CHAPTER 5

DNA Sequencing

Introduction

The goal of this experiment is to sequence human DNA using the same procedures employed in large sequencing projects. The process of DNA sequencing consists of three basic tasks. The first task is the generation of the individual fragments to be sequenced. The second involves running sequencing reactions and the third electrophoresis and compilation of the data. For this exercise you will use DNA isolated from your cheek cells. DNA sequencing will be carried out using the technique of random or shotgun sequencing. This requires preparation of a sequencing library. This technique was the main technique used in the human genome project, which encompasses sequencing approximately 30 million short DNA fragments (International Human Genome Sequencing Consortium, 2001; Vender et al., 2001). You will sequence only three such DNA fragments prepared from your genomic DNA. However, you will perform all steps for preparing a sequencing library and sequence these fragments. This technique was utilized in sequencing the entire human genome.

Sequencing DNA using the shotgun or random fragment sequencing method involves the following steps.

1. Fragmentation of DNA into random short fragments using a nebulization procedure. Experiment 1 describes this procedure.

2. Repairing the ends of the sheared DNA fragments in order to create blunt-ended DNA fragments. Experiment 2 describes this technique.

3. Blunt-end ligation of repaired fragments into sequencing vector (pUC 18 plasmid). The conditions of ligation are selected to permit ligation of only a single fragment into one vector molecule. The ligation procedure is the subject of experiment 3.

4. Transforming bacteria with chimeric plasmids using electroporation. This procedure will be described in experiment 4.

5. Isolation of the plasmid from a single bacterial colony and sequencing insert DNA using the cycle sequencing reaction. Samples are prepared

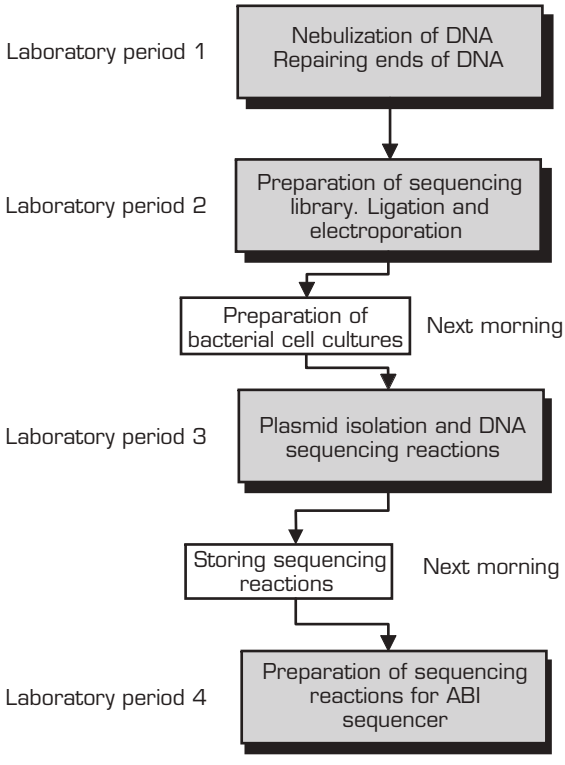


Figure 5.1 Schematic outline of the procedures used in a DNA sequencing laboratory.

for sequencing and sequenced in an ABI Sequencer. These procedures will be carried out in experiments 5–7.

The entire experiment will be done during four laboratory periods. Figure 5.1 presents the overall timetable for these experiments.

Background

Determination of a DNA sequence is the only method in biological science that generates data that are not biased by previous assumptions, hypotheses, or experimental designs. It is therefore not surprising that DNA sequencing has revealed many unexpected facts concerning gene structure, the regulation of gene expression, and organization of genomes, as well as discovering new genes never seen before. Advances in large-scale sequencing have also brought about new scientific disciplines, for example genomics and functional genomics, that are devoted to analyzing whole genomes and their function (Hieter and Boguski, 1997).

All of the present methods of DNA sequencing are based on the anchored-end principle. In these methods, one end of the sequenced DNA molecule remains unchanged (anchored) while the other is generated in a base-dependent way. This creates sets of DNA molecules of various lengths having one end common and the other end terminating at a specific base. Separation of these molecules according to length generates the base sequence of the fragment. The separation is usually accomplished by electrophoresis using a matrix capable of distinguishing between two DNA molecules differing only by a single nucleotide.

There are two methods that generate DNA molecules of different lengths in a base-specific manner. The first method, which was introduced by Maxam and Gilbert (1977), uses base-specific chemical cleavage of the DNA fragment. The second method uses the enzymatic synthesis of DNA fragments (Sanger et al., 1977).

In the Maxam and Gilbert (1977) method of chemical degradation, one end of the DNA is labeled and four separately run base modification reactions are performed. One of the bases (A, C, G, or T) is modified in each reaction. The DNA backbone is then cleaved at each modified residue, resulting in a "nested set" of fragments all labeled at one end and terminating at the location of a specific modified base. Fragments are then separated using gel electrophoresis. This method of DNA sequencing is rarely used at the present time.

The enzymatic method of DNA sequencing utilizes properties of DNA polymerase for implementing the anchored-end principle. DNA polymerase can synthesize a complementary strand from a single-stranded template. Initiation of this synthesis is dependent on the presence of a primer with a 3′-hydroxyl group. In addition, procaryotic DNA polymerases are able to incorporate a dideoxynucleotide instead of deoxynucleotide into the growing DNA chain. Thus, all synthesized DNA molecules will share an identical 5′-end and the 5′-end of the primer and the 3′-end will terminate at specific bases by incorporation of the substrate lacking a 3′-hydroxyl residue.

Four primer extension reactions are initiated using the same primer. Each reaction contains all four usual 2′-deoxynucleotides (dNTPs), but only one of the four 2′,3′-dideoxynucleotides (ddNTPs). By carefully controlling the ratio between dNTP and ddNTP in each of the four reactions, incorporation of the dideoxy nucleotide and, hence, chain termination is random. The end result is the generation of a set of DNA fragments of different lengths, each terminated at the 3′-end at a specific base. These fragments are then separated using electrophoresis.

The length of a DNA molecule that can be sequenced using the anchored-end principle depends not on the ability to create different

sized "anchored" fragments, but on the resolving power of the matrix used for separating these fragments. The resolving power of matrices presently used permits separation of DNA fragments up to approximately 1,000 bases, setting the limit on the length of DNA fragment that can be sequenced in a single reaction.

Sequencing strategies

Sequence determination of a DNA fragment smaller than 1,000 bases is relatively simple. It requires cloning the fragment into an appropriate single- or double-stranded DNA sequencing vector, sequencing the fragment in a single sequencing reaction, and running electrophoresis to separate these fragments. In order to sequence a large DNA molecule it is necessary to subdivide this fragment into smaller fragments of approximately 1,000 bases long. Each small fragment is sequenced separately in a single sequencing reaction and the sequence of the whole fragment is assembled. The way in which smaller fragments are generated from the large DNA fragment and then assembled into the sequence of the whole fragment is referred to as the **sequencing strategy**.

There are two groups of sequencing strategy: directed strategies, in which specific starting points are used for sequencing and random strategies, in which the starting points for sequencing are random (Hunkapiller et al., 1991). Directed strategies permit the direct sequencing of a large region by generating small fragments, the position of which in the whole molecule is known. Usually sequencing proceeds successively from one end of the large fragment to the other. Random strategies (shotgun strategies) involve sequencing of fragments generated by random shearing of a large piece of DNA or random insertions of a universal primer site into target DNA using a transposon.

Primer walking or primer-directed strategy is the most frequently used directed strategy and can be used for sequencing DNA fragments of 10,000 bases or longer. The entire fragment is cloned into a sequencing vector and the initial sequence data are obtained using a vector-based universal primer. The sequences obtained are used for synthesizing a new primer that hybridizes near the 3′-end of the newly elucidated sequence. This primer is used to sequence the next DNA fragment. The cycle of sequencing and primer synthesis is repeated until the whole fragment is sequenced. The technique is uniquely suited to the dideoxy DNA sequencing method and bypasses the need for subcloning smaller pieces of DNA.

In the random sequencing strategy randomly generated fragments are subcloned into a sequencing vector, forming a **sequencing library** of the fragment. Next, the fragments are randomly chosen for sequencing from this library. Identifying overlaps between small fragments and arranging them into the most probable order assembles the sequence of the original

piece of DNA. The number of clones necessary for assembling the whole fragment (**subclone coverage**) and the amount of raw data (**sequence coverage**) are directly proportional to the size of the target DNA. The relationship between these parameters (Deininger, 1983) is given by

$$S = 1 - \left(1 - \frac{i}{L}\right)^n \tag{5.1}$$

where S is the fraction of the fragment sequenced, i is the average number of bases read per clone, L is the length of the whole fragment (bp), and n is the number of clones of length i sequenced. Since the average length of sequence read per subclone is usually approximately 400 bases, to assemble 95 percent of the sequence requires $n = 3L/400$ subclones to be sequenced giving a sequence redundancy of 3.0. For example, for a 40,000 bp fragment sequencing 300 clones of 400 bases gives 95 percent of the whole sequence ($0.95 = 1 - (1 - 400/40{,}000)^n$). For both strand coverage of the same fragment the number of clones should be doubled so n will be equal to $n = 6L/400$ giving a redundancy of 6.0 and sequencing of 99.7 percent of the whole fragment.

Random strategy using the chain termination method of DNA sequencing is a method of choice for sequencing large DNA fragments or whole genomes. Directed strategies, such as primer walking and deletion strategy, are used for sequencing shorter DNA fragments or for filling gaps between contigs.

References

Deininger, P.L. (1983) Random subcloning of sonicated DNA: application to shotgun DNA sequence analysis. *Anal. Biochem.*, **129**, 216–23.

Hieter, P. and Boguski, M. (1997) Functional genomics: it's all how you read it. *Science*, **278**, 601–2.

Hunkapiller, T., Kaiser, R.J., Koop, B.F., and Hood, L. (1991) Large-scale and automated DNA sequence determination. *Science*, **254**, 59–67.

International Human Genome Sequencing Consortium (2001) Initial sequencing and analysis of the human genome. *Nature*, **409**, 860–921.

Maxam, A. and Gilbert, W. (1977) A new method for sequencing DNA. *Proc. Natl Acad. Sci. USA*, **74**, 560–4.

Sanger, F., Nicklen, S., and Coulson, A. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl Acad. Sci. USA*, **74**, 4298–5467.

Vender, J.C. et al. (2001) The sequence of human genome. *Science*, **291**, 1305–51.

FIRST LABORATORY PERIOD

In this laboratory period you will begin to prepare a sequencing library of your DNA. To sequence DNA using the shotgun or random fragment sequencing method, DNA should be first sheared into random short fragments. The fragmentation of DNA will be accomplished using a nebulization process. Next, you will "repair" the ends of the sheared DNA fragments in order to create blunt-ended fragments that can be cloned into sequencing plasmids.

Experiment 1: nebulization shearing of DNA

Introduction

To prepare a sequencing library, DNA must be fragmented into small fragments. In this experiment you will prepare DNA fragments using a novel DNA shearing method. You will shear your DNA that you prepared in a previous laboratory exercise.

A truly useful method for producing random subclones should have the following properties. First, it should produce truly random DNA fragments, i.e. shearing should be sequence independent. Second, it should be reproducible at any time and with any DNA. In order to achieve this, shearing should be reached in a steady-state manner; i.e. shearing to a particular size should not be dependent on the time of application of the shearing agent. Third, the method should allow the generation of DNA fragments in a size range of 500–2,000 bp. Fourth, the method should be efficient and the majority of the DNA treated should be converted into the desired size fragments. The method that fulfills all of these requirements uses dynamic shearing of DNA molecules in the process of nebulization (Surzycki, 1990).

Background

Application of a random strategy for sequencing large pieces of DNA requires the preparation of a sequencing library that contains DNA fragments of approximately 1,000–2,000 bp long that are randomly generated from the original DNA.

There are four methods used for generating random DNA fragments. The first method employs partial restriction enzyme digestion. The second involves fragmentation of DNA by DNase I in the presence of Mn^{2+}, the third relies on sonication to physically break the DNA, and the fourth uses a nebulization process for shearing DNA. However, there are a number of major disadvantages in the use of the first three methods.

The major drawback of the first method, the use of restriction enzymes, is

the lack of randomness in the clone bank because the distribution of restriction sites along the DNA is not random. This necessitates using a number of different restriction enzymes for the preparation of sequencing banks, which is a laborious and time-consuming process. This method also requires performing a number of carefully controlled restriction enzyme reactions that are difficult to reproduce with different enzymes and DNA preparations.

The use of DNase surmounts some of the difficulties in the first method because there is very little DNA sequence specificity in DNase cleavage. However, even to a larger extent than in the first method, the application of DNase in generating random fragments is difficult to reproduce and requires numerous test reactions. This is wasteful and necessitates having large amounts of starting material.

The advantage of the third method is that it is easier to reproduce and control than either of the enzymatic methods. However, its application requires large amounts of starting material because only a small portion of sheared DNA molecules are the required size. This method also involves laborious calibration of the sonicator and rigorous timing for subsequent treatments. Moreover, it has been shown that sonication shears AT-rich sequences preferentially and, thus, does not create truly random sequencing libraries. This is particularly evident if the DNA to be sheared is composed of long AT- and GC-rich stretches.

The nebulization method avoids all of these difficulties and is now used for preparation of random sequencing libraries (Surzycki, 1990). The method works on the following principle. In the process of nebulization or reducing liquid to a fine spray, small liquid droplets of uniform size are produced. In the process of droplet formation the liquid being nebulized flows from the liquid surface to the forming bubble. This creates a transient flow between the liquid surface and the droplet through a connecting small capillary. The diameter of this capillary channel is approximately half the diameter of the forming droplet and can be adjusted as desired by controlling the size of the droplets. The velocity of flow of the liquid in the capillary is not constant across the capillary due to frictional resistance between adjacent layers of flowing liquid. The velocity gradient generated causes liquid in the center to flow faster than liquid in the outer layers creating flow that is called **laminar flow**. Because the velocity of flow is not constant across the capillary tube, the DNA molecule that finds itself in two adjacent flow layers will have its ends moving at two different velocities. This results in stretching and rotating of the molecule until it is positioned in a single laminar layer or broken at the point where the stretching force is maximal.

For large, ridged, linear molecules, such as DNA, the stretching force is the greatest at the middle of the molecule. Consequently, the molecule will have the greatest probability of breaking in half. The nebulization of DNA will result in the breakage of each molecule almost exactly in half in the

repeated process of bubble formation. This will continue until the molecule reaches a small enough size that it cannot be positioned across two laminar flow layers. The final size of the broken molecules will depend only on the size of the droplet formed, i.e. the size of the capillary nebulization channel, but **not on the time of nebulization**.

The nebulization process permits the regulation of the size of the DNA fragments generated. The formation of droplets can be considered at two levels of nebulization: the primary nebulization process that results in the formation of primary droplets and the secondary nebulization process resulting in the formation of secondary droplets by the shattering of the primary droplets on the surface of the nebulization sphere. Droplets are formed at both sides by laminar flow of the liquid, generating force that is formally described by the equation of liquid capillary flow. Accordingly, this force is directly proportional to the gas pressure applied and the viscosity of the liquid and inversely proportional to the size of the droplets. Consequently, the smaller the droplets and the higher viscosity and greater gas pressure applied, the larger the force that creates smaller DNA fragments.

The droplet diameter that is necessary for shearing DNA molecules is in the order of 0.1–2.0μ. This droplet size is created only at the site of secondary nebulization. According to the equation describing the droplet size formed during the secondary nebulization process the size of the droplet depends on the velocity of the primary droplets, the absolute viscosity of the gas used, and the diameter of the droplets generated in the primary nebulization process.

In order to decrease the diameter of primary droplets, DNA is nebulized in a solution of 25 percent glycerol. Moreover, nitrogen or argon is used for achieving droplets with the correct diameter in secondary nebulization. Because the absolute viscosity of these gases is high and the viscosity of nitrogen is very close to the absolute viscosity of argon these gases can be used interchangeably. Use of air, that is a mixture of gases of different absolute viscosity, generates a different size droplet that consequently leads to a broad distribution of fragment sizes.

Safety precautions

Ethidium bromide is a mutagen and suspected carcinogen. Contact with the skin should be avoided. Wear gloves when handling ethidium bromide solution and gels containing ethidium bromide.

For safety purposes, the electrophoresis apparatus should always be placed on the laboratory bench with the positive electrode (red) facing away from the investigator, that is away from the edge of the bench. To avoid electric shock always disconnect the red (positive) lead first.

Ultraviolet (UV) light can damage the retina of the eye and cause severe

sunburn. Always use safety glasses and a protective face shield to view the gel. Work in gloves and wear a long-sleeved shirt or laboratory coat when operating UV illuminators.

Technical tips

Between 1 and 5 µg of DNA should be nebulized in order to create a complete sequencing library. The purity and size of DNA is not critical for successful nebulization. A very good library can be prepared using DNA as small as 30–50 kbp. The nebulization volume used here is 1 ml. The breathing hole of the inhalator should be closed in order to limit DNA lost with escaping mist. A cut end of a 15 ml plastic centrifuge tube can be used (Surzycki, 1990). The opening can also be covered with a QS-T cap (Roe and Crabtree, 1995; Hengen, 1997).

Nitrogen or argon gas should be used for nebulization in order to obtain a library of uniform fragment size. Substituting any other gas or compressed air is possible, but will result in a broader size distribution of fragments. If a gas pressure regulator is used, the pressure should be set to 30 psi. This pressure should generate a DNA fragment of approximately 1,000 bp. If larger fragments are desired lower pressure should be used. For example, to obtain a DNA fragment of 3,000 bp nebulization should be carried out at 10 psi (Surzycki, 2000).

The time of nebulization when using a lower gas pressure should not be changed. This is because the time of nebulization depends only on the volume of nebulized liquid.

The minimum amount of liquid that can be used in an inhalator type nebulizer is 1 ml. Size fractionation of nebulized DNA by gel electrophoresis is not necessary. More than 85 percent of DNA fragments are of the desired size because the nebulization process cannot generate DNA fragments smaller than that set by the droplet size. Larger fragments can be generated by incomplete nebulization, but even when they are present their cloning efficiency is low and these fragments are not present in the sequencing library.

Incomplete nebulization can result from a defective nebulizer, a volume that exceeds 1 ml of nebulized DNA, or low gas pressure. It is important to realize **that circular and supercoiled DNA** are not sheared by nebulization.

Running gel electrophoresis of nebulized samples is optional. Moreover, only one or two gels need to be prepared for an entire class since each student will have a single nebulized sample of her or his DNA. Even if the sheared DNA is not visible, as shown in Fig. 5.2, students should continue processing their sample in order to prepare their sequencing library. A sequencing library can be prepared successfully using a very small amount of sheared DNA that cannot be visualized on ethidium bromide-stained gels.

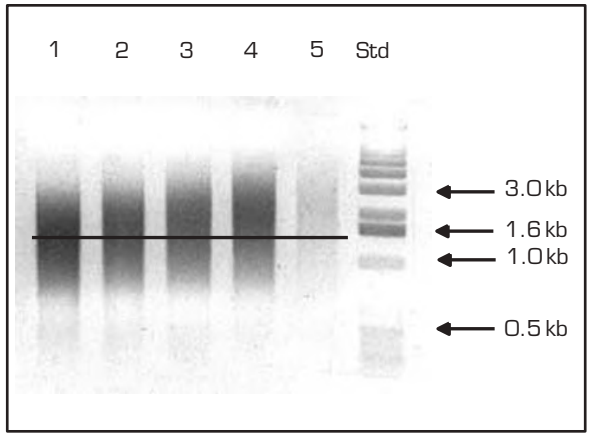


Figure 5.2 Agarose gel electrophoresis of human genomic DNA nebulized for various times using nitrogen at 30 psi. Lane 1 is nebulization for 90 seconds, lane 2 is nebulization for 75 seconds, lane 3 is nebulization for 55 seconds, lane 4 is nebulization for 35 seconds, and lane 5 is nebulization for 15 seconds. The STD lane is a molecular weight standard ladder of 1 kb. The line indicates the position of 1.5 kb DNA.

Protocol

1. Label two sterile 1.5 ml microfuge tubes SP and 0 and place them on ice. Add 150 µl of TE buffer to the SP tube. Next you will add 2–10 µg of your DNA to this tube. To do so you need first to calculate how many microliters of your DNA you should add. **Calculation example:** if the concentration of your DNA is 0.5 µg µl^{-1}, in order to obtain 2 µg of DNA you need to take 4 µl of your DNA (2/0.5 = 4.0 µl).

2. Add the calculated amount of DNA to the SP tube and mix by pipetting up and down.

3. You will be given a tube with 0.85 ml of TE buffer (pH 7.5) containing 25 percent glycerol. This is the solution for nebulization. Add the DNA prepared in the SP tube to it.

4. Place 20 µl of the DNA mixture into tube 0. This will constitute the control, non-nebulized DNA.

5. Transfer the remaining DNA solution into the bottom of the nebulization apparatus using a P1000 Pipetman. Close the nebulizer top and insert the "closing valve" into the breathing hole of the inhalator.

6. Attach the instrument to the nitrogen gas tank pressure regulator using the plastic tubing provided. **Note:** the nebulizer can be attached to a laboratory-compressed air line. To obtain the desired air pressure, adjust the laboratory air outlet to approximately one-third open.

7. Set the gas pressure regulator to 30 psi (2.0 atm). Open the gas pressure regulator valve and nebulize in 15 second intervals for a total time of

75 seconds. You will see a little mist escaping under the "closing valve." This indicates that the nebulizer is operating properly. After each 15 second interval tap the nebulization apparatus slightly on the laboratory bench in order to collect all of the droplets at the bottom of the reservoir. **Note:** if you are using laboratory-compressed air stop nebulization by removing **the hose from the air line and restart it by reconnecting it**. Do not close the air valve. This will assure constant air pressure during the entire nebulization process.

8. Transfer liquid from the nebulizer to a 1.5 ml microfuge tube. Place 15 µl of the nebulized DNA into a separate tube and add 2 µl of stop solution. Mix well by pipetting up and down. Prepare a mini-gel using a casting tray no larger than 7.5 cm × 7.5 cm and a thin gel (0.2 cm). Seal the ends of the gel-casting tray with tape. Regular labeling tape or electrical insulation tape can be used. Use a mini-gel well-casting comb with wells of 0.2–0.5 cm long and 1 mm (or less) wide. Check that the bottom of the comb is approximately 0.5 mm above the gel bottom. To adjust this height it is most convenient to place a plastic charge card (for example MasterCard) at the bottom of the tray and adjust the comb height to a position where it is easy to remove the card from under the comb. **Note:** a single gel can accommodate 12 samples including a standard. Thus, the samples of an entire class can be analyzed on one or two agarose gels.

9. Prepare 500 ml of one times TAE (Tris–acetate EDTA) buffer by adding 10 ml of a 50 times TAE stock solution to a final volume of 500 ml of deionized water.

10. Prepare 1 percent gel. Place 20 ml of the buffer into a 150 ml flask and add 200 mg of agarose. Melt the agarose by heating the solution in a microwave oven at full power for approximately 2 minutes. Carefully swirl the agarose solution in order to ensure that the agarose is dissolved, i.e. no agarose particles are visible. If evaporation occurs during melting adjust the volume to 20 ml with deionized water.

11. Cool the agarose solution to approximately 60°C and add 1 µl of ethidium bromide stock solution. Slowly pour the agarose into the gel-casting tray. Remove any air bubbles by trapping them in a 10 ml pipette.

12. Position the comb approximately 1.5 cm from the edge of the gel. Let the agarose solidify for approximately 20–30 minutes. After the agarose has solidified, remove the comb with a gentle back and forth motion, taking care not to tear the gel.

13. Remove the tape from the ends of the gel-casting tray and place the tray on the central supporting platform of the gel box. For safety purposes, the electrophoresis apparatus should be always placed on the laboratory bench with the positive electrode (red) facing away from the investigator, that is away from the edge of the bench.

14. Add electrophoresis buffer to the buffer chamber until it reaches a level of 0.5–1 cm above the surface of the gel.

15. Load 8 µl of 1 kb standards into the first well. Load samples prepared in step 8 onto the remaining wells and run the gel at 80–90 V for 30 minutes.
16. Photograph the gel. Use a setting of 1 second at F8 with Polaroid 667 film. One can also use a computer-imaging system for recording the results.

Expected results

Figure 5.2 presents gel electrophoresis of DNA nebulized for various times using nitrogen at 30 psi. Increasing the time of nebulization to longer than 75 seconds does not generate smaller fragments (wells 2 and 3). This is because the time of nebulization depends on the volume of the liquid to be nebulized. The size of the DNA fragments generated depends only on the gas pressure applied and, consequently, on the droplet size.

Experiment 2: repair of the ends of sheared DNA

Introduction

DNA molecules that have been sheared by the process of nebulization have three types of ends. Approximately 12 percent of the molecules have blunt ends at both ends of the molecules. The remainder of the DNA molecules contain various lengths of unevenly sheared ends with single-stranded DNA chains protruding for several nucleotides at the 5′- or 3′-strands. These ends cannot be ligated into blunt-ended, open vector molecules. To achieve high efficiency in library preparations, these protruding ends should be "repaired." To repair these ends, you will use T4 DNA polymerase. T4 DNA polymerase is capable of filling in recessed 3′-ends of DNA molecules in the presence of substrate by synthesizing short pieces of DNA. In addition to repair activity, T4 DNA polymerase possesses 3′-exonuclease activity that will remove protruding, single-stranded 3′-ends from sheared DNA, creating in the process a blunt-ended DNA fragment. DNA polymerase repaired ends are devoid of the 5′-phosphates that are needed to clone the fragment into a dephosphorylated vector. We will use T4 polynucleotide kinase to phosphorylate the 5′-ends lacking phosphate. The 5′-phosphates are necessary for ligase activity, in order to ligate the fragments into the sequencing vector.

Figure 5.3 presents a schematic representation of conversion of 5′- or 3′-recessed DNA fragment ends in the process of end repairing.

Safety precautions

PCI reagent is an equal mixture of phenol and chloroform:isoamyl alcohol (CIA). The reagent can be rapidly absorbed by and is highly corrosive to the

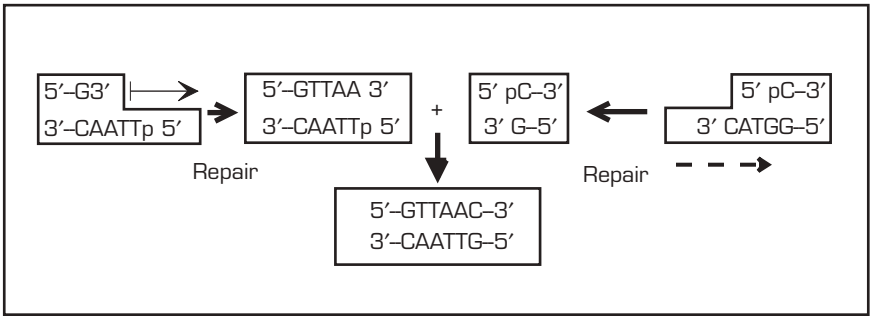


Figure 5.3 Schematic representation of a repair reaction and blunt-end ligation. The recessed 3′- and 5′-ends are converted to blunt ends by the action of T4 DNA polymerase.

skin. It initially produces a white softened area, followed by severe burns. Because of the local anesthetic properties of phenol, skin burns may not be felt until there has been serious damage. Gloves should be worn when working with this reagent. Because some brands of gloves are soluble or permeable to phenol, they should be tested before use. If PCI is spilled on the skin, flush off immediately with a large amount of water and treat with a 70 percent solution of PEG 4000 in water. Phase Lock Gel™ (PLG) tubes containing PCI should be collected in a tightly closed glass receptacle and stored in a chemical hood until proper disposal.

Technical tips

It is important to use T4 DNA polymerase rather than Klenow fragment enzyme in this experiment. The T4 DNA polymerase should be used instead of Klenow enzyme because of the following.

1. It has a much higher exonuclease activity than that of Klenow enzyme. This assures rapid removal of protruding 3′-ends.

2. It is active in many different buffers and the end repair reaction could be done simultaneously with phosphorylation of 5′-ends by polynucleotide kinase using the kinase-specific buffer.

3. The enzyme is inexpensive and much more stable than Klenow enzyme.

It is important to know the DNA concentration in your repair reaction because this is the concentration of blunt-ended DNA fragments. It is necessary to know this concentration in order to calculate the correct insert to plasmid ratio in the ligation reaction. The method described for removing enzyme from the repair reaction will recover nearly 90 percent of the DNA. It is not possible to measure the concentration of DNA fragments using a standard spectroscopic method of absorption at 260 nm. This is because the spectroscopic method requires a DNA concentration of at least 5 $\mu g\,ml^{-1}$ ($A_{260} = 0.1$) and a minimum sample volume of 100 µl. DNA

Table 5.1 DNA end-repair reaction

Ingredients	Add to reaction	Final concentration
Ten times polynucleotide kinase buffer	5.0 µl	one times
Ten times dNTP solution	5.0 µl	200.0 µM
Nebulized DNA fragments (2–10 ng µl^{-1})	30.0 µl	0.06–0.3 µg
ATP 10 mM	5.0 µl	1.0 mM
T4 polynucleotide kinase (10 u µl^{-1})	1.0 µl	10.0 units
T4 DNA polymerase (3 u µl^{-1})	1.0 µl	3.0 units
Water	To 50.0 µl	

fragments prepared for cloning can rarely be obtained at this concentration and volume.

The most important component of the repair reaction is ATP upon which the phosphorylation of 5′-ends of DNA and, consequently, successful ligation critically depends. The stock solution should be freshly prepared and neutralized.

It is critical to remove polynucleotide kinase after the repair reaction because even a small amount of this enzyme present during ligation will phosphorylate 5′-ends of dephosphorylated plasmid. This will result in religation of plasmid molecules and, consequently, very low frequency of plasmids with inserts.

To remove polynucleotide kinase, it is best to use phenol–CIA extraction and PLG microfuge tubes. The PLG tubes contain a proprietary compound that, when centrifuged, migrates to form a tight barrier between organic and aqueous phases. The interphase material is trapped in and below this barrier allowing the complete and easy collection of the entire aqueous phase without contaminating it with organic solvents. The PLG barrier also offers increased protection from exposure to organic solvents.

Alternatively, polynucleotide kinase can be heat inactivated at 65°C for 20 minutes. Inactivation of T4 polymerase is not necessary since its presence will not interfere with the ligation reaction.

Protocol

1. Place a 1.5 ml microfuge tube on ice and label it RE. You will be given two additional tubes, one containing ten times dNTP mixture and another with ten times polynucleotide kinase buffer. The DNA that will be repaired is your nebulized DNA.

2. The total reaction volume is 50 µl. Assemble the reaction mixture in the RE tube using the ingredients indicated in Table 5.1.

3. First add the required amount of water. Next add buffer, ten times dNTP solution, and DNA. Mix by pipetting up and down.

4. Start the reaction by the addition of both enzymes. Mix by pipetting up and down. Avoid creating air bubbles in the process. Centrifuge for 5–10 seconds in order to collect the liquid at the bottom of the tube.

5. Incubate at room temperature for 25 minutes.

6. Stop reactions by the addition of 1 µl 0.5 M EDTA and 50 µl of TE buffer. Mix well by pipetting up and down.

7. Add 100 µl of PCI solution and mix by inverting the tube several times to form an emulsion.

8. Centrifuge the empty PLG tube in a microfuge for 30 seconds at 10,000 r.p.m. in order to pellet the gel. Orient the tube in the centrifuge rotor with the lid connector pointing away from the center of rotation. Measure the time of centrifugation from the moment of **starting the microfuge**.

9. Transfer 100 µl of the repair reaction mixture prepared in step 7 into the PLG tube.

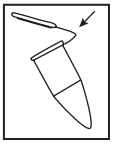
10. Centrifuge the PLG tube at 10,000 r.p.m. for exactly 30 seconds from starting the centrifuge. **Be sure to orient the tube in the centrifuge rotor with the lid connector pointing away from the center of rotation.** After centrifugation, the organic phase at the bottom of the tube will be separated from the aqueous phase at the top of the tube by the PLG barrier.

11. Remove the tube from the centrifuge and add 100 µl of CIA solution to the aqueous phase. Mix by repeated inversion to form an emulsion. Do not vortex or allow the bottom organic phase to mix with the upper aqueous phase.

12. Centrifuge the PLG tube at 10,000 r.p.m. for exactly 30 seconds from starting the centrifuge. **Be sure to orient the tube in the centrifuge rotor with the lid connector pointing away from the center of rotation.**

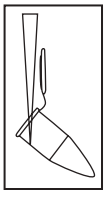
13. Transfer the aqueous phase, collected from the above the gel, to a fresh 1.5 ml microfuge tube. It should have a volume similar to the original volume of the aqueous phase (100 µl).

14. Add 50 µl of 7.5 M ammonium acetate to the tube. Precipitate nucleic acids by the addition of 320 µl of 95 percent ethanol. Mix well by inverting the tube six to ten times.



15. Place the tube in the centrifuge and orient the attached end of the lid pointing away from the center of rotation (see the icon for details). Centrifuge at maximum speed for 10 minutes at room temperature.

16. Remove the tube from the centrifuge and open the lid. Holding it by the lid, gently lift the end, touching the lip to the edge of an Erlenmeyer flask and drain the ethanol. You do not need to remove all the ethanol from the tube. **Place the tubes back into the centrifuge orienting them as before. Note:** when pouring off ethanol, do not invert the tube more than once because this can loosen the pellet.



17. Wash the pellet with 700 µl of cold 70 percent ethanol. Holding a P1000 Pipetman vertically, slowly deliver the ethanol to the side of the tube opposite the pellet. Hold the Pipetman as shown in the margin icon. **Do not start the centrifuge:** in this step the centrifuge rotor is used as a "tube holder" that keeps the tubes at an angle that is convenient for ethanol washing. Remove the tube from the centrifuge by holding it by the lid. Pour off the ethanol as described in step 16. **Note:** this procedure makes it possible to quickly wash a large number of pellets without centrifugation and vortexing. Place the tube back into the centrifuge and repeat the 70 percent ethanol wash one more time.

18. After the last wash, place the tube into the centrifuge. Make sure that the orientation is the same as before. Without closing the tube lids, start the centrifuge for 2–3 seconds to collect the remaining ethanol at the bottom of the tube. Remove all ethanol with a P200 Pipetman outfitted with a capillary tip.

19. Resuspend the DNA pellet (invisible) in 4 µl of water. This will be successful only if you know the position of the pellet on the side of the tube. It is important to realize that, for most microfuges, the pellet will be distributed on the side of the tube. To dissolve DNA, place 4 µl of water on the side wall in the middle of the tube and move the drop down to the bottom using the end of a yellow tip. Repeat this procedure several times in order to ensure that the invisible pellet at the side of the tube is dissolved. You will use all 4 µl for ligation. The final concentration of DNA solution will be 12 ng µl^{-1} (60 ng 5 µl^{-1}) to 60 ng µl^{-1} (300 ng 5 µl^{-1}) (see Table 5.1).

20. Label the tube "R- DNA" (repaired) and with your group number. Store it in a –20°C freezer. You will use this DNA for the ligation reaction in the next laboratory period.

References

Deininger, P.L. (1983) Random subcloning of sonicated DNA: application to shotgun DNA sequence analysis. *Anal. Biochem*., **129**, 216–23.

Hengen, P.N. (1997) Method and reagents. Shearing DNA for genomic library construction. *Trends Biochem. Sci*., **22**, 273–4.

Roe, B. and Crabtree, J.S. (1995) Protocols for recombinant DNA isolation, cloning and sequencing. In *DNA Isolation and Sequencing. Essential Techniques Series*, B.A. Roe, J.S. Crabtree and A.S. Khan (eds). John Wiley & Sons, New York.

Surzycki, S.J. (1990) A fast method to prepare random fragment sequencing libraries using a new procedure of DNA shearing by nebulization and electroporation. In *The International Conference on the Status and Future of Research on the Human Genome*. *Human Genome II*, p. 51. Human Genome Project, San Diego.

Surzycki, S. (2000) *Basic Techniques in Molecular Biology*. Springer-Verlag, Berlin, Heidelberg, and New York.

SECOND LABORATORY PERIOD

In this laboratory period you will carry out two of the next steps of the procedure for preparation of a sequencing library. These steps represent the procedure of DNA cloning. In the first step, you will ligate DNA fragments into the sequencing plasmid (cloning vector). In the second step, you will introduce the constructed plasmids into bacterial cells using an electroporation procedure (transformation of bacteria). A collection of bacterial cells, each carrying a single plasmid with only one DNA fragment, constitutes the sequencing library of your DNA.

Experiment 3: ligation to sequencing vector

Introduction

In vitro ligation is used for joining foreign DNA fragments to a linearized plasmid. Construction of highly representative, random fragment libraries of DNA is essential to successful sequencing using the shotgun strategy of DNA sequencing. In order to prepare these libraries it is necessary to clone repaired DNA fragments into pUC vectors, which are used as sequencing vehicles. In order to ensure that each fragment is represented in the library, the blunt-ended fragments are ligated to the *Sma*I cloning site of the pUC vectors. The *Sma*I restriction endonuclease cuts both DNA strands in the same position (CCC|GGG), thereby creating a DNA molecule with two blunt ends with a CCC sequence at the one end and a GGG sequence at the other.

Prepared vector DNA will be given to you. This vector will be restricted with *Sma*I restriction endonuclease. To prevent recirculization of the vector molecules and to select for intermolecular ligation, the vector DNA is dephosphorylated after digestion with *Sma*I restriction enzyme. Because ligase requires the presence of a 5′-phosphate group at the ends of the DNA molecules to be ligated, the dephosphorylated vector molecule ends cannot be ligated with themselves. However, repair molecules of the DNA fragment contain 5′-phosphate groups can be ligated to the vector.

Background

Principle of DNA cloning

DNA cloning can be defined in most general terms as a method of rapid isolation and amplification of DNA fragments that can be used in subsequent experiments. Cloning involves construction of hybrid DNA molecules that are able to self-replicate in a host cell, usually bacteria. This is accomplished

by inserting DNA fragments into a plasmid- or bacteriophage-cloning vector, introducing the vector into bacterial cells, and amplifying vector DNA using bacterial DNA replication machinery. The DNA fragment or insert can be derived from any organism and obtained from genomic DNA, cDNA, previously cloned DNA, PCR (polymerase chain reaction) products, or synthesized *in vitro*.

Typically cloning of any DNA fragment involves the following tasks.

1. Preparing the vector for cloning.
2. Preparing DNA fragments to be cloned.
3. Joining the fragments with the vector.
4. Introducing the hybrid vector into bacteria.
5. Selecting for cells with a vector.

To accomplish these tasks, the plasmid and DNA fragment are engineered to be linear molecules with termini compatible to be joined by ligation. Then the fragment and vector are ligated together to form a circular recombinant molecule. Ligated constructs are introduced into *Escherichia coli* cells by transformation. Finally, transformed *E. coli* cells are selected from cells without a vector.

Different procedures for carrying out each of these steps have been devised. In general, these procedures can be separated into two groups: the procedures for cloning many different fragments at once, e.g. procedures for the construction of DNA libraries and the procedures for cloning one or few DNA fragments at a time. In most instances preparation of a DNA library and in particular preparation of a sequencing library requires blunt-ended cloning of DNA fragments into plasmid.

Cloning vectors

The replication machinery of bacterial cells can be used for cloning and amplifying specific DNA fragments. Most DNA fragments are incapable of self-replication in bacterial cells. However, any DNA fragment can be easily amplified and replicated when it is part of an autonomously replicating bacterial element. Most cloning vectors can be categorized according to the purpose and type of extra-chromosomal element used (Brown, 1991). The vectors used in most cloning experiments are general-purpose plasmid vectors that were made from naturally occurring procaryotic plasmids, primarily *E. coli* plasmids. These plasmid vectors have the following characteristics.

1. Cloning vectors are small, circular, double-stranded DNA molecules. The vector DNA contributes as little as possible to the overall size of recombinant molecules. This assures that a cloned fragment constitutes a large percentage of amplified and isolated plasmid DNA, making it easier to prepare large quantities of insert DNA.
2. Cloning vectors contain a **replicon**, that is a stretch of DNA that permits DNA replication of the plasmid independent of replication of the host

chromosome. This element contains the site at which DNA replication begins or the origin of replication and genes encoding RNAs and/or proteins that are necessary for plasmid replication. The replicon largely determines the copy number of the plasmid, which is defined as the number of plasmid molecules maintained per bacterial cell. High copy number plasmids are plasmids that accumulate 20 or more copies per bacteria and low copy number plasmids are plasmids that have less than 20 copies per cell (Brent and Irwin, 1989). High copy number plasmids are the most frequently used plasmids in cloning. Low copy number plasmids are useful for cloning DNA sequences deleterious to host cells or DNA sequences that are prone to rearrangement by a host, such as inverted or direct repeats. Most recombinant plasmids contain either the ColE1 or closely related pMB1 replicon (Bernard and Helinski, 1980; Brown, 1991). Examples of ColE1 replicon-containing plasmids are pBluescript or pT series plasmids and plasmids containing the pMB1 replicon are pBR322, pGEM, and pUC series plasmids. An anti-sense RNA transcript and ROP protein regulate the copy number for ColE1 and pMB1 replicons. Mutations in either of these elements can result in a higher plasmid copy number. This feature was used in the construction of very high copy number, general-purpose plasmids such as pUC and pGEM series plasmids that lack the ROP gene.

3. Cloning vectors contain selectable markers for distinguishing cells transformed with the vector from non-tranformed cells. This marker also maintains the presence of the plasmid in cells, particularly in the case of low copy number plasmids. The selectable marker is usually a gene conferring resistance to antibiotics on the host cells. This **positive selection marker** in most plasmids is the β-lactamase gene, the product of which cleaves and inactivates penicillin or its more frequently used derivative ampicillin. When transformed cells are grown in the presence of antibiotic. Cells carrying the antibiotic-resistant plasmid survive, while host cells that do not contain the plasmid are eliminated.

4. Cloning vectors contain unique cloning sites for the introduction of DNA fragments. The cloning sites in most general-purpose vectors used today consist of a **multiple cloning site** or a polylinker cloning region where a number of restriction enzyme cleavage sites are immediately adjacent to each other. These sites are chosen to be unique in the vector sequences. DNA fragments can be easily introduced into the plasmid by linearizing the plasmid by digestion with one or two enzymes present only in the polylinker region and ligating the desired fragment into it. This procedure creates a chimeric molecule without disrupting the critical features of the vector. Polylinker sites are usually flanked by sequences that can be used for priming DNA synthesis with commonly available primers for DNA sequencing or a PCR. The general purpose vectors such as pUC, pGEM, or pBluescript contain M13 reverse, M13 forward –20 and –40 primers that can be used for amplification or sequencing any inserted DNA fragment.

5. Cloning vectors contain an element for screening for the recombinant clones. The screening procedure, as opposed to selection procedures, only permits recognition of colonies transformed with vectors containing an insert from those transformed with vector alone (Rodriguez and Tait, 1983). The universally used screening method is the α-complementation screening procedure for insertional inactivation of β-galactosidase enzyme activity. The α-complementing vectors have a multiple cloning site region inserted into a DNA sequence encoding the first 146 amino acids of the *lacZ* (β-galactosidase) gene α fragment. An intact α fragment, with a few additional amino acids, can complement the ω fragment of the *lacZ* gene in bacteria restoring a fully active enzyme. The β-galactosidase enzyme metabolizes a chromogenic substrate (X-gal) and causes the formation of blue colonies on indicator plates. Inserting a DNA fragment into the polylinker region disrupts the protein-coding region of the α-fragment protein (insertional inactivation) that cannot complement into an active enzyme. Thus, cells carrying plasmid with insert have a gal^- phenotype. This allows for rapid identification of bacteria containing plasmids with inserts as white colonies on indicator plates.

Preparation of plasmid for cloning

The first step in plasmid preparation is choosing the vector for cloning. The choice of vector largely depends on the experiments for which the recombinant clone will be used. For example, different types of vectors will be used for generating large quantities of DNA, expressing a fusion protein in bacteria, synthesis of mRNA, or preparation of RNA or DNA probes. Vectors used for the construction of sequencing libraries are high copy number, general-purpose vectors. A list of most of the commonly used cloning vectors is available at http://vectordb.atcg.com

The second step involves preparation of the plasmid for ligation with the insert. This step usually consists of linearization of the plasmid with an appropriate restriction enzyme and removing 5′-phosphate from the DNA ends. Because supercoiled DNA molecules are transformed at high frequencies, appropriate care must be taken to remove uncut plasmid from the preparation. Linearization of the plasmid is an enzymatic reaction and, as such, it never goes to completion. As a result, a small number of supercoiled molecules are always present and must be removed before the cloning procedure. In addition, removing the 5′-phosphate is necessary in order to limit religation of the plasmid to itself, which will create plasmid molecules lacking insert.

How plasmid and DNA fragments are prepared depends on the choice of ligation procedure to be used for joining them. There are two types of ligation procedure: ligation of fragments with compatible cohesive ends and ligation of fragments with blunt termini. Thus, there are two procedures for

cloning, cohesive-end cloning and blunt-end cloning. In the preparation of sequencing libraries the blunt-end procedure is used.

Blunt-end cloning requires creating blunt termini in both the plasmid and DNA fragment since any two blunt ends are compatible for ligation. Cleaving DNA with any two restriction enzymes that create blunt ends or cleaving DNA with any restriction enzyme and then converting the protruding ends to blunt ends can generate these termini.

The use of **blunt-end cloning** has several advantages. The most obvious advantage is that any DNA fragment can be cloned regardless of its origin. Only one type of plasmid needs to be prepared for cloning all DNA fragments with blunt termini. The ligation reaction is short (10–30 minutes) and is done at room temperature. A disadvantage of the method is the necessity for preparing most of the fragments for cloning by creating blunt termini. Recent improvements in blunt-end cloning technology have resulted in increases in blunt-end cloning efficiency to approximately 10^7–10^9 transformants per microgram of DNA, removing the most important disadvantage of this method.

During ligation, DNA ligase will catalyze the formation of phosphodiester bonds between adjacent nucleotides only if phosphate is present at the 5′-end of DNA molecules. Removing the 5′-phosphates from both ends of the linear plasmid DNA and leaving them on the fragment to be cloned will minimize recircularization of the plasmid. The enzyme used for this purpose is either bacterial alkaline phosphatase or calf intestinal phosphatase. The result of this treatment is that neither strand of the vector can form a phosphodiester bond if insert DNA is not present in the reaction mixture.

Ligation reaction

Ligation or joining of a foreign DNA fragment to a linearized plasmid involves formation of new bonds between the 5′-phosphate and 3′-hydroxyl ends of DNA. There are two enzymes available for catalysis of this reaction *in vitro*: *E. coli* DNA ligase and bacteriophage T4 DNA ligase. For almost all cloning purposes the bacteriophage enzyme is used. Ligation of dephosphorylated plasmid ends with a phosphorylated insert results in the formation of hybrid molecules with two phosphate bonds and two single-stranded gaps. These gaps are repaired after introduction of plasmid into bacterial cells.

The most significant factor in a ligation reaction is the concentration of DNA ends. Essentially, during ligation there are two competing reactions: bimolecular concatamerization and unimolecular cyclization. In order to achieve successful cloning, the first reaction to occur should be bimolecular concatamerization of the vector with insert followed by a recirculization reaction to form circular plasmid. Unfortunately these two reactions are mutually exclusive, so some form of compromise must be used.

The ratio of recirculization and concatameric ligation products is dependent on two factors *i* and *j*, where *i* is the total concentration of DNA termini in the reaction mixture and *j* is the effective concentration of one end of a DNA molecule in the immediate neighborhood of the other end of the same molecule. The value of *j* is constant for a linear DNA molecule of a given length and is **independent of DNA concentration**. For cohesive-end ligation and blunt-ended ligation, the value of *i* in ends per milliliter can be calculated from the equation

$$i = 2N_0 M \times 10^{-3} \text{ ends ml}^{-1} \tag{5.2}$$

where N_0 is the Avogadro number and M the molar concentration of the DNA molecules.

The *j* value can be determined using the equation (Dugaiczyk et al., 1975)

$$j = j_\lambda \left(\frac{\text{mw}_\lambda}{\text{mw}_X} \right)^{3/2} \text{ends ml}^{-1} \tag{5.3}$$

where, j_λ is 3.6×10^{11}, mw_λ is the molecular weight of the λ genome (30.8×10^6 Da), and mw_x is the molecular weight of unknown molecule. After converting *M* to DNA concentration in micrograms per milliliter [DNA] and mw_x to kbp, the ratio *j*:*i* for any given DNA is equal to (rearranged from Rodriguez and Tait, 1983)

$$j/i \approx \frac{51.1}{0.812(\text{kbp}_x)^{0.5}[\text{DNA}]} \tag{5.4}$$

Equation (5.4) indicates that, to achieve a low *j*:*i* ratio, the concentration of DNA in the reaction should be high. For any ligation reaction, three conditions apply.

1. When $i = j$ there is an equal chance of one end of the molecule making contact with the end of a different molecule and/or the end of the same molecule.

2. When $j > i$ there is a greater chance of one end of the molecule contacting the other end of the same molecule than of finding the end of another molecule. Therefore, under this condition the recirculization reaction predominates.

3. When $j < i$ there is a greater chance of one end of the molecule contacting the other DNA molecule rather then its own end. Therefore, under this condition, the concatamerization reaction predominates.

Although the theoretical consideration discussed above predicts that conversion from concatamerization to circulization should occur at $j:i = 1$, experimental observation indicates that this conversion actually occurs at $j:i = 2–3$ (Dugaiczyk et al., 1975).

To choose the correct compromise between two types of ligation one should realize that a ligation reaction is not an instantaneous event. This reaction proceeds through a progressive series of individual ligation events, where each event significantly alters the $j:i$ ratio for the remaining unligated molecules. Since the value of j is constant, as the reaction proceeds the value of i decreases resulting in increases of the $j:i$ ratio. The high $j:i$ ratio favors the formation of circular molecules rather than linear concatamers.

For cloning DNA insert into plasmid, the reaction conditions initially should favor joining the insert to one end of the plasmid vector. The second ligation event, occurring later, should be circularization of the plasmid–insert hybrid molecule to avoid joining the next linear fragment to the first one. To achieve this, the ratio of $j:\Sigma i$ at the beginning of ligation should be low to allow the concatamerization reaction and, as the reaction proceeds, this ratio should rise to a value greater than three to permit circularization of the plasmid. This can be done in three ways.

1. Starting the ligation reaction at a high concentration of vector DNA. As equation (5.4) indicates, a low $j:i$ value depends on the DNA concentration per milliliter. For the vectors commonly used for cloning (2,600–4,000 bp), the concentration of vector DNA is usually adjusted in the range 3–6 $\mu g\,ml^{-1}$ or 1–3 $pmol\,ml^{-1}$.

2. Adjusting the initial molar ratio of plasmid to linear fragments to be greater than 1.0. Since during the reaction the j value is not changed, but the i value will decrease as a result of the ligation of one end of the fragment to the plasmid, the $j:i$ ratio will increase towards the end of the reaction. At a high $j:i$ ratio the circularization reaction predominates, resulting in the formation of circular hybrid molecules. The insert concentration is usually adjusted to a two- to fourfold **molar** excess over plasmid DNA. The $j:i$ ratio for such a reaction can be approximately calculated using equation (5.4) and assuming a kbp average between vector and insert. For example, using pUC19 plasmid in the ligation reaction (2.68 kbp) at concentration 6 $\mu g\,ml^{-1}$ and 2.6 times molar excess of 1 kbp insert (6 $\mu g\,ml^{-1}$), the $j:i$ ratio at the beginning of the reaction is approximately 4.0 $(51.1/(2.68 + 1.0/2\,bp)^{0.5} \times 12\,\mu g\,ml^{-1} \times 0.812 = 3.8)$. This ratio will steadily increase as the reaction proceeds resulting in almost complete circulization of the hybrid plasmid molecules.

3. Using phosphatase-treated vector with a phosphorylated insert. Under this condition the vector cannot self-ligate at any $j:i$ ratio and, therefore, the initial concentration of plasmid is not important. The initial concentration of the insert is chosen so as not to allow its circulization at the beginning of the reaction. Ligation of fragment to vector results in the formation of a new molecule that can be self-ligated into a circle. Cyclization of a hybrid molecule depends on its j value and is independent of insert concentration as long as the molar concentration of the insert is equal to or higher than the molar

concentration of plasmid. For all useful molar ratios of vector to insert (1 : 1 to 1 : 3) the $j:i$ for insert is always high, favoring circularization of the insert rather than concatamerization. Thus, cloning of concatamers of the insert does not occur. Using an insert concentration higher than 1 : 3 is not recommended because it may lead to cloning inserts that have been ligated together. Following formation of the hybrid molecules, conditions favoring cyclization of the hybrid predominate if the insert is not larger than the vector (a high $j:i$ ratio for hybrid). Practically, lower concentrations of plasmid are preferred because this leads to nearly complete formation of circular hybrid molecules. For example, ligation of plasmid at 2.5 $\mu g\,ml^{-1}$ with a 2.68 kbp insert gives a $j:i$ ratio for the construct equal to 5.4, whereas the same ligation at 1 $\mu g\,ml^{-1}$ plasmid gives value $j:i$ equal to 13.6.

The efficiency of a ligation reaction depends not only on DNA and enzyme concentrations, but also on the purity of the vector and insert, the reaction pH and temperature, and the presence of inhibitors of ligase. In general, most deleterious components that frequently contaminate ligation reactions are as follows.

1. Monovalent salts (for example sodium chloride) or ammonium salts. Concentrations of sodium chloride above 50 mM and ammonium salt above 10 mM severely inhibit ligation reactions.

2. Phosphate. Blunt-end ligation is particularly sensitive to this salt and concentrations of phosphate greater than 25 mM should be avoided.

3. ATP concentration. Blunt-end ligation is inhibited by ATP concentrations above 0.1 mM.

Technical tips

Ligation reactions can fail for several reasons (listed in frequency of occurrence).

1. Too high a concentration of vector. This can happen because the concentration of fragments can be lower than expected due to low efficiency of the nebulization process. It is possible to repeat the ligation reaction using a lower amount of vector (2–5 ng per reaction) and the same amount of DNA fragments.

2. The presence of a high concentration of monovalent salts (for example sodium chloride or ammonium salts). The concentrations of these salts in the final reaction should not exceed 50 mM. Careful washing of DNA pellets with 70 percent ethanol should remove all salts from plasmid and insert preparations. Neglecting to do so is a frequent cause of failure in ligation reactions.

3. Too high a concentration of ATP. The blunt-end ligation reaction is inhibited by the presence of an ATP concentration above 0.1 mM. Buffers supplied by manufactures with T4 DNA ligase frequently contain ATP at optimal concentration for cohesive end ligation. The use of these buffers in

Table 5.2 Master reaction mixture

Ingredients	Add to reaction	Final concentration
Two times ligation buffer (vial 1)	50.0µl	One times
Five times DNA buffer (vial 2)	20.0µl	One times
pUC18 DNA (10ng/µl)	10.0µl	20ng (10fmol)

Table 5.3 Ligation reactions

Ingredients	C1	C2	3	4
Master reaction mixture	16.0µl	16.0µl	16.0µl	16.0µl
Your DNA (tube R)	–	–	4.0µl	–
Partner's DNA (tube R)	–	–	–	4.0µl
Water	5.0µl	4.0µl	–	–
T4 DNA ligase (vial 3)	–	1.0µl	1.0µl	1.0µl

blunt-end ligation reactions is a very frequent error, causing poor ligation efficiency. Carefully check the content of the ligation buffer supplied when performing blunt-end ligation.

4. Degradation of ATP in the stock solution or old ligation buffer. Always use freshly prepared 0.5 mM ATP.

Protocol

You will perform two ligation reactions using the repaired DNA from both you and your partner prepared during the first laboratory period. The protocol includes two control reactions. The first control reaction tests for the presence of undigested vector DNA in plasmid preparations. The second control tests the efficiency of dephosphorylation of vector ends.

1. Label one 1.5 ml microfuge tube RX. Also prepare two control reaction tubes labeled C1 and C2 and two ligation reaction tubes labeled 3 and 4. Place the tubes on ice.

2. Prepare the master reaction mixtures for five reactions in the RX tube as shown in Table 5.2.

3. Mix ingredients by pipetting up and down. Centrifuge for 5 seconds in a microfuge. Place tubes on ice. **Note:** it is absolutely necessary to mix the contents of vials 1 and 2 thoroughly directly before use.

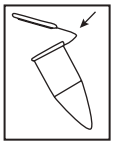
4. Place tubes C1, C2, 3, and 4 at room temperature and prepare reactions as indicated in Table 5.3. Add water, the master reaction mixture, and DNA first. Mix the ingredients gently by pipetting up and down several times. Centrifuge for 5–10 seconds to collect liquid at the bottom of the tubes.

5. Start the reaction by the addition of enzyme to tubes C2, 3, and 4. Mix

well by gently pipetting up and down several times. Incubate the reactions for 10 minutes at room temperature. **Note:** the expected molar ratio between vector (1.6 kb) and insert (1–2 kb) is approximately 3 : 1 to 15 : 1 assuming all repaired DNA was recovered.

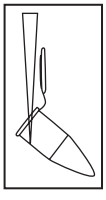
6. Stop the reactions with the addition of 1 µl of 0.5 M EDTA, pH 8.0. Add 80 µl of water to the reaction tubes and mix well by inverting the tubes several times.

7. Add 50 µl of 7.5 M ammonium acetate to each tube and mix well by inverting several times.



8. Add 300 µl of 95 percent ethanol to each tube and mix well by inverting four to five times. Place the tubes in the microfuge and centrifuge for 20 minutes at room temperature. **Be sure to orient each tube in the centrifuge rotor with the lid closing pointing away from the center of rotation.** This will "mark" the position of pelleted DNA since your pellet will not be visible.

9. Remove the tube from the centrifuge and open the lid. Gently lift the end, touching the tube to the edge of an Erlenmeyer flask and drain the ethanol. You do not need to remove all the ethanol from the tube. Place the tubes back into the centrifuge in the same orientation as above. **Note:** when pouring off ethanol do not invert the tube more than once because this can loosen the pellet.



10. Wash the pellet with cold 70 percent ethanol. Add 700 µl of 70 percent ethanol to the tube, using a P1000 Pipetman. Holding the P1000 Pipetman vertically (see the icon in the margin) slowly deliver the ethanol to the side of the tube opposite the pellet. **Do not start the centrifuge.** In this step the centrifuge rotor is used as a "tube holder" that keeps the tube at an angle convenient for ethanol washing. Withdraw the tube from the centrifuge by holding the tube by the lid. Remove ethanol as in step 9. **Note:** this procedure makes it possible to quickly wash a large number of pellets without centrifugation and vortexing. Vortexing and centrifuging the pellet are time-consuming and frequently lead to substantial loss of material.

11. Place the tube back into the centrifuge and repeat the 70 percent ethanol wash one more time.

12. After the last wash, place the tube into the centrifuge, making sure that the tube position in the rotor is the same as in steps 9 and 10. Without closing the tube lids, start the centrifuge for 2–3 seconds and collect the remaining ethanol at the bottom of the tube. Remove all ethanol with a P200 Pipetman outfitted with a capillary tip. **Note:** never **dry the DNA pellet** in a vacuum. This will make dissolving the DNA pellet very difficult if not impossible.

13. Resuspend the DNA pellet (invisible) in 5 µl of water. This will be successful only if you know the position of the pellet on the side of the tube. It is important to realize that, for most microfuges, the pellet will be distributed

on the side of the tube. To dissolve DNA, place 5 µl of the water on the side wall in the middle of the tube and move the drop down to the bottom using the end of a yellow tip. Repeat this procedure several times to assure that the invisible pellet at the side of the tube is dissolved.

References

Bernard, H.U. and Helinski, D.R. (1980) Bacterial plasmid cloning vectors. In *Genetic Engineering. Principles and Methods*, Vol. 2, J.K. Setlow and A. Hollaender (eds), pp. 133–67. Plenum Press, New York and London.

Brown, T.A. (1991) Cloning vectors. In *Molecular Biology Labfax*, B.D. Hames and D. Rickwood (eds), pp. 193–234. Bios Scientific Publishers Ltd and Academic Press Inc., Oxford.

Dugaiczyk, A., Boyer, H.W., and Goodman, H.M. (1975) Ligation of *Eco*RI endonuclease-generated DNA fragments into linear and circular structures. *J. Mol. Biol.*, **96**, 171–84.

Brent, R. and Irwin, N. (1989) Introduction to plasmid biology. In *Current Protocols of Molecular Biology*, F.M. Asubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Sith et al. (eds), pp. 1.5.1–1.5.8. John Wiley & Sons, Inc.

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

Experiment 4: transformation of bacteria by electroporation

Introduction

In this experiment you will prepare a sequencing library by transforming *E. coli* cells with plasmids containing random fragments of your DNA. You will use the entire product of the ligation reactions prepared in experiment 3. Each group will carry out four transformations using two control ligation products and two experimental ligation products. You will use 30 µl of cells for each transformation by electroporation (cell concentration 10^{10} cell ml^{-1}) and an electroporation cuvette with a 0.1 cm electrode gap. Using this method the frequency of transformation is 0.02. Low transformation frequency prevents co-transformation of a cell with two or more plasmid molecules.

The bacterial cells used in this experiment will be given to you ready for transformation. You will plate transformed cells on a selective medium that permits growth of transformed colonies and visual selection of cells with plasmid containing insert (ampicillin and X-gal IPTG). The colonies will be used to prepare plasmid for DNA sequencing.

The electroporation method of transformation has numerous advantages over other transformation methods and is always used for preparation of sequencing libraries. The advantages of this method are as follows.

1. Electroporation yields very high transformation frequencies that come close to the frequencies obtained when using bacteriophage vectors. At saturating DNA concentrations, 80–100 percent of the cells are transformed.
2. The technique makes it possible to transform very small volumes of cells. A volume of cells as small as 20 µl can be transformed yielding approximately 10^9 transformants.
3. Preparation of cells for transformation is very simple and does not use elaborate and time-consuming protocols. Moreover, the cells used for electroporation can be prepared ahead of the time and stored indefinitely without losing competence.
4. Transformation efficiency is practically independent of DNA size and form. Linear DNA molecules, supercoiled DNA, and circular DNA are transformed with equal efficiency.

Background

Electroporation is the most efficient transformation procedure that can be used for bacterial transformation. It involves a brief application of high-voltage electric field to the cells resulting in the formation of transient holes in the cell membrane through which plasmid DNA can enter the cell. The method was originally developed for animal and plant cells (Neumann et al., 1982) and later for bacteria (Böttger, 1988; Dower et al., 1988; Li et al., 1988; Smith et al., 1990). Transformation efficiencies as high as 10^{10} transformants μg^{-1} of plasmid have been achieved for *E. coli* cells (Dower et al., 1988; Smith et al., 1990).

The maximum efficiency of this process depends on many variables. The most important are electrical pulse shape, electrical field strength, and electrical pulse time. The relationship between these parameters is described by the equations (Shigekawa and Dower, 1988)

$$V_t = V_0\left[e^{-(t/\tau)}\right] \tag{5.5}$$

$$E = \frac{V_0}{d} \tag{5.6}$$

$$\tau = R \times C \tag{5.7}$$

where V_t is voltage at time t, V_0 is the initial voltage or peak of voltage, τ is the pulse time constant, R is the resistance of the circuit (ohms), C is the capacitance of the charged capacitor (farads), E is the electric field strength, and d is the distance between the electrodes.

In most instruments used today, charging a capacitor to a predetermined voltage and its subsequent discharge to the electroporation chamber

generates an electric pulse. Capacitor discharge results in an exponentially decaying pulse shape that is characterized by the value of τ. According to equation (5.5), τ can be defined as the time over which the voltage declines to 1/*e* or approximately 37 percent of the initial value (V_0). The electrical field is derived from the peak voltage (V_0) delivered to the chamber at the moment of discharge. At this moment the difference in potential in the chamber is at its maximum, generating membrane depolarization and pore formation. The formation of pores is largely dependent on the field strength parameter determined by equation (5.6). The introduction of external compounds (e.g. plasmids, DNA, proteins, etc.) into the cell critically depends on the voltage drop determined by τ.

The time constant τ is specific for each cell type and, in general, the smaller the cell size, the shorter the τ needed to introduce external elements. The optimal time constant for *E. coli* was determined to be 5 milliseconds. The field strength for optimal electroporation of different bacterial strains differs and must be experimentally determined.

Transformation results are usually described by the values representing the frequency of transformation (f_{tr}) and efficiency of transformation (E_{tr}). Equation (5.8) describes the frequency of transformation and the efficiency of transformation is described by equation (5.9).

$$f_{tr} = \frac{Tr}{S} \tag{5.8}$$

$$E_{tr} = \frac{Tr}{[\text{pDNA}]} \tag{5.9}$$

where *Tr* is the number of transformants, *S* is the number of cells at 80 percent survival, and [pDNA] is the concentration of plasmid DNA used in micrograms. The frequency of transformation is directly dependent on DNA concentration in the range of 10 pg ml^{-1} to 7.5 μg ml^{-1} and, at these DNA concentrations, 80 percent of the cells survive electroporation (Dower et al., 1988). Moreover, because from equation (5.8), $Tr = f_{tr} \times S$, the efficiency of transformation can be rewritten as

$$E_{tr} = \frac{f_{tr} \times S}{[\text{pDNA}]} \tag{5.10}$$

Equation (5.10) indicates that the transformation efficiency (E_{tr}) is high when the cell concentration is high (10^9 to 3×10^{10}) and the DNA concentration is low (1–10 ng rx^{-1}). These conditions are commonly used in electroporation of *E. coli* in order to achieve high transformation efficiencies and avoid co-transformation. For example, for a standard electroporation reaction, 30 μl of cells at a concentration of 10^{10} cells ml^{-1} are used with 5 ng

of plasmid DNA. The transformation efficiency commonly achieved is 10^9 transformants μg^{-1} of DNA, giving a transformation frequency of two transformants per 100 surviving cells ($f_{tr} = E_{tr} \times [pDNA]/S$) ($f_{tr} = 10^9 \times 0.005/2.4\ 10^8 = 0.02$). At this transformation frequency, transformation of a single cell with two or more plasmids is very rare.

In addition to the parameters described above, electroporation is affected by a number of other variables such as temperature, the components of the electroporation medium, and the method of cell recovery after electroporation.

Electroporation at low temperature is approximately 100 times more efficient than when cells are pulsed at room temperature. Low temperature presumably affects the fluidity of the membrane, aiding in pore formation and slowing their closure. Similarly quick restoration of membrane fluidity and closing pores is crucial for cell survival after the pulse. Thus, cells should be transferred to prewarmed growth medium as fast as possible after pulse application. Delaying this transfer by 1 minute will cause a threefold drop in the transformation efficiency and cell survivability. Moreover, cells should be given some time to recover and rebuild their membrane before plating on solid medium. The recovery time should be short enough to prevent cell division that will result in the "cloning" of transformed cells. This is particularly important when electroporation is used for the preparation of genomic and sequencing libraries. A recovery time of 45–50 minutes at 37°C appears to be the best for cell recovery and short enough to not allow any cell division.

Technical tips

In this experiment we will use a special electrocompetent strain of *E. coli*, ElectroMax DH10B. Many other electrocompetent strains can be used, as long as they support α-complementation, for distinguishing bacterial cells containing plasmid with insert from those without it. The bacterial strain should also be ampicillin sensitive.

Low or no transformation results if arcing occurs during pulsing. Arcing can occur for the following reasons.

1. There is residual salt or buffer in the sample due to inadequate washing of ligation reactions with 70 percent ethanol.

2. There is the presence of air bubbles in the sample due to incorrect pipetting into the electrophoresis chamber.

3. There is too high a concentration of cells used in electroporation. This would result in arcing of all samples. Electroporate cells without plasmid are added to test this possibility.

4. There are old cell preparations, incorrectly stored cells, or the cells are thawed too fast. All of these can bring about partial lysis of cells causing arcing.

5. There is too small a volume of cells in the electroporation chamber. A cuvette with a 0.1 cm electrode gap requires at least 25 µl of sample.

A lower than expected frequency of transformation can result from the following.

1. Incorrect setting of the electroporation apparatus with too high or too low a voltage than that recommended for a given bacterial strain. The voltage recommended with ElectroMax DH10B is 1850 V.

2. An incorrect τ constant. This value can be adjusted on some electroporation units. Choose the correct capacitance of the capacitor (in farads) and resistor (in ohms) to give a τ constant close to 5 milliseconds. Use equation (5.7) for calculating this value. For example, if using an Invitrogen Electroporator II choose a 50 µF capacitor and 150 Ω resistor in order to obtain a τ constant of 7.5 ms ($50 \times 10^{-6}\,\text{F} \times 150\,\Omega = 0.0075$ s).

3. A warm electroporation chamber or sample during pulsing. Warming frequently occurs during loading of the samples. It is better to chill the electroporation cuvettes after loading the sample even if this prolongs incubation of the cells with DNA over the recommended time.

4. Excessive volume of the cells in the electroporation chamber. Cell volume should not exceed 40 µl.

Another problem frequently met is the presence of all blue colonies on experimental plates 3 or 4 with concomitant absence of blue colonies on both control plates (C1 and C2). This can happen when the insert is cloned in a frame with the α-peptide or when the insert DNA is small (<200 bp). In the last instance, color development will be weak and the colonies may appear pale blue or white. Colonies with recombinant plasmid can be sometimes distinguished from those with plasmid alone by colony morphology. Recombinant colonies appear translucent while non-recombinant colonies are opaque. The cells from such colonies can be used for plasmid isolation and sequencing.

Small colonies surrounding a single colony (satellite colonies) will frequently appear on ampicillin plates. These colonies appear near ampicillin-resistant colonies because the β-lactamase enzyme, which is responsible for antibiotic resistance, is secreted from the cell, thereby removing antibiotic from the agar in the vicinity. This usually results from two low a concentration of antibiotic in the plate or the plate being incorrectly stored. Ampicillin-containing plates should be stored at 4°C for no longer than three to four weeks. The satellite colonies can be eliminated by short incubation of the plates (less than 14 hours). In this case in order to enhance blue color development without further bacteria growth the plates can be incubated at 4°C for several hours. Because the cells for plasmid isolation will be grown in the medium supplemented with ampicillin, satellite cells will not grow even if accidentally transferred together with transformed cells.

Electroporation cuvettes can be reused three to five times. The efficiency

of transformation progressively decreases with successive use of a cuvette. This is probably due to some cells being "baked" onto the electrode surface or pitting of the electrode surface by washing procedures. Electroporation cuvettes can be washed before reusing using the following procedure.

1. Immediately after use immerse the cuvette in 1 percent Alconox solution. This will prevent drying of bacterial cells onto the electrode surface.

2. Rinse the cuvette six to eight times with distilled or deionized water. Do not keep cuvettes in Alconox solution for more than 1 hour.

3. Rinse the cuvette three times with 70 percent ethanol. Fill the cuvette with ethanol, cap it, and invert it several times. This treatment should sterilize the cuvette.

4. Dry the cuvette by filling it with 95 percent ethanol, inverting it several times, pouring off the ethanol, and drying it upside-down on a paper towel.

"Falcon 2059" polypropylene culture tubes should be used for cell recovery after electroporation. The use of glass tubes will result in poor recovery. It is possible to use other brands of polypropylene tubes, but some batches can occasionally be contaminated with surfactants that inhibit transformation. The manufacturer tests Falcon 2059 tubes for such contamination.

Protocol

Before starting the electroporation procedure, make the following preparations: (i) label four electroporation cuvettes C1, C2, 3, and 4 and cool them on ice for at least 5 minutes; (ii) label four Falcon 2059 tubes with the numbers corresponding to those of the electroporation cuvettes and place these tubes in the tube rack at room temperature; (iii) thaw the ligation reaction (3 and 4) and two control reactions (C1 and C2) prepared previously; (iv) set the electroporator voltage to 1.85 kV; and (v) warm up 10–50 ml of TB medium to 37°C.

1. Remove the bacterial cells from –70°C storage and gently thaw them on ice. Tap the tube gently to mix the cells. **Note:** do not leave cells on ice for an extended time. Use the cells as soon as possible. Cells can be refrozen for later use, but the transformation frequency will be significantly lower.

2. Add 30 µl of cells to the tube labeled C1 (control). Pipette the cells up and down two times to mix the DNA with the cells. Be very careful not to create air bubbles during this procedure.

3. Transfer 30 µl of this mixture to a cuvette labeled C1. Holding the cuvette at a 45° angle, deliver the mixture to the lower end of the electroporation chamber of the cuvette. Deliver liquid slowly and do not operate the Pipetman beyond the first stop. This procedure will prevent the formation of air bubbles in the electroporation chamber. Tap the cuvette on the laboratory bench several times to distribute the liquid on the bottom of the chamber. Close the cuvettes with the cap provided and place it back on

ice for 45 seconds. **Note:** an electroporation chamber with a 0.1 cm gap is very narrow. Frequently the cell sample will stay at the top of the chamber. Vigorous tapping of the cuvette on the laboratory bench will make sample flow to the bottom. It is important to do this as quickly as possible and not warm up the cuvette and cells.

4. Place the cuvette into the electroporation machine and initiate electroporation. **Note:** if a loud "snap" is heard while pulsing, arcing occurred inside the electroporation chamber. Continue with the protocol as usual; some transformation may still have occurred. See the section on technical tips for how to prevent arcing.

5. As quickly as possible add 1 ml of warm TB medium directly into the cuvette using a P1000 Pipetman. Pipette slowly up and down two times. Gently transfer all the cells from the electroporation cuvette into the appropriately labeled Falcon tube. **Treat the cells very gently: they are very fragile after electroporation.** Leave the tube at **room temperature** until all samples have been electroporated. **Note:** do not discard electroporation cuvettes. They can be reused after washing for less critical transformations (for example control transformations). A protocol for washing electroporation cuvettes is given in the section on technical tips.

6. Electroporate samples C2, 3, and 4 following the procedure described in steps 2–5.

7. Transfer all Falcon tubes to an orbital shaker and incubate the cells at 37°C for 45 minutes in order to allow the cells to recover and to express antibiotic resistance. The rotating speed of the shaker should not exceed 240 r.p.m.

8. Plate two plates, one with undiluted cells (1 : 1) and one with diluted cells (1 : 10), respectively. To prepare the 1 : 10 dilution add 900 µl of PBS to four microfuge tubes and label each tube with the sample name and dilution factor (for example 3 1 : 10, 4 1 : 10, C1 1 : 10, etc.). Prepare two plates for each electroporation reaction labeled on the bottom of the plate with the sample name and appropriate dilution (for example C1 1 : 1, C1 1 : 10, 3 1 : 1, etc.). **Note:** for optimal cell survival, use only PBS for dilution. Diluting the cells in growth medium instead of PBS will lower cell viability by approximately 20 percent.

9. Mix the cells in the tube labeled C1 by gently tapping with fingers. Transfer 100 µl to the dilution tube labeled C1 1 : 10. This will constitute 1 : 10 dilution of the original cell culture. Mix well by pipetting up and down several times.

10. Using the same yellow tip, immediately pipette 100 µl of cells from the 1 : 10 dilution onto the corresponding plates.

11. Using the same tip, transfer 100 µl of cells from Falcon tube C1 onto the plate labeled C1 1 : 1. **Note:** make one set of dilutions at a time. Finish plating the bacteria from a single electroporation tube before preparing the next dilution set. This prevents cross-contamination and plating errors.

12. Sterilize the cell spreader by dipping it into a beaker of 95 percent ethanol and briefly pass it through a flame to ignite the alcohol. Burn off the ethanol keeping the spreader **away** from the burner flame.
13. Cool the spreader by touching it to the agar away from the cells. Spread the cells on a plate with a 1 : 10 dilution first by dragging the cell suspension across the agar surface with the spreader back and forth several times. Spread the cells on a plate labeled 1 : 1 next. Return the spreader to the beaker with ethanol.
14. Replace the plate lids and let them stand until all liquid is absorbed into the agar.
15. Dilute and plate the cells from the remaining electroporation tubes using the procedure described in steps 9–13. Plate the cells from the 3 and 4 tubes last.
16. Place the plates upside down in a 37°C incubator and incubate them for 15–18 hours.

Next day

1. Count the cells on plates 3 and 4. Count only white colonies. Calculate the efficiency of transformation, which is expressed as the number of transformants per microgram of plasmid used. Use the following equation for this calculation: E_{tr} = number of transformed colonies/nanograms of plasmid DNA in the ligation rx × dilution factor.
2. Inspect two control plates C1 and C2. They should contain a few blue colonies. The presence of a large number of colonies on both plates indicates a failure to linearize the plasmid during vector preparation and/or incomplete dephosphorylation of its 5′-ends. If this is the case, the plate with the ligated insert and plasmid (3 and 4) will also contain mostly blue colonies.
3. Prepare five bacterial cell cultures for plasmid preparation. Each student should prepare five cultures. You will be given 10 ml glass tubes containing 2 ml of TB-amp medium (100 μg ml^{-1}). Inoculate each tube with a single white colony from plates 3 and 4. Using a sterile toothpick, touch the colony and drop the toothpick into the tube. Grow cells overnight on an orbital shaker and store cells at 4°C until the next laboratory period.

Expected results

Figure 5.4 shows a plate with a 1 : 10 dilution of a human sequencing library prepared using the protocol described. The expected efficiency of transformation is approximately 10^6–10^7 transformants per microgram of plasmid used. This will result in approximately 100–1,000 colonies on a 1 : 10 dilution plate. If the transformation efficiency is very low there should be at least ten to 100 colonies on a plate with a 1 : 1 dilution.

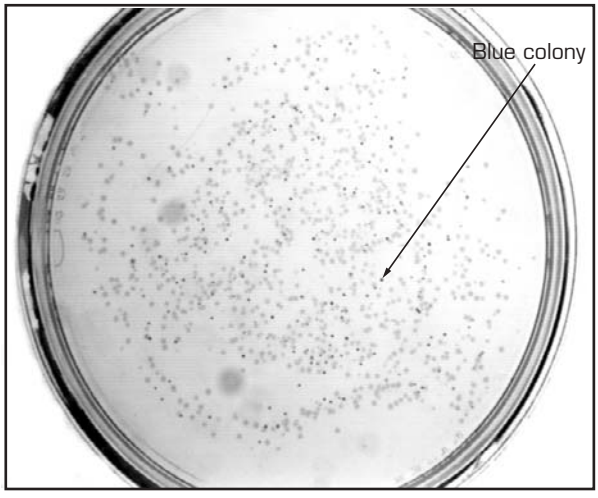


Figure 5.4 Human sequencing library plated at 1 : 10 dilution. After electroporation, cells were grown for 45 minutes in 1 ml of TB medium. Before plating the cells were diluted 1 : 10 in one times PBS and 100 µl were plated onto LB agar medium containing X-gal and IPTG and 100 µg ml^{-1} ampicillin. The plates were incubated overnight at 37°C.

References

Böttger, E.C. (1988) High-efficiency generation of plasmid cDNA libraries using electrotransformation. *BioTechniques*, **6**, 878–80.

Dower, W.J., Miller, J.F., and Ragsdale, C.W. (1988) High efficiency transformation of *E. coli* by high voltage electroporation. *Nucleic Acids Res.*, **16**, 6127–45.

Li, S.J., Landers, T.A., and Smith, M.D. (1988) Electroporation of plasmids into plasmid-containing *Escherichia coli*. *BioTechniques*, **12**, 72–4.

Neumannn, E., Schaefer-Ridder, M., Wang, Y., and Hofschneider, P.H. (1982) Gene transfer into mouse L-cells by electroporation in high electric field. *EMBO J.*, **1**, 841–5.

Shigekawa, K. and Dower, W.J. (1988) Electroporation of eukaryotes and prokaryotes: a general approach to the introduction of macromolecules into cells. *BioTechniques*, **6**, 742–51.

Smith, M., Jesse, J., Landers, T.A., and Jordan, J. (1990) High efficiency bacterial electroporation: 1×10^{10} *E. coli* transformants/µg. *Focus*, **12**, 38–40.

THIRD LABORATORY PERIOD

In this laboratory period you will isolate plasmid using modification of alkaline procedure (Surzycki, 2000) and run sequencing reactions. In the second laboratory period you prepared a sequencing library and grow bacterial cells from a single bacterial colony. This colony started from as a single transformed cell in your sequencing library. Consequently, all of the cells contain an identical plasmid. This plasmid, when purified, can be used for sequencing the cloned insert.

Experiment 5: preparation of plasmid for DNA sequencing

Introduction

In this experiment you will isolate plasmids that will be used for DNA sequencing. The plasmids will be purified from liquid cultures prepared in the second laboratory period. These cells contain plasmids from your genomic DNA sequencing library. The method used will be a modification of the alkaline lysis procedure that is suitable for the fast preparation of plasmid template for sequencing. The modification encompasses neutralization of the alkaline lysate with ammonium acetate rather than potassium or sodium salts and removal of the remaining proteins by precipitation with 1.87 M ammonium acetate. This allows purification of plasmid DNA without the use of toxic organic solvents. The procedure is quick and yields plasmid DNA of a purity comparable to the CsCl-prepared DNA.

Background

Isolation of plasmid DNA from bacterial cells is an essential step for many molecular biology procedures. Many protocols for large- and small-scale isolation of plasmids (mini-preps) have been published. The plasmid purification procedures, unlike the procedures for purification of genomic DNA, should involve removal not only of protein, but also another major impurity: bacterial chromosomal DNA. The task of plasmid purification differs substantially from that of the preparation of genomic DNA. Most plasmid DNA purification methods start from the preparation of a crude bacterial lysate and eventually employ standard protein removal procedures. To achieve separation of plasmid from chromosomal DNA, these methods exploit the structural differences between plasmid and chromosomal DNA. Plasmids are circular supercoiled DNA molecules substantially smaller than bacterial chromosomal DNA.

There are three basic methods of plasmid preparation.

1. Alkaline lysis, which was introduced by Birnboim and Dolly (1979) and Birnboim (1983). In this method cells are lysed and DNA denatured by sodium deodecyl sulfate (SDS) and NaOH. Neutralization of the solution results in a fast reannealing of covalently closed plasmid DNA due to the interconnection of both single-stranded DNA circles. Much more complex bacterial chromosomal DNA cannot reanneal in this short time and forms a large insoluble DNA network largely due to interstrand reassociation at multiple sites along the long linear molecules. In the next step of the procedure, lowering the temperature results in the precipitation of protein–SDS complexes. Subsequently, both complexes, DNA and protein, are removed by centrifugation leaving plasmid molecules in the supernatant. If cleaner plasmid is desired, the remaining protein and RNA are removed by standard methods.
2. Lysis by boiling in the presence of detergent, which was introduced by Holmes and Quigley (1981). In this method high temperature and detergent lyse bacteria cells. Bacterial chromosomal DNA under these conditions remains attached to the bacterial membrane. Subsequent centrifugation pellets chromosomal–DNA complexes while plasmid DNA remains in the supernatant. A recent modification of this procedure involves the lysis of bacterial cells using a microwave oven rather than a boiling water bath (Hultner and Cleaver, 1994; Wang et al., 1995). Further plasmid purification, if desired, can be carried out using standard deproteinization procedures and RNase treatment.
3. Application of affinity matrixes for plasmid or proteins, which was introduced by Vogelstein and Gillespie (1979).

Technical tips

The success of plasmid isolation using alkaline lysis depends on three critical steps.
1. Resuspension of cells in solution II. Solution II should be added to all tubes at once at room temperature. The cells should be resuspended one tube at the time and placed on ice. The time of incubation on ice is not critical and the stipulated time is the minimal time necessary. Cells should be resuspended in solution II thoroughly, resulting in a uniform, very viscous solution.
2. The addition of ammonium acetate solution. This solution should be as fresh as possible in order to assure complete neutralization of the lysate. Incomplete neutralization will result in contamination with bacterial chromosomal DNA. This will make sequencing of the plasmid impossible.
3. Cells should be grown in TB medium overnight, i.e. 17–20 hours. Prolonged growth of the cells on TB medium can result in lysis of the cells. If a medium other than TB is used for growth (e.g. L-broth), the volume of cells used for preparation should be increased to 500–600 µl.

Some plasmid preparations can be contaminated with RNA. This

contamination appears as a diffuse band running together or slightly ahead of the bromophenol blue dye during gel electrophoresis of plasmids. For most sequencing protocols, some contamination with RNA is not deleterious. However, it is better to sequence a plasmid preparation with little or no RNA.

It is important to follow the ethanol washing of a pellet as described in the procedure. Do not use microfuge tubes manufactured by Eppendorf Co. for this procedure. Never **dry the DNA pellet** in a vacuum. This is an unnecessary step that can make hydration of the DNA very difficult if not impossible.

Protocol

1. Transfer 400 µl of cells to a 1.5 ml microfuge tube and collect the cells by centrifugation at **room temperature** for 3 minutes. Discard the supernatant. Recentrifuge the cells for a few seconds and remove the remaining medium using a P200 Pipetman outfitted with a capillary micro-tip.

2. Add 400 µl of freshly prepared **solution II** to the cell pellet. Using the flat end of a toothpick break the pellet up into small pieces by swirling the toothpick. Close the tube and mix by inverting four to six times. Place the tube on ice and incubate for 15 minutes.

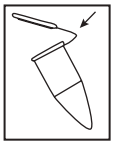
3. Meanwhile prepare the ammonium acetate–RNase solution. You will need 3 ml of this solution for ten plasmid preparations. Add 3.5 ml of 7.5 M ammonium acetate to a plastic 15 ml conical centrifuge tube and place it on ice. Add 45 µl of RNase A stock solution, close the tube, and mix by inverting two to four times. Place the solution on ice.

4. Add 300 µl (0.75 volume) of ice cold ammonium acetate–RNase solution to each microfuge tube with lysed cells and mix by gently inverting the tube five to eight times. Place the tube back on ice and incubate for 10 minutes.

5. Centrifuge the lysate at **room temperature** for 5 minutes.

6. Pour off the supernatant into a fresh 1.5 ml microfuge tube. Be careful not to transfer the gelatinous pellet.

7. Add 420 µl (0.6 × total volume) of isopropanol to the supernatant. Mix well by inverting the tube three to four times. Incubate at room temperature for exactly 10 minutes.

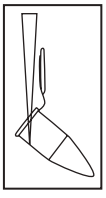


8. Place the tube in the centrifuge, with the attached side of the lid away from the center of rotation. Centrifuge at maximum speed for 10 minutes at room temperature.

9. Remove the tube from the centrifuge and open the lid. Gently invert and touch the lip of the tube to the rim of an Erlenmeyer flask. Drain the isopropanol. You do not need to remove all of the isopropanol from the tube. Place the tubes back into the centrifuge

in the same orientation as before. **Note:** when pouring off alcohol, do not invert the tube more than once because this may dislodge the pellet.

10. Place the tubes back into the centrifuge in the same orientation as before. **Do not start the centrifuge:** in this step the centrifuge rotor is used as a "tube holder" that keeps the tube at an angle convenient for ethanol washing.



11. Wash the pellet with 700 µl of cold 70 percent ethanol. Holding a P1000 Pipetman vertically, slowly deliver ethanol to the side of the tube opposite the pellet, i.e. the side facing the center of the rotor. Hold the Pipetman as shown in the margin icon. Remove the tube from the centrifuge by holding it by the lid. Gently invert the tube and touch the lip to the rim of an Erlenmeyer flask. Hold the tube to drain the ethanol. You do not need to remove all of the ethanol from the tube. Place the tubes back into the centrifuge in the same orientation as before and wash with 70 percent ethanol one more time. **Note:** this procedure makes it possible to quickly wash a large number of pellets without centrifugation and vortexing. Vortexing and centrifuging the pellet are time-consuming and frequently lead to substantial loss of plasmid DNA.

12. Place the tube back into the centrifuge making sure that the side with the pellet is away from the center of rotation. Without closing the tube lid, start the centrifuge for 2–3 seconds in order to collect the remaining ethanol at the bottom of the tube. The centrifuge should not reach maximum speed. A speed of 500–1,000 r.p.m. is sufficient for collecting all the ethanol remaining on the sides of the tube. Remove all of the ethanol using a P200 Pipetman micropipette outfitted with a capillary tip.

13. Add 15 µl of sterile deionized water to the tube and dissolve the pelleted DNA. Gently pipette the liquid up a down, directing the stream of liquid towards the pellet. **Note:** centrifugation in a microfuge with a fixed angle rotor will deposit most of the DNA pellet on the side of the tube rather than on the bottom. To dissolve all plasmid DNA when these centrifuges are used, it is necessary to direct the stream of the dissolving solution **towards the lower two-thirds of the bottom side of the tube**.

ABI sequencing reactions require at least 1–3 µg of pure plasmid DNA. The concentration of plasmid and its quality can easily be established using mini-gel electrophoresis. Alternatively the concentration of plasmid DNA can be measured by absorbance at 260 nm using a spectrophotometer.

Mini-gel electrophoresis

1. Prepare a mini-gel using a casting tray no larger than 7.5 cm × 7.5 cm and a thin gel (0.2 cm). Seal the ends of the gel-casting tray with tape. Regular labeling tape or electrical insulation tape can be used. Use a mini-gel

well-casting comb with wells 0.2–0.5 cm long and 1 mm (or less) wide. Check that the bottom of the comb is approximately 0.5 mm above the gel bottom. To adjust this height it is most convenient to place a plastic charge card (for example MasterCard) at the bottom of the tray and adjust the comb height to a position where it is easy to remove the card from under the comb.

2. Prepare 500 ml of one times TAE by adding 10 ml of a 50 times TAE stock solution to 490 ml of deionized water.

3. Prepare 1 percent agarose gel. Place 15 ml of the buffer into a 100 ml Erlenmeyer flask and add 150 mg of agarose powder. Melt the agarose by heating the solution in a microwave oven at full power for 1–2 minutes until the agarose is fully dissolved. If evaporation occurs during melting, adjust the volume to 15 ml with deionized water.

4. Cool the agarose solution to approximately 60°C and add 1 µl of ethidium bromide stock solution. Slowly pour the agarose into the casting tray. Remove any air bubbles by trapping them in a 10 ml pipette. Place the comb 1 cm away from one end of the gel. Allow the gel to solidify for 20–30 minutes.

5. Spot 5 µl of TE buffer onto the virgin side of a piece of parafilm. Add 1 µl of stop solution (with dye) to it. Prepare as many "spots" as you have samples. Add 2.0 µl of sample to each aliquot and mix the buffer and plasmid DNA pipetting up and down with a Pipetman. Immediately load the suspension onto the gel. Repeat the above procedure for each sample using a fresh tip for each preparation.

6. Electrophorese the samples at 70–90 V for 25–40 minutes.

7. View the gel with a UV light box and photograph it. Plasmid bands should be visible as strong single or double bands. A typical result of gel electrophoresis of plasmid preparations is shown in Fig. 5.5. If the intensity of the bands of your preparation are similar to those in Fig. 5.5, you will need 1–2 µl of your plasmid preparation for a single sequencing reaction.

Experiment 6: sequencing reactions for an ABI 3700 sequencer

Introduction

In this experiment you will use a modification of dideoxy sequencing called "cycle sequencing." Cycle sequencing uses heat-stable DNA polymerase and a thermal cycler for sequencing. This makes it possible to generate sequence information from a relatively small amount of template. In this modification of PCR amplification, only a single primer (sequencing primer) is used rather than the two primers used in normal PCR reactions. Thus, the amplification is linear rather than exponential.

The newly synthesized DNA will be labeled with dideoxy nucleotides (terminators) that are labeled with fluorescent dye. Each terminator is

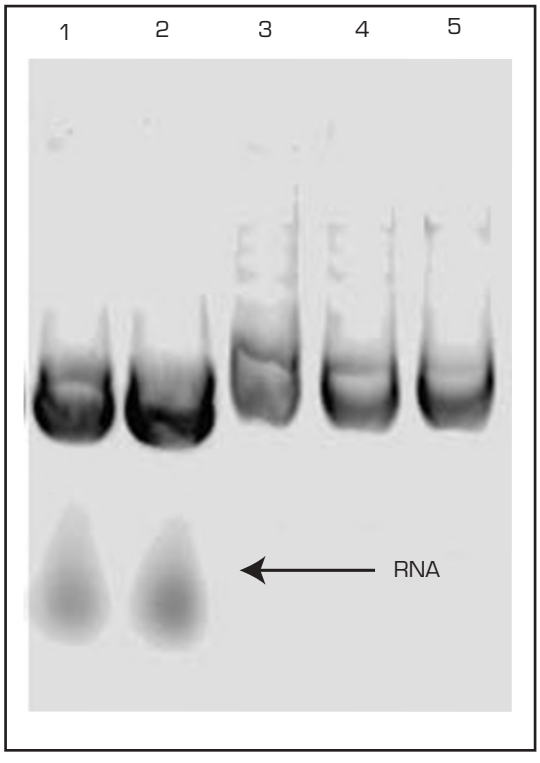


Figure 5.5 Agarose gel electrophoresis of a plasmid preparation. Computer imaging exposure for 1 second. Plasmids were purified from 300 µl of cells grown for 19 hours in TB medium containing 100 µg ml^{-1} ampicillin and 2 µl of plasmid samples were loaded onto a 1 percent agarose gel and run for 25 minutes at 5 V cm^{-1}.

labeled with a different color dye. A color sensor in the automatic sequencer can detect these labels. Sequencing will be carried out using the most modern sequencing machine. It uses a capillary rather than a gel to separate the products of the sequencing reactions. You will sequence three of the best plasmids you prepared in experiment 5.

Technical tips

The thermal cycler for sequencing should be able to accommodate thin-walled 0.2 ml PCR tubes. Since the sequencing reaction volume is very small (10 µl) and use of oil in order to prevent evaporation is not recommended, the thermal cycler used for running sequencing reactions should be equipped with a heated lid. The Idaho Technologies air thermal cycler used in other PCR experiments described in this book is also an excellent instrument for running sequencing reactions. It should be equipped with a thin tube adapter available from Idaho Technologies. The time required for

running sequencing reactions using this instrument is shorter than the time necessary for running these reactions in a standard PCR instrument, making it an ideal instrument for class use.

Protocol

1. Label one 1.5 ml microfuge tube RX and place it on ice. Add the reagents shown in Table 5.4 to this tube starting **from the addition of water**.
2. Label three 0.2 ml thin-walled PCR tubes with your group number and tube number (e.g. 1.1, 1.2, and 1.3). Add 8 µl of the reaction mixture prepared in step 1 to each tube.
3. Add 2.0 µl of your first plasmid to the first tube. Mix well by pipetting up and down. Repeat this procedure for tubes 2 and 3, adding the remaining plasmid DNAs. Use a fresh yellow tip for each transfer.
4. Insert the four tubes into the heating block of the thermal cycler. Start the following temperature cycling program: 95°C for 10 seconds, 50°C for 10 seconds, and 60°C for 3.5 minutes. Use 40 cycles.

Table 5.4 Sequencing reaction mix

Ingredient	Single reaction	Four reactions
ABI buffer mix	4.0 µl	16.0 µl
Primer M13 F (3.2 pm μl^{-1})	2.0 µl	8.0 µl
Water	2.0 µl	8.0 µl

Next day

1. Remove the tubes from the thermal cycler and store them in a –20°C freezer.

FOURTH LABORATORY PERIOD

In this laboratory period you will perform the final step in the preparation of the DNA fragment for sequencing. This step is removal of dideoxy terminators from the sequencing reactions prepared in the third laboratory period.

Experiment 7: removing dideoxy terminators

In this experiment you will remove any remaining unincorporated dideoxy nucleotides from your sequencing reactions. Big Dye-labeled terminators (ddNTPs) can contaminate capillaries of the ABI 3700 sequencer and render them unusable. You will use Edge Gel Filtration Cartridges for removing fluorescent dye, which are specially designed for this task. You should carry out this procedure very carefully because even a small amount of fluorescent dye in your preparation will permanently damage the capillary tubes of the DNA sequencer.

Protocol

1. Place the cartridge assembly (cartridge and bottom tube) into a microfuge. Centrifuge for **exactly 2 minutes at 3000 r.p.m. Note:** this is important because a shorter centrifugation time and lower speed will result in an elution volume greater than the input volume.
2. Transfer the cartridge to a clean microfuge tube that will be provided to you. Discard the bottom tube, which should contain some liquid.
3. Holding the cartridge up to the light, transfer the reaction mixture to the top of the cartridge. Carefully dispense the sample directly onto the center of the gel bed at the top of the cartridge without disturbing the gel surface.
4. Close the cap and centrifuge the cartridge for 2 minutes at 3000 r.p.m.
5. Collect the tube containing sample and discard the cartridge. Up to 4 µl of the sample may be lost during sample processing. This is normal. However, if the volume loss is greater than 4 µl this is an indication that you centrifuged your cartridge too fast or for too long.
6. Label your tubes on the lid with numbers that will be given to you by your instructor. Store the tubes at –20°C until samples are ready for sequencing.

References

Birnboim, H.C. (1983) A rapid alkaline extraction method for the isolation of plasmid DNA. *Methods Enzymol.*, **100**, 243–55.

Birnboim, H.C. and Dolly, J. (1979) A rapid alkaline lysis procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.*, **7**, 1513–23.

Holmes, D.S. and Quigley, M. (1981) A rapid boiling method for the preparation of bacterial plasmids. *Anal. Biochem*., **114**, 193–7.

Hultner, M.I. and Cleaver, J.E. (1994) A bacterial plasmid DNA miniprep using microwave lysis. *BioTechniques*, **16**, 990–4.

Surzycki, S. (2000) *Basic Techniques in Molecular Biology*. Springer-Verlag, Berlin, Heidelberg, and New York.

Vogelstein, B. and Gillespie, D. (1979) Preparative and analytical purification of DNA from agarose. *Proc. Natl Acad. Sci. USA*, **76**, 615–19.

Wang, B., Merva, M., Williams, W.V., and Weiner, D.B. (1995) Large-scale preparation of plasmid DNA by microwave lysis. *BioTechniques*, **18**, 554–5.