amplification can be partially degraded. As long as a few DNA molecules are intact between the two primers, amplification can occur.
   b. In developmental biology, PCR is very sensitive and can be used to examine which genes are turned on in early development (which mRNAs are made). Even if only a few transcripts are made, PCR can detect them.
   c. Because PCR is so rapid and easy to do, it may replace cloning as the amplification method of choice to obtain large amounts of material for sequencing.

One potential drawback to PCR is that one must have some sequence information about the piece of DNA to be amplified to make the appropriate specific primers. In the standard PCR, one needs sequence information from both ends of the DNA to be amplified. Inverse PCR is a variation of the standard PCR that requires sequence information from only one part of the DNA to be amplified. In inverse PCR (Figure 6.2), the DNA flanking (outside) the one primer region is amplified. DNA to be amplified is cut with a restriction endonuclease and circularized at the restriction endonuclease site. Primers to direct DNA synthesis outward from one known sequence are then amplified by PCR.

Many variations on the basic PCR procedure have been devised for specific uses. See the references at the end of the chapter.

As stated in *Science* (Appenzeller, 1990), PCR created a revolution and "new uses for PCR are developing almost as rapidly as the *Taq* poly-

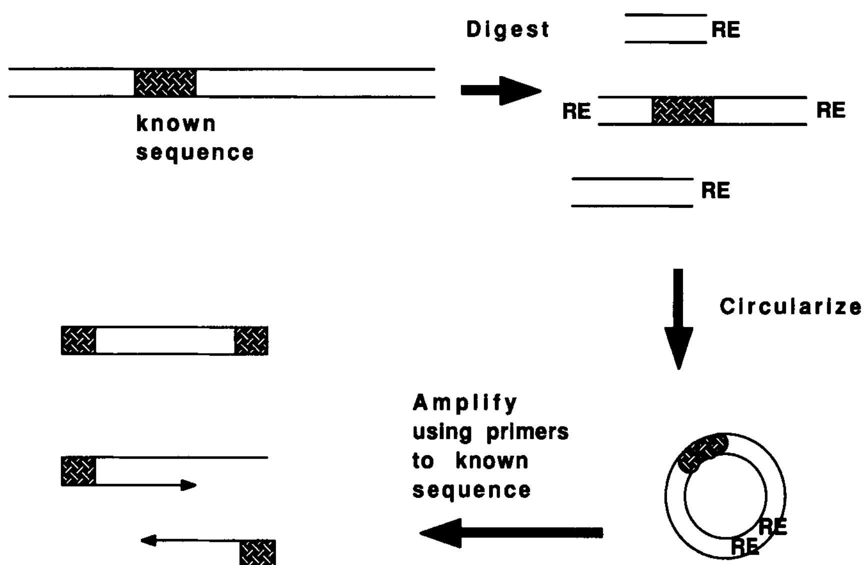


Figure 6.2 Inverse polymerase chain reaction. This process results in the amplification of the DNA outside (flanking) a known sequence. DNA is digested with a restriction endonuclease and circularized. A pair of primers directed outward from each end of the known sequence is used for PCR.

merase can replicate target sequences." Mullis (1990) has written an interesting personal account of his initial PCR work. The tremendous significance of this discovery was recognized by the awarding of the 1993 Nobel Prize in Chemistry to Kary B. Mullis for invention of the PCR method (Dagani, 1993). The 1993 Nobel Prize in Chemistry was awarded jointly to Michael Smith for his work on oligonucleotide-based site-directed mutagenesis.

PROTOCOL 6.1: PCR Experiment

This laboratory exercise uses PCR to amplify a sequence of an isolated DNA. Specifically, in this exercise, the T-DNA or transfer DNA of *Agrobacterium tumefaciens* is amplified.

Acting at a wound site, the *A. tumefaciens* infects many types of plants and causes a proliferation of plant cells, resulting in a tumor or crown gall. A plasmid of *Agrobacterium*, called the Ti or tumor-inducing plasmid, causes the crown gall disease. A part of the Ti-plasmid, the T-DNA, is excised from the plasmid and transferred to the infected plant

cell, where it integrates into the plant nuclear DNA. Genes in the T-DNA encode enzymes for the biosynthesis of hormones that are expressed in the plant. The overproduction of plant hormones then stimulates plant cell division and results in the production of a gall. The vir (virulence) genes, also on the Ti-plasmid, are required for the transfer of the T-DNA but are not themselves transferred. There is a 25-bp sequence (the T-DNA border) present at each end of the T-DNA. Any DNA between the T-DNA borders is transferred and integrated into the plant nuclear DNA during the course of the *A. tumefaciens* infection.

A. tumefaciens is a very efficient vector for introducing DNA into plants. Through recombinant DNA methods, the tumor-causing genes of the T-DNA can be deleted and genes of interest and a gene for a selectable marker are cloned into the T-DNA between the T-DNA borders. Any DNA inserted between the T-DNA borders transfers and integrates into plant DNA by the normal *Agrobacterium* infection process.

In this exercise, the template DNA can be a clone of the T-DNA border region of the Ti-plasmid or it can be the entire Ti-plasmid from *Agrobacterium*. The DNAs can be purified or *Agrobacterium* cells can be lysed and the DNA directly amplified.

Other options for PCR studies for students include the following:

1. Students could amplify any DNA for which the sequence is known. For example, students could amplify a specific DNA fragment of bacteriophage λ. λ DNA can be purchased, and the sequence of λ DNA is available (O'Brien, 1993).
2. Students could do their own PCR fingerprint. Primers for a hypervariable locus in humans are available. Students can obtain enough of their own cells for the PCR reaction by simply washing the mouth with a saline solution and collecting the wash in a paper cup. Mark Bloom and others of the Cold Spring Harbor DNA Learning Center have developed a protocol for this. See Bloom *et al.* (1994), Nakamua *et al.* (1988), and the DNA Fingerprinting section of suggested readings.
3. Depending on the experience of the students and the resources available, students could do a PCR project using the auxotrophs or transposon insertions generated in experiments in Chapter 1. Using inverse PCR with primers to the ends of the transposon, it is possible to amplify the sequences that flank the transposon. Those sequences could then be used as a hybridization probe to the Kohara ordered λ clones of the *E. coli* chromosome (Kohara, 1987) to identify where the transposon inserted in the *E. coli* genome.
4. After amplifying the specific DNA fragment using PCR, students could

then clone the DNA generated into a plasmid. Chapter 2 describes general cloning procedures.

Materials

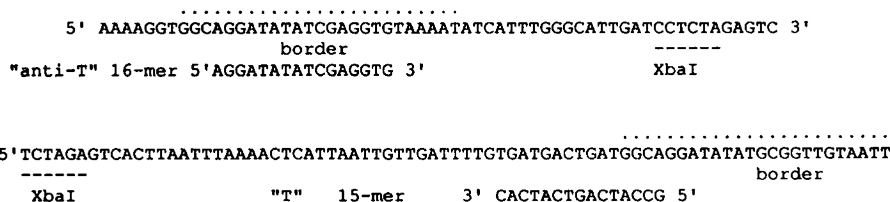
- thermocycler
- primers
- template DNA
- thermal-stable DNA polymerase
- 10× buffer
- dNTPs
- agarose gel
- DMSO
- $MgSO_4$
- electrophoresis equipment

Timetable

- 1 hr to set up the PCR reaction. Allow the reaction to proceed outside the scheduled class time.
- 1–2 hr, at a later date, to run a gel with the PCR samples and analyze the results.

Procedure

In this experiment the polymerase chain reaction is used to amplify a piece of DNA. Specifically, in this case, the T-DNA is amplified using primers for sequences near the T-DNA borders. The sequence of the primers used is:



NOTE: While performing the PCR reaction, it is crucial to *keep the reaction on ice;* add the components in the order indicated to a *small* microfuge tube (0.5 ml, not the usual 1.5-ml microfuge tube).

H_2O	To bring final reaction volume to 100 μl after the following items are added:
10× buffer	10 μl (10× buffer is 100 m*M* Tris–HCl (pH 8.3), 500 m*M* KCl, 15 m*M* $MgCl_2$)
dNTPs	20 μl of 1 m*M* stock of each dNTP (final concentration of 200 μ*M*)
Template DNA	10 μl (10 ng)
T_R primer	10 μl (10 μ*M*)
Anti T_L primer	10 μl (10 μ*M*)
DMSO (dimethylsulfoxide)	To give 0.5 to 10% final concentration of DMSO. Add 0, 1, 2, 10 μl, etc.
Thermal DNA polymerase	1 μl (2 units). For example, use Hot Tub polymerase from Amersham or *Taq* polymerase.

1. Be sure to keep the reaction on ice until ready to start because the 3′ to 5′ exonuclease activity of the DNA polymerase can begin to degrade the primers if incubated for a long time. The buffer used here contains 2 m*M* Mg^{+2}; for primer extension of DNA longer than 2 kb to be amplified, it is suggested that the concentration of Mg^{+2} be increased, up to 6 m*M*, using a stock solution of 1 *M* $MgCl_2$.
2. Once the above components are added and mixed, add 70 μl of sterile mineral oil (paraffin oil) to the top of the sample.
3. Place about 200 μl of paraffin oil in a well of the heating block on the thermal cycler. Place the reaction tube in the well. When all groups have added their samples to the heating block, the program will be started.

CAUTION: When the thermal cycler is on, the heating block may become very hot!!!

The cycles used are:

- 2 min 92°C—denaturing
- 2 min 55°C—annealing of primers to target sequences
- 2 min 75°C—extension of primers

4. Run thirty cycles of the above program, followed by 10 min at 72°C to allow filling in of the fragments.
5. After the amplification, run 15 μl of the sample on an 0.8% agarose gel to examine the size of the PCR products. How well does the size of the fragment observed match the expected size? If the template DNA is the plasmid pTDC npt, the expected size of the amplified fragment is 1.86 kb (Filichkin and Gelvin, 1992).

NOTES

1. If a thermal cycler is unavailable: (a) Some instructors have reported seeing some amplification when doing a PCR reaction and manually moving the microfuge reaction tubes between water baths set at the different temperatures. (b) See the reference list at the end of this chapter for references that describe the making of a thermal cycler.
2. For any specific PCR reaction, the temperature of each step in a cycle may need to be adjusted to give optimal amplification.
3. Mineral oil is added to the top of the reaction to prevent evaporation. Mineral oil in the well of the heating block of the thermal cycler ensures efficient heating and cooling of the microfuge tube.
4. During a PCR amplification, minor nonspecific products can be formed. The Mg^{+2} concentration or the DMSO concentration can be varied to optimize the production of the specific product.
5. The selection of primer sequences is somewhat empirical. Ideally, the primers should have a random base distribution with a 50% GC content or a GC content similar to that of the fragment to be amplified. The primers should not contain extensive secondary structure. The primers should not be complementary to each other (Erlich, 1989).

References

Appenzeller, T. (1990). Research news: Democratizing the DNA sequence. *Science* **247,** 1030–1032.

Bloom, M. V., Freyer, G. A., and Micklos, D. A. (1994). "Laboratory DNA Science: An Introduction to Recombinant-DNA Technology and Methods of Genome Analysis." Cold Spring Harbor Laboratory and Carolina Biological Supply Co.

Dagani, R. (1993). Nobel prize in Chemistry: Two biochemists win for DNA work. *Chem. Eng. News* **71,** 6–7.

Erlich, H. A. (1989). "PCR Technology: Principles and Applications for DNA Amplification." Stockton Press, New York.

Filichkin, S. A., and Gelvin, S. B. (1992). Effect of dimethyl sulfoxide concentration on specificity of primer matching in PCR. *Biotechniques* **12,** 5–6.

Guyer, R. L., and Koshlawd, D. E. (1989). *The Molecule of the Year. Science* **246,** 1541–1546.

Kohara, Y., Akiyama, K., and Isono, K. (1987). The physical map of the whole *E. coli* chromosome: Application of a new strategy for rapid analysis and sorting of a large genomic library. *Cell* **50,** 495–508.

Landegren, U. (1993). Ligation-based DNA diagnosis. *BioEssays* **15,** 761–765.

Mullis, K. B. (1990). The unusual origin of the polymerase chain reaction. *Sci. Am.* **262,** 56–65.

Nakamua, Y., Carlson, M., Krapco, K., and White, R. (1988). Isolation and mapping of a polymorphic DNA sequence (pMCT118) on chromosome 1p [D1S80]. *Nucleic Acids Res.* **16,** 9346.

O'Brien, S. J., ed. (1993). "Genetic Maps–Locus Maps of Complex Genomes," 6th ed. Cold Spring Harbor Press, Cold Spring Harbor, New York.

Suggested Reading

Overviews

Blair, G. E., and Zajdel, M. E. B. (1992). The polymerase chain reaction—already an established technique in biochemistry. *Biochem. Educ.* **20,** 87–91.
Dagani, R. (1993). Nobel prize in chemistry: Two biochemists win for DNA work. *Chem. Eng. News* **71,** 6–7.
Gibbs, R. A. (1990). DNA amplification by the polymerase chain reaction. *Anal. Chem.* **62,** 202.
Guyer, R. L., and Koshland, D. E. (1989). The molecule of the year. *Science* **246,** 1541–1546.
Kumar, R. (1989). The technique of polymerase chain reaction. *Technique J. Methods Cell Mol. Biol.* **1,** 133–152.
Mullis, K. B. (1990). The unusual origin of the polymerase chain reaction. *Sci. Am.* **262,** 56–65. [A highly personal story of the discovery/invention of PCR.]
White, T., Arnheim, N., and Erlich, H. (1989). The polymerase chain reaction. *Trends Genet.* **5,** 179–184.

Early Papers

Delidow, B. C., Peluso, J. J., and White, B. A. (1989). Quantitative measurement of mRNAs by polymerase chain reaction. *Genet. Anal. Technol.* **6,** 120–124.
Higuchi, R., von Beroldingen, C. H., Sensabaugh, G. F., and Erlich, H. A. (1988). DNA typing from single hairs. *Nature (London)* **332,** 543–546.
Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., and Erlich, H. A. (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239,** 487–491.
Saiki, R. K., Scharf, S., Faloona, F., Mullis, K. B., Horn, G. T., Erlich, H. A., and Arnheim, N. (1985). Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* **230,** 1350–1354.
Sommer, R., and Tautz, D. (1989). Minimal homology requirements for PCR primers. *Nucleic Acids Res.* **17,** 6749.

Monographs

Erlich, H. A., Gibbs, R., and Kazazian, H. H. (1989). "Current Communications in Molecular Biology—The Polymerase Chain Reaction." Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Innis, M. A., Gelfand, D. H., Sninsky, J. J., and White, T. J. (1990). "PCR Protocols: A Guide to Methods and Applications." Academic Press, San Diego.

Applications

Arnheim, N., White, T., and Rainey, W. E. (1990). Application of the polymerase chain reaction to organismal and population biology. *Bioscience* **40,** 174–182.
Jones, J. L. (1991). DNA probes: Applications in the food industry. *Trends Food Sci. Technol.* **2,** 28–32.
Erlich, H. A., and Arnheim, H. (1992). Genetic analysis using the polymerase chain reaction. *Annu. Rev. Genet.* **26,** 479–506.
Lassna, M. W., Peterson, P., and Yoder, J. I. (1989). Simultaneous amplification of multiple DNA fragments by polymerase chain reaction in the analysis of transgenic plants and their progeny. *Plant Mol. Biol. Reporter* **7,** 116–128.
Ross, P. E. (1992). Eloquent remains. *Sci. Am.* **266,** 114–125.

Mutagenesis

Hemsley, A., Arnheim, N., Toney, M. D., Cortopassi, G., and Galas, D. J. (1989). A simple method for site-directed mutagenesis using the polymerase chain reaction. *Nucleic Acids Res.* **17,** 6545–6551.

Herlitze, S., and Koenen, M. (1990). A general and rapid mutagenesis method using polymerase chain reaction. *Gene* **91,** 143–147.

Shyamala, V., and Ferro-Luzzi Ames, G. (1991). Use of exonuclease for rapid polymerase-chain-reaction-based in vitro mutagenesis. *Gene* **97,** 1–6.

Detection of Methylation and Strand Breaks

Steigerwald, S. D., Pfeifer, G. P., and Riggs, A. D. (1990). Ligation-mediated PCR improves the sensitivity of methylation analysis by restriction enzymes and detection of specific DNA strand breaks. *Nucleic Acids Res.* **18,** 1435–1439.

Inverse PCR

Ochman, H., Gerber, A. S., and Hartl, D. L. (1988). Genetic applications of an inverse polymerase chain reaction. *Genetics* **120,** 621–623.

Genomic Mapping

Chamberlain, J. S., Gibbs, R. A., Ranier, J. E., Nguyen, P. N., and Caskey, C. T. (1988). Deletion screening of the Duchenne muscular dystrophy locus via multiplex DNA amplification. *Nucleic Acids Res.* **16,** 11,141–11,156.

DNA Fingerprinting

Cooper, N. G., ed. (1994). "The Human Genome Project—Deciphering the Blueprint of Heredity." pp. 186–187. University Science Books, Mill Valley, Ca.

Gill, P., Jeffreys, A. J., and Werrett, D. J. (1985). Forensic application of DNA "fingerprints". *Nature* **318,** 577–579.

Herrmann, B., and Hummel, S., eds. (1994). "Ancient DNA". pp. 22–25. Springer-Verlag, New York.

Jeffreys, A. J., Wilson, V., and Thein, S. L. (1985). Hypervariable "minisatellite" regions in human DNA. *Nature* **314,** 67–73.

Jeffreys, A. J., Wilson, V., Neumann, R., and Keyte, J. (1988). Amplification of human minisatellites by the polymerase chain reaction: Towards DNA fingerprinting of single cells. *Nucleic Acids Res.* **16,** 10953–10971.

Nakamura, Y., Carlson, M., Krapcho, K., and White, R. Isolation and mapping of a polymorphic DNA sequence (pMCT118) on chromosome 1p [D1S80]. *Nucl. Acids Res.* **16,** 9364.

Nelson, D. L., Ledbetter, S. A., Corbo, L., Victoria, M. F., Ramirez-Solis, R., Webster, T. D., Ledbetter, D. H., and Caskey, C. T. (1989). *Alu* polymerase chain reaction: A method for rapid isolation of human-specific sequences from complex DNA sources. *Proc. Natl. Acad. Sci. U.S.A.* **86,** 6686–6690.

Weber, J. L., and May, P. E. (1989). Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction. *Amer. J. Human Genetics* **44,** 388–396.

Keohavong, P., Wang, C. C., Cha, R. S., and Thilly, W. G. (1988). Enzymatic amplification and characterization of large DNA fragments from genomic DNA. *Gene* **71,** 211–216.

Random Amplified Polymorphic DNA–RAPDs

Welsh, J., and McClelland, M. (1990). Fingerprinting genomes using PCR with arbitary primers [RAPDs]. *Nucleic Acids Res.* **18,** 7213–7218.

Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalski, J. A., and Tingey, S. W. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. [RAPD markers, Random Amplified Polymorphic DNA]. *Nucleic Acids Res.* **18,** 6531–6535.

PCR Cloning

Larrick, J. W., Danielsson, L., Brenner, C. A., Wallace, E. F., Abrahamson, M., Fry, K. E., and Borrebaeck, C. A. K. (1989). Polymerase chain reaction using mixed primers: Cloning of human monoclonal antibody variable region genes from single hybridoma cells. *Biotechnology* **7,** 934–938.

mRNAs

Wang, A. M., Doyle, M. V., and Mark, D. F. (1989). Quantitation of mRNA by polymerase chain reaction. *Proc. Natl. Acad. Sci. U.S.A.* **86,** 9717–9721.

Apparatus

Cao, T. M. (1989). A simple and inexpensive system to amplify DNA by PCR. *Biotechniques* **7,** 566–567.

Foulkes, N. S., Pandolfi de Rinaldis, P. P., Macdonnell, J., Cross, N. C. P., and Luzatto, L. (1988). Polymerase chain reaction automated at lost cost. *Nucleic Acids Res.* **16,** 5687–5688.

Rollo, F., Amici, A., and Salvi, R. (1988). A simple and low cost DNA amplifier. *Nucleic Acids Res.* **16,** 3105–3106.

Weier, H. U., and Gray, J. W. (1988). A programmable system to perform the polymerase chain reaction. *DNA* **7,** 441–447.

Technical Aspects

Carbonari, M., Sharigia, D., Cibati, M., and Fiorilli, M. (1993). Optimization of PCR performance. *Trends Genet.* **9,** 42–43.

Chester, N., and Marshak, D. R. (1993). Dimethyl sulfoxide-mediated primer T_m reduction: A method for analyzing the role of renaturation temperature in polymerase chain reaction. *Anal. Biochem.* **209,** 284–290.

Kainz, P., Schmiedlechner, A., and Strack, H. B. (1992). *In vitro* amplification of DNA fragments > 10 kb. *Anal. Biochem.* **202,** 46–49.

Krishnan, B. R., Kersulyte, D., Brikun, I., Berg, C. M., and Berg, D. E. (1991). Direct and crossover PCR amplification to facilitate *Tn5supF*-based sequencing of λ phage clones. *Nucleic Acids Res.* **19,** 6177–6182.

Lennon, G. G., Drmanac, R., and Lehrach, H. (1991). Easy removal of oil from microtiter dishes following PCR. *Biotechniques* **11,** 185–186.

Schmidt, T. M., Pace, B., and Pace, N. R. (1991). Detection of DNA contamination in *Taq* polymerase. *Biotechniques* **11,** 176–177.

Shen, W.-H., and Hohn, B. (1992). DMSO improves PCR amplification of DNA with complex secondary structure. *Trends Genet.* **8,** 227.

Sugimoto, T., Fujita, M., Taguchi, T., and Morita, T. (1993). Quantitative detection of DNA by coamplification polymerase chain reaction: A wide detectable range controlled by the thermodynamic stability of primer template duplexes. *Anal. Biochem.* **211,** 170–172.

Yap, E. P. H., and McGee, J. O'D. (1992). Non-isotopic SSCP (single-stranded conformation polymorphism) detection in PCR products by ethidium bromide staining. *Trends Genet.* **8,** 49.

Amplification of Larger DNAs

Cheng, S., Fockler, C., Barnes, W. M., and Higuchi, R. (1994). Effective amplification of long targets from cloned inserts and human genomic DNA. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 5695–5699.

Kainz, P., Schmiedlechner, A., and Strack, H. B. (1992). *In vitro* amplification of DNA fragments > 10 kb. *Anal. Biochem.* **202,** 46–49.

Krishnan, B. R., Kersulyte, D., Brikun, I., Berg, C. M., and Berg, D. E. (1991). Direct and crossover PCR amplification to facilitate Tn5*sup*F-based sequencing of λ phage clones. *Nucleic Acids Res.* **19,** 6177–6182.

Maga, E. A., and Richardson, T. (1991). Amplification of a 9.0-kb fragment using PCR. *Biotechniques* **11,** 185–186.

Ligation Reactions

Landegren, U. (1993). Ligation-based DNA diagnosis. *Bioessays* **15,** 761–765.

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APPENDIX 1: TEMPLATES FOR STREAKING COLONIES

Photocopies of the circles with numbered grids can be taped to the bottom of petri plates to serve as templates for streaking colonies onto plates (see Figure A.1).

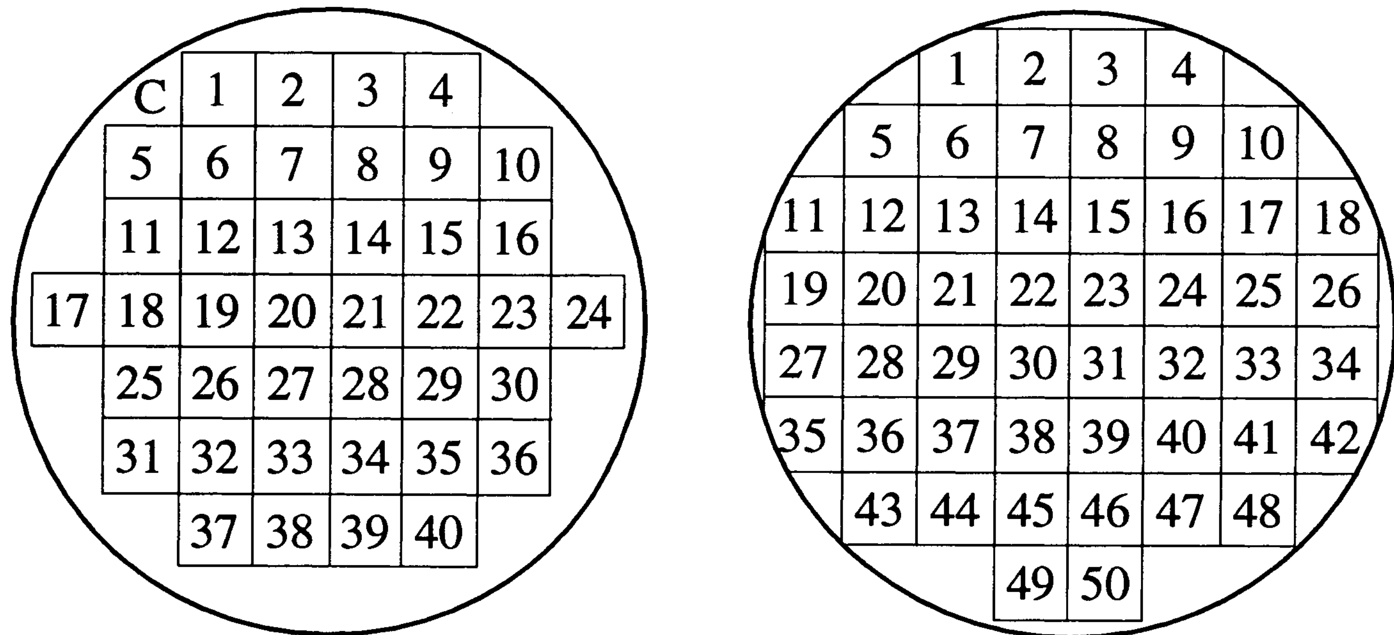

Figure A.1 Examples of templates to use to streak colonies onto plates. The C shows an area where a control bacterial strain should be streaked.

APPENDIX 2: STORING BACTERIAL STRAINS: MAKING PERMANENTS

Once a new mutant or strain has been made, it is very important to be able to store that bacterial strain indefinitely. More than one vial of each strain should be stored. Careful records of the strains and their genotypes should be kept.

Bacterial strains can be stored for short times (typically, up to a month) on plates wrapped with Parafilm and either kept at room temperature or refrigerated. Davis *et al.* (1980) report that *E. coli* strains remain viable for more than 10 years when stored on slants kept at room temperature. Bacterial strains containing plasmids, such as pBR322-based plasmids, should not be kept in slants at room temperature for long-term storage because the strains can lose the plasmids. Frozen glycerol stocks at −20°C work well for 1- or 2-year storage. For long-term storage, −70°C is recommended. See Sanderson and Zeigler (1991) for information on storage of bacterial strains by lyophilization or freeze-drying.

After a bacterial strain is removed from storage, check that the strain has maintained its phenotype.

The following procedures describe how to prepare bacteria for long-term storage (called permanents of bacterial strains).

Escherichia coli Strains

1. For slants to be stored at room temperature:
   a. Add 1 ml of melted L agar to each small screw-capped glass vial. Autoclave the vials with the caps not completely tightened. After

autoclaving, tip the vials slightly and allow the agar to solidify on a slant.

b. Using an inoculation loop, streak the bacteria across the agar surface. Also stab the loop with bacteria on it into the agar.
c. Grow the bacteria overnight at 37°C with the cap not tightened completely.
d. Melt pieces of paraffin in a beaker on a hot plate.

CAUTION: Be careful not to overheat the wax.

e. Tighten the caps on the vials completely. Dip the top of each vial into the melted wax to seal the vial.

2. For frozen glycerol stocks to be stored at −20 or −70°C:
   a. Grow an inoculum of the bacterial strain overnight in 1 ml of L broth with antibiotics if applicable.
   b. Add 1 ml of a saturated culture of bacteria to 2 ml of sterile glycerol in a small screw-capped vial. Mix thoroughly and freeze in a dry ice–ethanol bath. Store at −20 or −70°C.
3. For frozen dimethyl sulfoxide (DMSO) stocks to be stored at −70°C:
   a. Add 1 ml of a saturated culture of bacteria to 0.5 ml of sterile DMSO in a sterile, small screw-capped vial.
   b. Mix thoroughly and freeze immediately in a dry ice–ethanol bath. Store at −70°C.

Storage of Phage Stocks

Some types of phage are more sensitive to degradation than other types; some phage are more sensitive to chloroform than others.

For storage at 4°C, store λ lysates in SM buffer or Tris phage buffer at 4°C over a drop of chloroform. The titer of phage lysates stored in the refrigerator will slowly decline and may decrease by as much as a factor of 10 per year. For long-term storage, keep phage lysates at −70°C.

For storage at −70°C:

1. Prepare vials for storage of phage: aliquot 0.15 ml of glycerol into vials; loosely cap the vials and autoclave them. Alternatively, aliquot DMSO into sterile vials.
2. Add 1 ml of phage lysate to a vial. Mix thoroughly. Quick freeze vials in a dry ice–ethanol bath or in liquid nitrogen.
3. Store phage vials at −70°C.

4. To remove phage from −70°C, pick up phage by scratching the surface of the frozen lysate with a sterile inoculation loop. Streak the phage onto a plate containing a lawn of host bacteria. Alternatively, a loop full of phage can be added to a liquid culture of succeptible bacteria.

Storage and Handling of Nucleic Acids

- DNA should not be kept in a solution containing heavy metal ions because phosphodiester bonds may break under such storage conditions. DNA should not be stored in phenol because oxidation products formed in phenol may lead to the breakage of phosphodiester bonds.
- DNA can be stored in ethanol.
- DNA can be stored in 1 m*M* Tris and 0.1 ml EDTA, pH 8.5, at 4°C.
- DNA keeps very well at 4°C. I have used a DNA sample stored at 4°C for more than 6 years that still showed the expected clear, sharp restriction fragments.
- Davis *et al.* (1980) say that DNA stores well at 4°C in the CsCl–ethidium bromide solution from a buoyant density gradient. The container with the DNA should be wrapped in aluminum foil to keep the DNA in the dark.
- DNA does not store well at −20°C because single- and double-stranded breaks can occur at this temperature.
- Davis *et al.* (1980) suggest that DNA may be frozen at −70°C, although breakage of DNA can occur.
- If nucleic acid is to be frozen for storage, the nucleic acid can be divided into small aliquots before freezing. Repeated freezing, thawing, and refreezing of nucleic acid can cause breakage of the nucleic acid strands.

References

Davis, R. W., Botstein, D., and Roth, J. R. (1980). "Advanced Bacterial Genetics." Cold Spring Harbor Laboratory, Cold Spring Habor, NY.

Gherna, R. L. (1994). Culture preservation. *In* "Methods for General and Molecular Bacteriology" (P. Gerhardt, R. G. E. Murray, W. A. Wood, and N. R. Krieg, eds.), pp. 278–292. American Society for Microbiology, Washington, DC.

Sanderson, K. E., and Zeigler, D. R. (1991). Storing, shipping, and maintaining records on bacterial strains. *In* "Methods in Enzymology—Bacterial Genetic Systems" (J. H. Miller, ed.), Vol. 204, pp. 248–263. Academic Press, San Diego.

Requena, J. M., Soto, M., and Alonso, C. (1993). Bacteriophage λ: Long-term stored stocks ready for lysis. *Trends Genet.* **9**, 4.

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APPENDIX 3: REPORTER GENES

A number of different genes are available for use as selectable markers or reporter genes in bacteria, plant, and animal systems. This appendix identifies and briefly describes some of the markers and reporter genes available.

The gene for the bacterial enzyme chloramphenicol acetlytransferase (CAT) is frequently used as a reporter gene, fused to the transcriptional regulatory signals of the gene to be studied. The *cat* gene can be especially useful in mammalian systems since mammalian cells lack this enzyme and therefore have no endogenous CAT activity. A common assay for CAT activity uses the substrate [^{14}C]acetyl-CoA. The acetylated forms are separated on thin-layer chromatography. A nonradioactive, fluorescent chloramphenical substrate is also available (Hruby and Wilson, 1992).

The firefly (*Photinus pyralis*) luciferase gene is a useful reporter gene (Brasier and Ron, 1992; Luehrsen *et al.*, 1992). This enzyme (luciferin 4-monooxygenase or luciferase) produces a yellow-green light (560 nm) by the oxidation of luciferin. This reaction is ATP dependent. When luciferin is present in excess, the amount of light given off is proportional to the amount of enzyme. The light produced is quantitated using a luminometer. The luciferase messenger RNA and protein turn over rapidly. The luciferase assay is very sensitive, perhaps as much as 10–1000 times more sensitive than a CAT assay, and does not require radioactive materials. However, a luminometer is expensive.

The *bar* (bialaphos resistance) gene is a useful selectable and screenable marker in plants. Bialaphos is a tripeptide antibiotic made up of phosphinothricin with two alanine groups and is produced by *Streptomyces*. Phosphinothricin inhibits glutamine synthetase, which is a vital enzyme in the regulation of nitrogen metabolism in plants. Bialaphos and

other phosphinothricins are used as general herbicides. The *bar* gene codes for phosphinothricin acetlytransferase, which acetylates phosphinothricin, making it no longer active as an herbicide. The *bar* gene product can be detected immunologically. The *bar* gene enzyme activity can be determined by the acetylation of [^{14}C]phosphinothricin, which is separated by thin-layer chromatography. In some plant species a background acetylation of other substrates can sometimes interfere with this assay (D'Halluin *et al.*, 1992).

The *gusA* gene encodes β-glucuronidase and was originally cloned from the *gus* operon of *E. coli*. The *gus* gene is a useful reporter gene in plants and other organisms (Jefferson, 1989; Gallagher, 1992) and is particularly useful in plants because of the lack of endogenous background activity in higher plants. Sensitive, quantitative assays are available for β-glucuronidase using a fluorescent substrate such as 4-MUG (4-methylumbelliferyl-β-glucuronide) or a chromogenic substrate such as X-gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide). X-gluc has been used to study cell and tissue specificity.

The *lacZ* gene, part of the well-characterized *lac* operon of *E. coli*, encodes the enzyme β-galactosidase. Substrates for β-galactosidase include the chromogenic X-gal (5-bromo-4-chloro-3-indoly-β-D-galactoside) and MUG (4-methylumbelliferyl-β-D-galactoside), used in fluorescent assays (Miller, 1992).

References

Brasier, A. R., and Ron, D. (1992). Luciferase reporter gene assay in mammalian cells. *In* "Methods in Enzymology" (R. Wu, ed.), Vol. 216, pp. 386–397. Academic Press, San Diego.

D'Halluin, K., de Block, M., Denecke, J., Janssens, J., Leemans, J., Reynaerts, A., and Botterman, J. (1992). The *bar* gene as selectable and screenable marker in plant engineering. *In* "Methods in Enzymology" (R. Wu, ed.), Vol. 216, pp. 415–426. Academic Press, San Diego.

Hruby, D. E., and Wilson, E. M. (1992). Use of fluorescent chloramphenicol derivative as a substrate for chloramphenicol acetyltransferase assays. *In* "Methods in Enzymology" (R. Wu, ed.), Vol. 216, pp. 369–376. Academic Press, San Diego.

Gallagher, S. R. (1992). "GUS Protocols—Using the GUS gene as a Reporter of Gene Expression." Academic Press, San Diego.

Jefferson, R. A. (1989). The GUS reporter gene system. *Nature* **342,** 837–838.

Miller, J. H. (1992). The lac system. *In* "A Short Course in Bacterial Genetics—A Laboratory Manual and Handbook for *Escherichia coli* and Related Bacteria." pp. 45–80. Cold Spring Laboratory Press, Cold Spring Harbor, NY.

Suggested Reading

Caplan, A., Dekeyser, R., and van Montagu, M. (1992). Selectable markers for rice transformation. *In* "Methods in Enzymology" (R. Wu, ed.), Vol. 216, pp. 426–441. Academic Press, San Diego.

Gould, J. (1992). Nondestructive assay for β-glucuronidase in culture media of plant tissue cultures. *In* "Methods in Enzymology" (R. Wu, ed.), Vol. 216, pp. 357–362. Academic Press, San Diego.

Jefferson, R. A. (1987). Assaying chimeric genes in plants: The GUS gene fusion system. *Plant Mol. Biol. Rep.* **5**, 387–405.

Luehrsen, K. R., de Wet, J. R., and Walbot, V. (1992). Transient expression analysis in plants using firefly luciferase reporter gene. *In* "Methods in Enzymology" (R. Wu, ed.), Vol. 216, pp. 397–414. Academic Press, San Diego.

Punt, P. O. J., and van den Hondel, C. A. M. J. J. (1992). Transformation of filamentous fungi based on hygromycin B and phleomycin resistance markers. *In* "Methods in Enzymology" (R. Wu, ed.), Vol. 216, pp. 447–452. Academic Press, San Diego.

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APPENDIX 4: ANTIBIOTIC INFORMATION

Mode of Action

Ampicillin (Figure A.2)

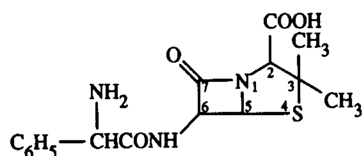
Penicillins, including ampicillin, and cephalosphorins are antibiotics containing a β-lactam ring that is fused to a second sulfur-containing ring. These classes of antibiotics function as substrate analogues that interfere with cell wall synthesis to kill actively growing and dividing bacteria. In the cell wall synthesis of bacteria, a peptidoglycan network is made by crosslinking linear polysaccharide chains with short peptides. This addition of crosslinking peptides, called "transpeptidation," is inhibited by the β-lactam antibiotics. These antibiotics bind irreversibly to the active site of the transpeptidases that catalyze the crosslinking (Riddihough, 1992).

The penicillins and cephalosporins are produced by a number of different organisms, including filamentous fungi, gram-positive bacteria such as the *Streptomycetes*, and some gram-negative bacteria. Interestingly, all these organisms produce the β-lactam antibiotics by essentially the same biosynthetic pathway. The genes of this biosynthetic pathway have been well characterized (Aharonowitz *et al.*, 1992) because of the clinical importance of these antibiotics.

Tetracycline

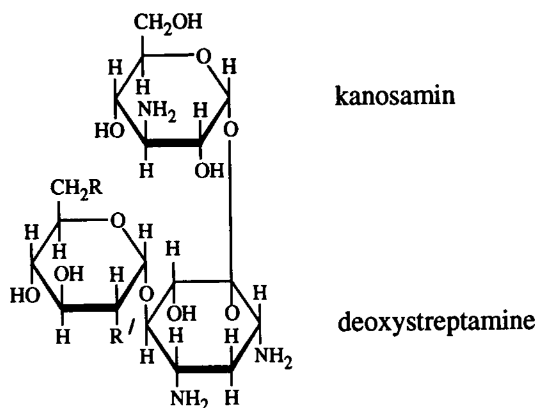
Tetracycline is a bacteriostatic agent that binds to a ribosomal protein in the 30 S subunit of the ribosome and prevents protein synthesis by inhibiting ribosomal translocation.

a



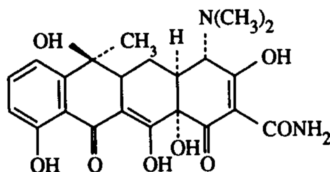
ampicillin

b



kanamycin A, $C_{18}H_{36}N_4O_{11}$ R = NH_2, R′ = OH

c



tetracycline

Figure A.2 Chemical structures of three commonly used antibiotics. (a) The structure of ampicillin. (b) The structure of kanamycin A. (c) The structure of tetracycline.

Kanamycin

Kanamycin is a deoxystreptamine aminoglycoside that binds to ribosomal components and inhibits protein synthesis (Sambrook *et al.*, 1989).

Method of Preparing and Storing the Antibiotic

To prepare stock solutions of antibiotics, sterile water and sterile containers should be used. Although that is usually all that need be done to prepare stock solutions, the solutions can be filter-sterilized using a 0.22-μm filter. The stock solutions should be stored in the dark at −20°C.

Ampicillin

Make a stock solution of 50 mg/ml in water; store stock solution at −20°C. Use on agar plates at concentrations of 50–100 μg/ml. Plates should be stored at 4°C until needed. Ampicillin plates stored at room temperature can lose ampicillin activity. It is not recommended that ampicillin plates be stored more than 1 month in the refrigerator because ampicillin can break down even at 4°C. Certainly, older ampicillin plates should be carefully checked for activity before use. Carbenicillin is another penicillin-like antibiotic that is more stable than ampicillin at room temperature storage . Carbenicillin is recommended for use with more slowly growing bacteria such as *Agrobacterium* because of its greater stability.

Tetracycline

The Merck Index indicates that tetracycline at 28°C is soluble at 1.7 mg/ml in water and more than 20 mg/ml in methanol. However, tetracycline hydrochloride is freely soluble in water and moderately soluble in methanol or ethanol.

For tetracycline hydrochloride make a stock of 5 mg/ml in water; store stock solution at −20°C. For *E. coli*, use on rich agar plates, such as L agar, at 15 μg/ml. On minimal agar plates, use at 10 μg/ml. For *Agrobacterium*, use on YEP plates at 5 μg/ml.

When tetracycline-resistant bacteria are being selected, the medium used should not contain magnesium ions because those ions can interfere with the tetracycline.

Light can increase the rate of breakdown of tetracycline; tetracycline-containing plates should be wrapped to protect them from light and kept at 4°C.

Kanamycin

Make a stock solution of 10 mg/ml in water; store stock solution at −20°C. Use on agar plates at 30 to 50 μg/ml. Store kanamycin-containing plates at 4°C.

Mechanism of the Antibiotic Resistance Gene

Ampicillin

Certain bacteria are resistant to penicillins and related antibiotics. This ampicillin resistance (amp^r) is due to the presence of the plasmid-encoded gene for β-lactamase, an enzyme that cleaves the β-lactam ring of penicillin and related antibiotics. The enzyme hydrolyzes the amide bond of the β-lactam ring to produce penicilloic acid, which has no antibiotic activity. Recently, crystallographers have determined the structure of a specific β-lactamase interacting with penicillin G to high (1.7 Å) resolution (Strynadka *et al.*, 1992). β-Lactamase is secreted into the periplasmic space of the bacterium, where it hydrolyzes the β-lactam ring and thereby detoxifies ampicillin.

Because β-lactamase is a periplasmic enzyme, it can diffuse into the agar surrounding an amp^r bacterial colony and degrade the ampicillin in the agar plate. After long periods of incubation, this allows ampicillin-sensitive bacterial colonies to grow around the ampicillin-resistant bacterial colony. When selecting amp^r cells, do not incubate ampicillin-containing agar plates so long that amp^s satellite colonies begin to grow up around the amp^r colonies.

Tetracycline

The tetracycline resistance (tet^r) gene from the plasmid pBR322 is constitutively expressed and codes for a membrane-associated protein that prevents tetracycline from entering the bacterial cell.

The tetracycline resistance (tet^r) gene from the transposon Tn*10* is inducible. The tetracycline resistance determinant (*tet*) from Tn*10* has been shown to contain four genes: *tetA*, the structural gene for tetracycline resistance; *tetR*, which codes for a repressor; *tetC*; and *tetD*. Deletion of *tetC* and *tetD* has no effect on the level of tetracycline resistance of the transposon in *E. coli* K-12. The *tetA* gene codes for the resistance protein, which is a 43.2-kDa inner membrane protein thought to bring about an energy-dependent export of tetracycline from the cytoplasm. The *tetR* gene codes for a 23.3-kDa repressor protein that regulates the expression of itself and the *tetA* gene. The *tetA* and *tetR* genes are transcribed in opposite

directions from overlapping promoters. In the absence of tetracycline, the *tetR* gene product, the repressor protein, binds to *tet* DNA and inhibits initiation of transcription at the *tetA* and *tetR* promoters. In the presence of tetracycline, the repressor protein does not bind to the *tet* DNA and transcription of the *tetA* and *tetR* genes occurs.

Nguyen *et al.* (1983) determined that the Tn*10* *tetA* gene and the pBR322 tet^r gene share 49% nucleotide homology and 44% amino acid homology. The two genes apparently diverged from a common ancestor.

Cloning Tn*10* tet Resistance Gene

In attempts to clone the tetracycline resistance determinant (*tet*) of Tn*10*, it was discovered that when the *tet* determinant is on a high-copy-number plasmid, the level of resistance to tetracycline is not as great as when *tet* is on a low-copy-number plasmid (Moyed *et al.*, 1983). Overexpression of the *tetA* gene product inhibits bacterial growth.

Eckert and Beck (1989) observed that when the *tetA* gene was overexpressed in *E. coli* the cell membrane potential was affected, cell growth was stopped, and cells were killed. They thought that these effects were not due to the destruction of the membrane by the TetA protein but were due to the unrestricted movement of protons and ions across the membrane.

Selection for Loss of Tetracycline Resistance

Methods for selecting tetracycline-sensitive *E. coli* cells among a population of tetracycline-resistant cells have been worked out by Bochner *et al.* (1980) and by Maloy and Nunn (1981). Such selection methods make it easy to identify revertants of Tn*10*-generated mutations in which a precise excision of the Tn*10* and one of the target site duplications has occurred.

Kanamycin

The kanamycin resistance gene codes for an aminoglycoside phosphotransferase (APH[3′]-II) that phosphorylates kanamycin and is thought to interfere with the uptake of kanamycin into the bacterial cell.

References

Aharonowitz, Y., Cohen, G., and Martin, J. F. (1992). Penicillin and cephalosporin biosynthetic genes: Structure, organization, regulation, and evolution. *Annu. Rev. Microbiol.* **46,** 461–495.

Bochner, B. R., Huang, H.-C., Schieven, G. L., and Ames, B. N. (1980). Positive selection for loss of tetracycline resistance. *J. Bacteriol.* **143,** 926–933.
Maloy, S. R., and Nunn, W. D. (1981). Selection for loss of tetracycline resistance by *Escherichia coli*. *J. Bacteriol.* **145,** 1110–1112.
Moyed, H. S., Nguyen, T. T., and Bertrand, K. P. (1983). Multicopy Tn*10 tet* plasmids confer sensitivity to induction of *tet* gene expression. *J. Bacteriol.* **155,** 549–556.
Nguyen, T. T., Postle, K., and Bertrand, K. P. (1983). Sequence homology between the tetracycline-resistance determinants of Tn*10* and pBR322. *Gene* **25,** 83–92.
Riddihough, G. (1992). Drug ring bust (News and Views). *Nature* (*London*) **359,** 674.
Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). "Molecular Cloning: A Laboratory Manual," 2nd Ed., pp. 1.5–1.6, A-6. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Strynadka, N. C. J., Adachi, H., Jensen, S. E., Johns, K., Sielecki, A., Betxel, C., Sutoh, K., and James, M. N. G. (1992). Molecular structure of the acyl-enzyme intermediate in β-lactam hydrolysis at 1.7Å resolution. *Nature* (*London*) **359,** 700–705.

Suggested Readings

Tetracycline

Altschmied, L., and Hillen, W. (1984). TET repressor·*tet* operator complex formation induces conformational changes in the *tet* operator DNA. *Nucleic Acids Res.* **12,** 2171–2180.
Bertrand, K. P., Postle, K., Wray, L. V., and Reznikoff, W. S. (1983). Overlapping divergent promoters control expression of Tn*10* tetracycline resistance. *Gene* **23,** 149–156.
Bertrand, K. P., Postle, K., Wray, L. W., and Reznikoff, W. S. (1984). Construction of a single-copy promoter vector and its use in analysis of regulation of the transposon Tn*10* tetracycline resistance determinant. *J. Bacteriol.* **158,** 910–919.
Braus, G., Argast, M., and Beck, C. R. (1984). Identification of additional genes on transposon Tn*10*:*tet*C and *tet*D. *J. Bacteriol.* **160,** 504–509.
Hillen, W., Schollmeier, K., and Gatz, C. (1984). Control of expression of the Tn*10*-encoded tetracycline resistance operon II: Interaction of RNA polymerase and TET repressor with the *tet* operon regulatory region. *J. Mol. Biol.* **172,** 185–201.
Kleinschmidt, C., Tovar, K., Hillen, W., and Porschke, D. (1988). Dynamics of repressor-operator recognition: The Tn*10*-encoded tetracycline resistance control. *Biochemistry* **27,** 1094–1104.
Meier, I., Wray, L. W., and Hillen, W. (1988). Differential regulation of the Tn*10*-encoded tetracycline resistance genes *tet*A and *tet*R by the tandem *tet* operators O_1 and O_2. *EMBO J.* **7,** 567–572.
Mendez, B., Tachibana, C., and Levy, S. B. (1980). Heterogeneity of tetracycline resistance determinants. *Plasmid* **3,** 99–108.
Nguyen, T. T., Postle, K., and Bertrand, K. P. (1983). Sequence homology between the tetracycline-resistance determinants of Tn*10* and pBR322. *Gene* **25,** 83–92.
Schollmeier, K., and Hillen, W. (1984). Transposon Tn*10* contains two structural genes with opposite polarity between *tet*A and $IS10_R$. *J. Bacteriol.* **160,** 499–503.
Tovar, K., and Hillen, W. (1989). Tet repressor binding induced curavture of *tet* operator DNA. *Nucleic Acids Res.* **17,** 6515–6522.

Cloning Tn*10* tet Resistance Gene

Eckert, B., and Beck, C. F. (1989). Overproduction of transposon Tn*10*-encoded tetracycline resistance protein results in cell death and loss of membrane potential. *J. Bacteriol.* **171,** 3557–3559.

Moyed, H. S., Nguyen, T. T., and Bertrand, K. P. (1983). Multicopy Tn*10* *tet* plasmids confer sensitivity to induction of *tet* gene expression. *J. Bacteriol.* **155**, 549–556.

Selection for Loss of Tetracycline Resistance

Bochner, B. R., Huang, H.-C., Schieven, G. L., and Ames, B. N. (1980). Positive selection for loss of tetracycline resistance. *J. Bacteriol.* **143**, 926–933.

Maloy, S. R., and Nunn, W. D. (1981). Selection for loss of tetracycline resistance by *Escherichia coli*. *J. Bacteriol.* **145**, 1110–1112.

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APPENDIX 5: X-gal AND IPTG

Use of X-gal

X-gal (5-bromo-4-chloro-indolyl-β-D-galactoside) is a chromogenic substrate for β-galactosidase. When X-gal is cleaved by β-galactosidase, the blue 5-bromo-4-chloro indigo is produced. X-gal is used in plates at a concentration of 40 μg/ml. X-gal is soluble in dimethyl formamide. The amount of X-gal needed is dissolved in the smallest volume of dimethyl formamide possible and then added to agar media before plates are poured.

Use of IPTG

IPTG (isopropyl-β-D-thiogalactoside) is an inducer of the *lac* operon. IPTG is water soluble and is used at 120 μg/ml. The *lac* operon consists of a promoter, P, an operator, O, and the *lacZ* (β-galactosidase), *lacY* (permease), and *lacA* (acetylase) genes. The *lacI* gene encodes a repressor protein that binds the operator and prevents transcription of the *lac* operon. For transcription of the *lac* operon to occur, the repressor cannot be bound to the operator. In addition, a positive regulator, a complex of cyclic AMP and catabolic activating protein, is required for RNA polymerase binding to the promoter.

The Lac repressor protein interacts with IPTG and no longer binds the operator region of the *lac* operon. Also note that in the absence of glucose, cyclic AMP is produced and interacts with the catabolic activating protein to form a complex that binds to the promoter of the *lac* operon to allow RNA polymerase to transcribe the *lac* operon. Thus, even if IPTG

were present, if glucose were also present in the medium, the *lac* operon would not be expressed.

pUC Vectors and Need for IPTG

Note that pUC cloning vectors contain *lacI′* (Hackett *et al.*, 1984). *lacI′* refers to only a piece of the *lacI* gene. pUC contains part of the *lac* regulatory region—the operator and promoter but not a functional repressor. When cloning into pUC vectors is being performed and pUC is being introduced into *E. coli* strains, the genotype of the *E. coli* strain should be examined. The recipient *E. coli* strain should be lac^-. When cloning into strains such as DH5α is being performed, the inducer IPTG is not needed in the plates. Also check to see if the strain is $lacI^q$. $lacI^q$ is a mutation that causes an overproduction of the *lac* repressor protein. To allow transcription of the *lac* operon in a $lacI^q$ strain, an inducer (IPTG) must be added. The $lacI^q$ strain is useful to regulate the transcription of a gene cloned into the pUC polylinker.

Reference

Hackett, P. B., Fuchs, J. A., and Messing, J. W. (1984). "An Introduction to Recombinant DNA Techniques: Basic Experiments in Gene Manipulation." 1st Ed., p. 38. Benjamin/Cummings, New York.

APPENDIX 6: MORE INFORMATION ON MOLECULAR BIOLOGY PROTOCOLS

This laboratory manual covers a range of introductory molecular biology and molecular genetics topics, including transposon mutagenesis, recombinant DNA cloning, and nucleic acid hybridization techniques. Because this manual is intended as an introduction to molecular biology, there are many topics in molecular biology that have not been included.

For other protocols in molecular biology, the reader should refer to several manuals listed below:

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. A., Smith, J. A., and Struhl, K. (1989). "Current Protocols in Molecular Biology." Green Publishing Association and Wiley-Interscience, New York.

This is a two-volume manual with protocols and relevant references. This series is especially useful because it publishes yearly updates.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). "Molecular Cloning: A Laboratory Manual," 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

This is a three-volume set.

For techniques dealing specifically with complex genomes see the following:

Anand, R., ed. (1992). "Techniques for the Analysis of Complex Genomes." Academic Press, San Diego.

Saluz, H. P., and Jost, J. P. (1987). "A Laboratory Guide to Genomic Sequencing: The Direct Sequencing of Native Uncloned DNA." Birkhauser, Basel, Switzerland.

In addition, several journals focus on new techniques:

- *Trends in Genetics*, Technical Tips section. In every issue, this journal has a few short new protocols or new modifications of protocols used in molecular biology.
- *Trends in Biochemical Sciences (TIBS)*
- *Biotechniques*

It is also possible to use the Internet to get "up-to-the-minute" information on molecular biology protocols by joining a Bionet newsgroup. For more information on the newsgroup BIOSCI, contact: biosci@net.bio.net. See *Trends Biochem. Sci.* (1993). **18**, 447.

APPENDIX 7: SOURCES OF STRAINS

To obtain bacterial or phage strains:

1. Write directly to the author of the journal article in which the bacterial strain or phage is described.
2. Order a strain from
   1. "The American Type Culture Collection Catalogues of Bacteria and Bacteriophage" (1992). 18th ed.
      12301 Parklawn Dr.
      Rockville, MD 20852-1776
      Phone: 1-800-638-6597
   2. Dr. Barbara Bachmann
      E. coli Genetic Stock Center
      Department of Biology, 255 OML
      Yale University, P.O. Box 6666
      New Haven, CT 06511-7444
      Submit requests in writing.
   3. The Kohara *et al.* (1987) λ library of the *E. coli* chromosome can be obtained by contacting:
      Drs. Donna Daniels and Fred Blattner
      Department of Genetics
      University of Wisconsin, Madison
      Madison, WI 53705
3. Purchase plasmid vectors or bacterial strains from molecular biology suppliers. (See the list of molecular biology suppliers.)

For more information about cloning vectors, see Pouwels *et al.* (1987) and Rodriquez and Denhardt (1988).

References

Kohara, Y., Akiyama, K., and Isono, K. (1987). The physical map of the whole *E. coli* chromosome: Application of a new strategy for rapid analysis and sorting of a large genomic library. *Cell* **50**, 495–508.

Pouwels, P. H., Enger-Valk, B. E., and Brammar, W. J. (1987). "Cloning Vectors." Elsevier, Amsterdam. There are updates published in later years.

Rodriquez, R. L., and Denhardt, D. T. (1988). "Vectors: A Survey of Molecular Cloning Vectors and Their Uses." Butterworths, Boston.

APPENDIX 8: LIST OF SUPPLIERS

For a more complete list of suppliers, see American Chemical Society Biotech Buyer's Guide (1994–1995), American Chemical Society, 1155 16th St., NW, Washington, DC 20036, phone 202–872–4600; or the American Laboratory 1994 Buyer's Guide Edition (1994), Vol. *26*(4).

Name	Address	Toll-free number
	Restriction enzymes	
Boehringer-Mannheim	9115 Hague Road P.O. Box 50414 Indianapolis, IN 46250	1–800–262–1640
Fisher Scientific	1600 West Glen Lake Ave. Itasca, IL 60143	1–800–766–7000
Gibco/BRL	3175 Staley Road Grand Island, NY 14072	1–800–828–6686
New England Biolabs	32 Tozer Road Beverly, MA 01915-5599	1–800–632–5227
Pharmacia LKB Biotechnology	P.O. Box 1327 Piscataway, NJ 08855–9836	1–800–526–3593
Promega	2800 Woods Hollow Road Madison, WI 53711–5399	1–800–356–9526
Stratagene	11011 N. Torrey Pines Road La Jolla, CA 92037	1–800–424–5444
United States Biochemical	P.O. Box 22400 Cleveland, OH 44122	1–800–321–9322
	Photobiotin	

(*Continued*)

Appendix—*Continued*

Name	Address	Toll-free number
Research Organics, Inc.	4353 E. 49th St. Cleveland, OH 44125	1–800–321–0570
Gibco/BRL	3175 Staley Road Grand Island, NY 14072	1–800–828–6686
Resin for rapid plasmid DNA isolation		
Qiagen, Inc.	11712 Moorpark St. P.O. Box 7401-737 Studio City, CA 91604	1–800–426–8157
Agarose		
Pharmacia	P.O. Box 1327 Piscataway, NJ 08855–1327	1–800–558–7110
Note: NA agarose, for electroelution: Cat. No. 17-0554-01/02/03 (10/100/1000 g)		
Dialysis tubing		
VWR Scientific Co.	1400 N. Providence Rd. Suite 100 Media, PA 19063	1–800–932–5000
Note: Spectra/por Molecular porous membrane tubing: Cat. No. 25225-226 (25 mm × 100-ft roll, with a diameter of 15.9 mm)		
Rocking platform		
Bellco Glass, Inc.	P.O. Box B 340 Edrudo Rd. Vineland, NJ 08360	1–800–257–7043
Gel electrophoresis apparatus		
Fisher Scientific	1600 W. Glenlade Ave. Itasca, IL 60143	1–800–331–7341
Hoefer Scientific Instruments	654 Minnesota St. P.O. Box 77387 San Francisco, CA 94107	1–800–227–4750
IBI (International Biotechnologies, Inc.)	275 Winchester Ave. P.O. Box 9558 New Haven, CT 06535	1–800–243–2555
Owl Scientific Plastics, Inc.	P.O. Box 522 Cambridge, MA 02139	1–800–242–5560
Ultraviolet transilluminator		
Fotodyne, Inc.	16700 W. Victor Rd New Berlin, WI 53151-4131	1–800–362–3686
Fisher Scientific	1600 W. Glenlade Ave. Itasca, IL 60143	1–800–331–7341
Chemicals		
Aldrich Chemical Co., Inc.	940 W. St. Paul Ave. Milwaukee, WI 53223	1–800–558–9160
Calbiochem Corp.	P.O. Box 12087 San Diego, CA 92112	1–800–854–3417

Sigma Chemical Co.	P.O. Box 14508 St. Louis, MO 63178	1–800–325–3010
	X-gal	
Gold Biotechnology, Inc.	7166 Manchester Rd. St. Louis, MO 63143	1–800–248–7609

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APPENDIX 9: ADDITIONAL INFORMATION

Units

mega = M = 10^6
kilo = k = 10^3
milli = m = 10^{-3}
micro = μ = 10^{-6}
nano = n = 10^{-9}
pico = p = 10^{-12}
femto = f = 10^{-15}
atto = a = 10^{-18}

Conversion Kilobases to Mass

1 kb of double-stranded DNA = 6.6×10^5 Da
1 MDa double-stranded DNA = 1.52 kb

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APPENDIX 10: MOLECULAR WEIGHT STANDARDS

λ *Hind*III (bp)	λ *Hind*III + *Eco*RI, double digest (bp)
23,130	21,226
9,416	5,148
6,557	4,973
4,361 (sometimes faint)	4,268
2,322	3,530 (sometimes faint)
2,027	2,027
564	1,904
125	1,584
	1,375
	947
	831
	564
	125

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GLOSSARY

AdoMet-*S*-Adenosyl-L-methionine (also called SAM) The molecule that acts as a methyl group donor for modification enzymes that add methyl groups to DNA.

A-Form of DNA A conformation of double-stranded DNA that is a right-handed double helix with approximately 11 bp per turn of the helix. See B- and Z-forms of DNA.

Alkaline Phosphatase An enzyme, with optimal activity at alkaline pH, that cleaves a phosphate group off a substrate molecule.

α-Acceptor A DNA sequence coding for the carboxy-terminal region of β-galactosidase. An example is deltaM15, which encodes a peptide containing a deletion of the operator proximal region of β-galactosidase, lacking amino acids 11 to 41, producing a defective protein that can function as an α-acceptor peptide. See α-complementation; α-donor.

α-Complementation The ability of two nonoverlapping deletions of the *lacZ* gene to complement each other. Peptides produced by the DNA sequences coding for the α-donor peptide and the α-acceptor peptide interact to give β-galactosidase activity. See α-donor; α-acceptor.

α-Donor A DNA sequence coding for the first 145 amino acids of the *lacZ* gene. This part of the *lacZ* gene codes for the α-donor polypeptide of β-galactosidase. See α-complementation; α-acceptor.

Amber Mutation A mutation in a DNA sequence that causes the mRNA transcribed from that sequence to have a stop codon UAG.

Amber Suppressor A mutation in a gene coding for a tRNA that changes the anticodon so that the tRNA inserts an amino acid at an amber stop codon during translation.

Amino Group NH_2.

Angstrom 10^{-8} centimeter.

Annealing The pairing of complementary single strands of nucleic acid to form a double helix (double-stranded) nucleic acid.

Anode The electrode where oxidation occurs. In gel electrophoresis, the anode is the positive terminal of the power supply.

Antibiotic A chemical substance that can kill or inhibit the growth of bacteria or microorganisms.

Archaeo A more primitive type of prokaryote than eubacteria. The Archaeo include methanogens, extreme halophiles, and thermoacidophiles. Archaeobacteria and eubacteria differ in ribosomal DNA and in the composition of cell walls. See eubacteria.

Autonomously Replicating Sequence (ARS) The sequence of DNA that allows DNA replication to occur; a sequence able to act as an origin of DNA replication.

Artificial Transformation Procedure to introduce DNA into a cell that requires some manipulation of the cell; see natural transformation.

Adenosine Triphosphate (ATP) The major carrier of chemical energy in cells.

Att site Regions in a phage and in a bacterial genome where recombination integrates the phage into the host bacterial chromosome or excises the phage from the bacterial chromosome.

Atto One quintillionth part of; the factor 10^{-18}.

Autoradiography The detection of radioactively labeled molecules by the image they produce on a photographic film.

Auxotroph A strain of an organism with a mutation that prevents the synthesis of a molecule required for growth, a molecule normally synthesized by wild-type strains. For the mutant organism to grow, the molecule must be supplied in the growth medium. From Latin *auxilium* meaning "help." See prototroph.

Avidin A basic glycoprotein of 68,000 Da isolated from egg white that binds strongly and noncovalently to biotin with a K_d of 10^{-15} M^{-1}. This strong and specific interaction between avidin and biotin is used as a means of detecting biotin. See biotin; streptavidin.

B-Form of DNA A conformation of double-stranded DNA that is a right-handed double helix with approximately 10 bp per turn of the helix. DNA in solution usually is in the B-form. See A- and Z-forms of DNA.

Biolistics From a combination of biological and ballistics. Methods of introducing DNA into a cell using DNA-coated projectiles.

Biotin A water-soluble vitamin, also called vitamin H. Biotin is involved in carboxylation reactions in the cell and is a co-enzyme in (S)-methyl-malonyl-CoA carboxytransferase. Biotin is used in some procedures for the nonradioactive labeling of DNA. See avidin; streptavidin.

Blunt-Ended Ligation The ligase-mediated joining of two double-stranded nucleic acid molecules that have blunt ends. Nucleic acid molecules with blunt ends do not have single-stranded cohesive or sticky ends.

Buffer A solution used to maintain the pH of a system; made from a conjugate acid and base.

CAAT Box Part of a DNA sequence that is involved in signaling eukaryotic RNA polymerase II where to start transcribing a gene. It is a conserved sequence found approximately 75 bp before the start of a eukaryotic transcription unit.

Carboxyl Group -COOH.

Catalysis Modifying the rate of a chemical reaction by the addition of a substance that itself does not undergo a permanent chemical change.

Cathode The electrode where reduction occurs. In gel electrophoresis, the cathode is the negative terminal of the power supply.

cDNA DNA complementary to RNA, usually mRNA; can be made by reverse transcriptase using RNA as a template.

Chain-Termination Mutation A mutation whose base pair sequence codes for a stop codon in the messenger RNA. A polypeptide chain made from such an mRNA will be prematurely terminated at the new stop codon.

Chaperones Proteins that facilitate the folding and assembly of other proteins.

Chelating Agent A compound that binds metal ions to form a chelate, in which the metal ion—the central atom—is attached to neighboring atoms by at least two coordinate bonds. EDTA is an example of a chelating agent.

Chemiluminogenic Substrate A substrate that when acted upon by an enzyme will give off light.

Chromogenic Substrate A substrate that when acted upon by an enzyme will produce a colored, frequently insoluble product.

cis-Acting A genetic element that influences the activity of DNA sequences contained on the same DNA molecule as the element. See trans-acting.

Cistron A genetic unit of function; equivalent to a gene.

Clone A population of cells descended from one cell and hence genetically identical. To clone DNA means to combine a piece of DNA into a plasmid, virus, or phage cloning vector in order to obtain many identical copies of that DNA.

Cloning Vector A plasmid or a phage into which foreign DNA can be inserted to amplify the number of copies of the foreign DNA.

Codon A triplet of nucleotides that encodes an amino acid or translation termination signal.

Cofactor A nonprotein structure required for the activity of an enzyme.

Cohesive End The 12-bp-long complementary single-stranded region (*cos* site) at each end of the linear bacteriophage λ genome that is in the phage head. When λ DNA is introduced into the *Escherichia coli* host cell, the two complementary sequences hydrogen bond to circularize the λ genome. Also, any single-stranded region at the end of a double-stranded nucleic acid. See sticky end.

Colony Hybridization The binding of bacteria to a membrane and hybridization of a nucleic acid probe to identify bacteria containing that sequence.

Compatibility Group Group of plasmids that are not able to coexist in the same bacterial cell.

Competence The ability of a cell to take up exogenous DNA.

Complementary Sequence The sequence of bases of one strand of a double helix of DNA that exactly defines the sequence of bases of other strand of the helix because A base pairs with T (U in RNA); G base pairs with C. The sequences of the two strands are complementary.

Concatemer of DNA A series of unit length genomes repeated in tandem.

Concatenated Circles of DNA Circular DNA molecules that are interlocked like rings on a chain.

Conjugation The joining of two bacterial cells during which DNA is transferred from the donor to the recipient cell.

Cosmid A cloning vector that contains a plasmid origin of replication and the *cos* site (cohesive ends) of bacteriophage λ. Large pieces of DNA can be cloned into a cosmid and packaged *in vitro* into λ phage particles, using the *cos* site. Once introduced into the host *E. coli* via the λ phage infection process, the cosmid vector replicates via its plasmid origin of replication.

Covalently Closed Circle A double-stranded circular DNA molecule that has two unbroken (circular) complementary single strands.

C Value The DNA content of the haploid set of chromosomes of an organism.

Dalton A unit of mass; equal to the mass of one hydrogen atom.

Digoxygenin (DIG) A cardenolide steroid isolated from *Digitalis* plants, used in a nonradioactive nucleic acid labeling system. The DIG incorporated into a nucleic acid is detected by an antibody to DIG.

DNA Fingerprint The specific pattern of DNA fragments on a gel generated by digesting the DNA with restriction endonucleases.

Electroelution The use of an electric current to remove a nucleic acid fragment from a gel.

Electrophoresis The movement of charged particles through a solution in an electric field.

Electroporation The use of a high-voltage electric discharge to induce the uptake of DNA into a cell. The electric discharge forms pores in the cell membrane through which molecules can enter or exit the cell. Electroporation can be a very efficient transformation procedure.

Endonuclease An enzyme that cleaves phosphodiester bonds within a nucleic acid molecule.

Enzyme A biological molecule that catalyzes a reaction. The molecule carries out the reaction but is not itself consumed in the reaction. The vast majority of enzymes are proteins. RNA molecules with enzymatic activity have been found; such molecules are called "ribozymes."

***Escherichia coli* DNA Polymerase I (pol I)** The first DNA polymerase of *E. coli* discovered by Arthur Kornberg. Pol I functions in the *E. coli* cell in DNA synthesis to remove the RNA primer. Used in the nick-translation reaction to label DNA probes.

Eubacteria (true bacteria) Prokaryotes including gram-positive bacteria, nonphotosynthetic gram-negative bacteria, and cyanobacteria (blue-green algae). Eubacteria contain murein in their cell walls while archebacteria do not. See archebacteria.

Eukaryote An organism made up of cells with true nuclei.

Exogenous Originating from outside.

Exonuclease An enzyme that cleaves phosphodiester bonds one nucleotide at a time from an end of a nucleic acid molecule.

Femtogram (fg) 10^{-15} g; one quadrillionth of a gram.

5′ end The end of a strand of nucleic acid with a free phosphate group bound to the 5′ carbon of the terminal backbone ribose or deoxyribose.

F Plasmid The fertility plasmid of *E. coli*. The presence of the F plasmid allows conjugation or mating of the donor, F-containing, bacterium with a recipient bacterium lacking the plasmid.

β-Galactosidase Enzyme that hydrolyzes lactose into two sugar molecules, glucose and galactose.

Gene The unit of DNA that codes for a polypeptide chain or an RNA.

Generalized Transduction The transfer (transduction) of DNA from any part of the bacterial host's genome to another bacterium mediated by a phage particle.

Genomic Library A set of cloned DNA fragments that include the entire genome of an organism.

Genotype The set of genes of an organism.

Gram Stain A staining procedure used to classify bacteria as gram-positive or gram-negative based on their retention of a dye such as crystal violet after treatment with a decolorizing agent such as acetone

or alcohol. Gram-positive bacteria retain the crystal violet color. Gram-negative bacteria do not.

GUS β-Glucuronidase, an enzyme often used as a reporter gene.

Helium Gun An apparatus used to introduce DNA into a cell. In a helium gun, helium gas under pressure is used to propel DNA-coated spheres toward the target tissue.

Heteroduplex Mapping Identification of regions where two nucleic acid molecules are not identical. Two related duplex DNAs are denatured, mixed, and allowed to reanneal. Some of the reannealed double-stranded molecules contain one strand from each type of DNA and are called heteroduplexes. When examined by electron microscopy, regions that do not base pair because they are not homologous will be visible as single-stranded loops.

High Frequency of Recombination (HFR) Strain A male *E. coli* bacterial that has the F factor integrated into the bacterial chromosome. When the F factor begins conjugation with an F^- (female) *E. coli* cell, bacterial genes on one side of the site of the F factor integration are transferred to the F^- cell at high frequency.

Hybridization The formation of a double-stranded nucleic acid molecule from single-stranded nucleic acid molecules that have complementary base sequences.

Hydrogen Bonding A weak chemical bond involving a hydrogen atom; important in formation of base pairing of double-stranded nucleic acid.

Intracistronic Complementation Ability of two mutations in the same cistron (gene) to complement each other and restore function. α-Complementation of the *lacZ* gene is an example.

In Vitro From Latin, meaning "in glass"; done in a test tube, not in a living organism.

In Vivo Occurring within a living organism.

Ionic Strength Defined as $1/2 \Sigma c_i z_i^2$, where Σ is the sum of the products of the concentration c_i and the square of the charge z_i of each ion in a solution.

IS Element Insertion sequence element. The simplest prokaryotic transposable genetic element. The IS element consists of terminal repeated DNA sequences and genes required for the movement and insertion of the element into different locations in the host genome.

Kilobase Pairs (kb) 1000 nucleotide (base) pairs of nucleic acid.

Klenow Fragment The large fragment of *E. coli* DNA polymerase I; consists of a single polypeptide chain of molecular weight 76,000 produced by the cleavage of intact *E. coli* DNA polymerase I by the protease subtilisin.

Klett A type of colorimeter that uses colored filters and light scattering to measure the density of a cell culture in "Klett units."

β-Lactamase Enzyme produced by the ampicillin resistance gene that inactivates penicillin and other closely related compounds such as ampicillin by cleaving the β-lactam ring of these compounds.

lacZ Gene in the *lac* operon of *E. coli* that is involved in lactose utilization. *lacZ* codes for the enzyme β-galactosidase, which cleaves lactose. The *lacZ* gene can be used as a reporter gene and as a marker in the pUC cloning vectors.

Lag Phase The initial period of slow growth immediately after inoculating a culture of microorganisms into growth medium.

Latent Image The image formed on a light-sensitive material that is not visible until the material is developed.

L broth or LB (for Luria and Bertani)—A rich medium for culturing bacteria.

Library A collection of cloned fragments in a cloning vector.

Ligase An enzyme that utilizes ATP to catalyze the formation of bonds. In molecular cloning, T4 DNA ligase is used to join recombinant DNA molecules.

Linkers Synthetic oligonucleotides that are used to convert one restriction endonuclease site to another.

Log Phase The phase of the growth cycle of a microorganism in which growth increases exponentially.

Lysogen From Greek meaning lysis inducing; a bacterium that has a prophage integrated into its DNA. At some later point, the prophage may be excised from the bacterial genome and begin the lytic cycle.

Lysogeny A state where a phage is maintained in a bacterium as a prophage integrated into the bacterial genome.

Lytic Phage A phage that lyses the bacteria it infects.

Mainband DNA The major broad peak of an organism's DNA separated on a density gradient; distinct from satellite DNA.

Microfuge A small desktop centrifuge (microcentrifuge) that is used to centrifuge small (e.g., 1.5 ml) tubes (microfuge tubes).

mRNA Messenger RNAs; the RNA molecules transcribed from genes (DNA) that encode the sequences of amino acids in polypeptide chains.

Mutation A change in the sequence of an organism's DNA.

Natural Transformation The ability of an organism to take up exogenous DNA as part of the organism's normal growth cycle; see artificial transformation.

NBT–BCIP [Nitroblue tetrazolium (NBT); 5-bromo-4-chloro-3-indoyl phosphate (BCIP)]—A chromogenic substrate for alkaline phosphatase; part of a nonradioactive DNA labeling system.

Nick A discontinuity in one strand of a double-stranded DNA produced by the breakage of a phosphodiester bond.

Nick Translation A method of labeling DNA using *E. coli* DNA polymerase I, DNase I, and double-stranded DNA. DNA polymerase I acts on nicks generated by the DNase I, removing nucleotides on one side of the nick, while filling in nucleotides on the other. If labeled nucleotide triphosphates are present, they will be incorporated into the DNA by DNA polymerase I. There is a net movement—or translation—of the nick from one point to another on the double-stranded DNA.

Northern Blot The transfer of RNA that has been separated on a gel from the gel to a membrane (the Northern blot). The membrane is then hybridized with a labeled nucleic acid probe to detect specific RNAs that are homologous to the probe.

Nucleoside A purine or pyrimidine base attached to a pentose or a deoxypentose sugar.

Nucleotide A nucleoside with a phosphate group; a phosphoric ester of a nucleoside.

Oligo Labeling A method of labeling DNA that uses single-stranded DNA to be labeled, random, short oligonucleotides (6–14 nt) as primers, and Klenow fragments as the DNA polymerase. Also called random primer labeling. See Klenow fragment.

Oligonucleotide A short chain of nucleotides; polynucleotide refers to a long chain of nucleotides.

Open Circle Conformation of a circular double-stranded nucleic acid molecule in which one strand of the double helix has at least one nick.

Operator A region of DNA at the beginning of an operon where a repressor protein can bind to inhibit transcription of the operon.

Operon A set of contiguous structural genes that are transcribed into a single mRNA and the adjacent regulatory regions that control expression of the genes.

Particle Gun An apparatus used to introduce DNA into an organism. In particle bombardment, small beads are coated with DNA. The beads are placed on a macroprojectile that is propelled toward the target tissue by the force of gun powder. See helium gun.

pBR322 A plasmid cloning vector of 4363 bp containing antibiotic resistance genes for ampicillin and tetracycline.

Phage Lysate The progeny phage released by the lysis of phage-infected bacteria.

Phenotype The observable traits of an organism that result from the expression of that organism's genes.

Plasmid An autonomously replicating extrachromosomal molecule.

Polar Mutation A mutation that affects the transcription or translation of downstream genes in an operon.

Poly(A) Addition Site The 3′ end of a mRNA onto which 50 to 250 adenine nucleotides are added during the post-transcriptional modification of the mRNA.

Polycistronic Messenger RNA that codes for more than one polypeptide chain.

Polylinker or Multiple Cloning Site A region of clustered unique restriction endonuclease sites in a cloning vector, such as the pUC vectors.

Polymerase Chain Reaction (PCR) Method of *in vitro* DNA amplification.

PPD [4-Methoxy-4-(3-phosphatephenylspiro [1,2-dioxetane-3,2′-adamantane])]—A chemiluminescent substrate for alkaline phosphatase; part of a nonradioactive DNA labeling system.

Primer The short, preexisting polynucleotide chain that is needed for DNA synthesis to which new nucleotides are added. The primer pairs with one strand of DNA and provides a free 3′-OH end from which DNA polymerase begins synthesis of a DNA chain.

Probe A specific nucleic acid used in hybridization to identify nucleic acid molecules that are complementary to it.

Prokaryote An organism that lacks true nuclei in the cell.

Promoter The DNA sequence of a gene that is recognized by RNA polymerase and other DNA binding proteins and is the start site of transcription to produce messenger RNA (mRNA) complementary to that gene.

Prophage A phage genome integrated into a bacterial genome. A prophage maintained in the viral genome wil be replicated along with the bacterial genome.

Prototroph A microorganism that can grow on a minimal medium. (From Greek *protos* meaning the first.)

pUC13 A cloning vector of about 2.7 kbp containing an ampicillin resistance gene and a series of unique restriction endonuclease sites within the α-donor part of the *lacZ* gene.

Pulsed Field Gel Electrophoresis A method of separating very large DNA molecules in which the electric field is varied (pulsed) between different pairs of electrodes.

Random Primer Labeling *See* Oligo Labeling.

Randomly Amplified Polymorphic DNA (RAPD) DNA polymorphisms found by using arbitrary primers in a PCR amplification; useful as markers for genetic mapping.

Reading Frame The sequence of codons determined by reading nucleotides three at a time from a specific start codon.

Recombinant DNA A DNA molecule formed by the joining of two differ-

ent DNAs. This is generally accomplished *in vitro* by recombinant DNA cloning methods.

Replica Plating A procedure used to transfer the pattern of colonies from a master plate to a different plate. A sterile piece of velvet or filter paper is pressed lightly against the surface of the master plate to pick up a few cells from each colony to inoculate onto a different plate in the exact pattern as on the original plate.

Restriction Endonuclease An enzyme that recognizes a specific nucleotide sequence and causes cleavage of the DNA.

RFLP (Restriction Fragment Length Polymorphism) Variations in the length of a DNA restriction fragment among individuals of a species. Used as a marker in gene mapping.

Ribosomal DNA The DNA coding for the RNAs that are part of ribosomes.

Ribulose Bisphosphate Carboxylase The enzyme found in the chloroplast stroma that catalyzes the fixation of CO_2 into sugars during photosynthesis. Claimed to be the most abundant protein on earth.

SAM (*S*-Adenoxyl-L-methionine) See AdoMet.

Second-Site Suppressor A mutation at one site that partially or completely restores the function lost because of a mutation at a different site. For example, *supE* and *supF* are mutations in genes encoding tRNA molecules that cause an amino acid to be incorporated at the position of a stop codon.

Satellite DNA In an equilibrium density centrifugation of DNA, the DNA that bands at a density distinct from that of the majority of the DNA of the organism under study. The majority of the DNA is called mainband DNA.

Sequenase An engineered form of T7 DNA polymerase that is used in DNA sequencing.

Southern Blot The transfer of DNA that has been separated by gel electrophoresis to a membrane (the Southern blot). The membrane is then hybridized with a labeled DNA or RNA probe to detect specific DNAs homologous to the probe.

Southwestern Blot The transfer of proteins that have been separated on a gel to a membrane. The membrane is subsequently probed with an oligonucleotide used to examine protein–DNA binding.

Specialized Transduction A process by which a phage particle transduces (transfers) DNA only from a specific part of the bacterial host's genome to another bacterium. Specialized transduction occurs when the excision of a prophage is not precise; host chromosomal DNA on either side of the prophage integration site may be transduced by the phage.

Speed-Vac A microfuge to which a vacuum can be attached; useful to dry or to concentrate samples.

Stationary Phase The period of microbial growth when the cell number remains constant because no further growth is possible.

Sticky End A short (typically, 2–12 bp) single-stranded region at the end of a piece of double-stranded DNA. The single-stranded region can hydrogen bond with its complementary sequence on another piece of single-stranded DNA. See cohesive end.

Sticky-Ended Ligation Two DNA molecules or two ends of the same DNA molecule come together because complementary single-stranded sequences at the end of the double-stranded nucleic acid molecules hydrogen-bonded. T4 DNA ligase then joins the molecules by forming a phosphodiester bond at the nicks.

Stop Codons The codons UAA, UAG, UGA, which do not specify an amino acid and therefore end translation of the polypeptide chain.

Streptavidin An extracellular protein from *Streptomyces avidinii* that is very similar to avidin. Streptavidin has a molecular weight of 60,000 and tightly binds biotin. Streptavidin has fewer nonspecific background binding problems than does avidin. Streptavidin is used as a means of detecting biotin and is used in a type of nonradioactive labeling of nucleic acids. See biotin; avidin.

Stringency As originally defined by Britten and Davidson, stringency is the difference between the temperature of melting, T_m, of a DNA duplex and the temperature of incubation, T_i. High stringency is a T_i close to the T_m. Stringency is more commonly used to refer to the degree of mismatch that is allowed to remain in a nucleotide duplex. A low-stringency wash allows a great deal of mismatch; a high-stringency wash allows only those sequences that are perfectly or nearly perfectly base paired to remain as duplex molecules.

Supercoiled The conformation of a covalently closed circular double-stranded DNA such that the molecule coils back around itself.

Suppressor Gene A gene that encodes a product that overcomes the effects of a mutation in another gene.

***Taq* Polymerase** A thermostable DNA polymerase isolated from the thermophilic bacterium *Thermus aquaticus*. *Taq* polymerase or other thermostable DNA polymerases are used to amplify DNA *in vitro* by the polymerase chain reaction.

TATA Box A DNA sequence that is involved in signaling eukaryotic RNA polymerase II where to start transcribing a gene. It is a conserved AT-rich sequence found about 25 bp before the start of the eukaryotic transcription unit. (Also called a Hogness box.)

T-DNA Transferred DNA, that part of the Ti (tumor-inducing) plasmid of *Agrobacterium tumefaciens* that is transferred from the bacterial plasmid and integrates into the nuclear DNA of the infected host plant.

Temperate Phage A phage that is capable of either integrating into the bacterial genome to form a prophage or producing phage progeny and lysing the host bacterial cell.

3′ end The end of a strand of nucleic acid with a free hydroxyl group bound to the 3′ carbon of the terminal backbone ribose or deoxyribose.

Ti Plasmid Tumor-inducing plasmid from *Agrobacterium tumefaciens*, the causative agent of the plant disease "crown gall." See T-DNA

T_m Temperature of melting; the midpoint in a heat denaturation curve of double-stranded nucleic acid. The temperature at which half of the base pairs of a nucleic acid molecule are still base paired and half are not base paired. The T_m depends on the base composition of the nucleic acid.

trans-Acting Able to act on any piece of DNA in the cell; implies a diffusable product is produced.

Transduction The transfer of bacterial DNA from one bacterium to another via a phage particle.

Transfection In eukaryotic cells, this term means the uptake and incorporation of exogenous DNA.

Transformation In genetics and molecular biology, this term means the uptake and incorporation of exogenous DNA. In animal cell culture, this term also means the change in a cell line such that the cells now show unrestrained growth in culture.

Transgenic Containing foreign or exogenous DNA.

Transposase An enzyme involved in the insertion of a transposon into a new genomic site.

Transposon A nucleic acid sequence that is able to insert itself into a new location in a genome, resulting from the action of the enzyme transposase.

Ultracentrifuge A centrifuge capable of attaining very high speeds, often in excess of 50,000 rpm (280,000 times gravity). In ultracentrifuges a rotor is operated in an evacuated chamber to reduce heating by air friction.

Unblot Term referring to use of a dried gel to be hybridized to a labeled probe instead of transferring the nucleic acid from a gel to a membrane before hybridizing with a probe (see Southern, Northern, Western blots).

Vent Polymerase A thermostable DNA polymerase isolated from the archaebacterium *Thermococcus litoralis*. Useful in PCR. This DNA polymerase makes fewer errors than does *Taq* polymerase.

Virulent Phage A phage that always progresses through the lytic cycle when it infects a bacterium.

Western Blot The transfer of proteins, separated by gel electrophoresis,

onto a membrane for the subsequent detection of specific proteins by an antibody.

Wild Type The genotype or phenotype of an organism as it is found in nature or in the standard laboratory stock of the organism.

X-Gal 5-Bromo-4-chloro-indolyl-β-D-galactoside, a chromogenic substrate for β-galactosidase (the *lacZ* gene product); when cleaved, a blue precipitate is produced.

X-Gluc 5-Bromo-4-chloror-3-indolyl-β-D-glucuronide, a chromogenic substrate for β-glucuronidase (GUS).

Yeast Artificial Chromosome (YAC) A cloning vector useful for cloning very large DNA fragments.

Z-Form of DNA A conformation of double-stranded DNA that is a left-handed double helix with 12 bp per turn of the helix. This form was so named because of the zigzag structure of this helix. See A- and B-forms of DNA.

Zoo Blot A Southern blot used to test the ability of a DNA probe from one species to hybridize with the DNA from the genomes of a number of other species.

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