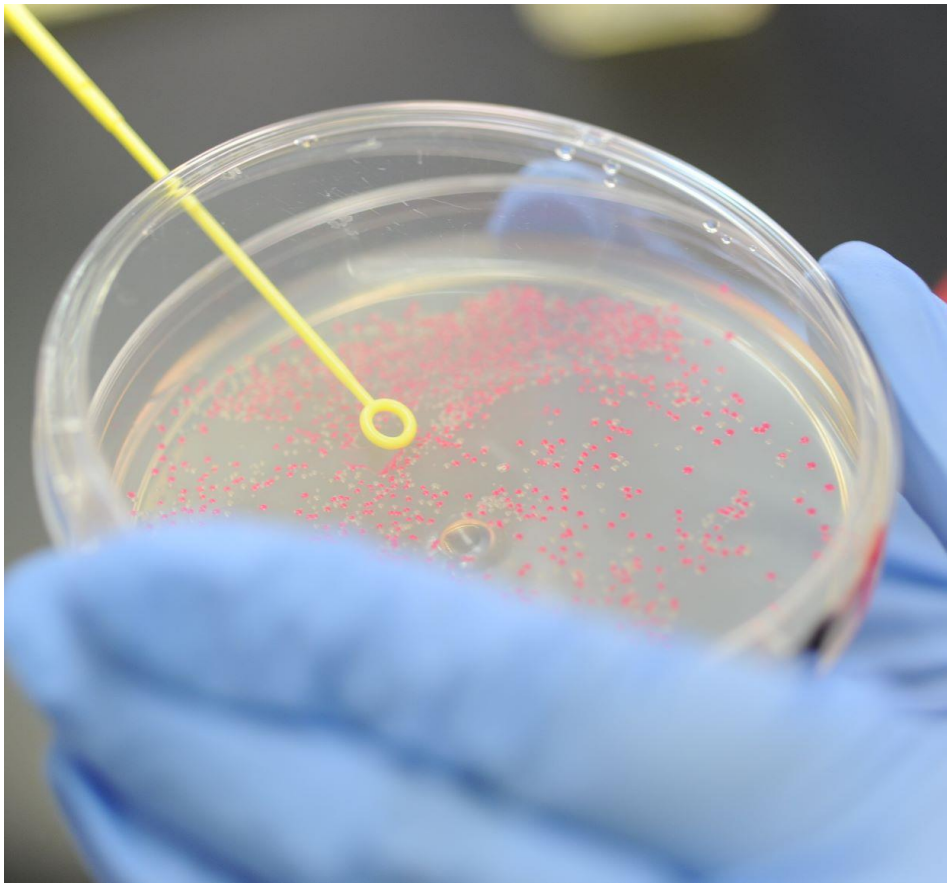
AMGEN® Biotech Experience

Scientific Discovery for the Classroom

Student Guide



30 YEARS

TSN

TEACHER SCIENTIST NETWORK

About the Amgen Biotech Experience

Genetic engineering is a branch of biotechnology that uses special procedures and techniques to change an organism's *DNA*. This ability has had a huge impact on the field of medicine, as genetically modified bacteria can make human *insulin* (the hormone responsible for regulating glucose levels in the blood) and other life-saving products. It's rare for secondary school or college students to have the chance to learn about and actually practice the procedures and techniques that are the foundation of the biotechnology industry—but in this program, you will have just that opportunity. As you work in the laboratory and carry out the very experiments that led to breakthroughs in biotechnology, you will gain hands-on experience of producing genetically modified bacteria.

The procedures in this program were developed through a series of discoveries that led to important breakthroughs in biotechnology. Some of the pioneering scientists who made these discoveries received Nobel Prizes, the highest distinction awarded to scientists in these fields from around the world. Werner Arber, Daniel Nathans and Hamilton Smith received the Nobel Prize in Physiology or Medicine in 1978 for their work with restriction enzymes. Stanley Cohen, Paul Berg and Herb Boyer received the Nobel Prize for Chemistry in 1980 for making the first recombinant DNA molecule. Kary Mullis received the Nobel Prize for Chemistry in 1993 for his discovery of the Polymerase Chain Reaction. The work that you are about to do is based on this Nobel Prize-winning science—science that is significant and will continue to play an important role in the development of biotechnology and medicine. You will follow in the footsteps of the many scientists who have pushed and continue to push the boundaries of biotechnology. There are many advances still to be made—and students who decide to continue studying this field may contribute to those advances.

The Amgen Biotech Experience (formerly Amgen-Bruce Wallace Biotechnology Lab Program) had humble beginnings almost 25 years ago with visionary scientists and teachers who shared passion and energy for sharing their knowledge with students. Bruce Wallace, one of Amgen's first staff members, wanted all students to experience the joy of discovery and the excitement of having science at their fingertips. A desire for more robust science education at schools near Amgen's global headquarters led to involving local teachers and, later, a college professor, in developing curriculum and training educators in biotechnology. The program grew through word of mouth and teacher interest and expanded over time to other states and countries.

Your Amgen Biotech Experience challenge

Your challenge in the Amgen Biotech Experience is to successfully carry out the steps of the genetic engineering process that are used to make insulin and other genetically engineered products. You will learn and practice the techniques and procedures that are part of this process. If you carry out all the steps in the program, you can create your own genetically modified bacteria.

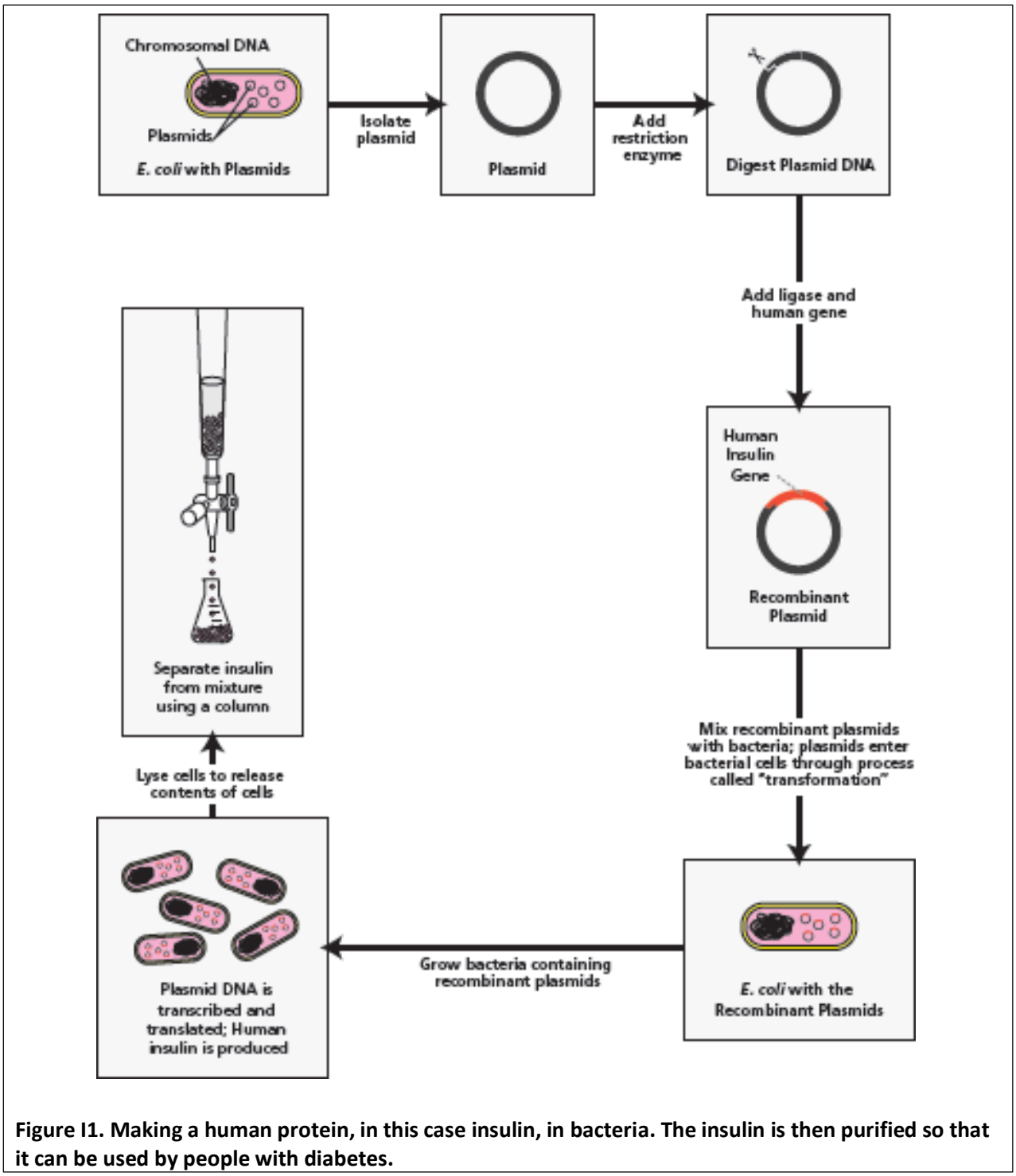


Figure I1. Making a human protein, in this case insulin, in bacteria. The insulin is then purified so that it can be used by people with diabetes.

Instead of cloning insulin or another human gene, you will work with a gene from a *sea anemone*, a soft-bodied animal related to coral and jellyfish. The gene is called *rfp* and the protein made by this gene is called red fluorescent protein. It has been altered by directed evolution to fluoresce even more brightly than the original wild-type would have done. If you are successful the bacteria you create will have a new and highly visible trait: they will produce red fluorescent protein and glow red when viewed on agar plates!

Figure I2. A fluorescing sea anemone.

Contents

Chapter 1: Some tools of the trade

Introduction

The year 1978 marked a major breakthrough in medicine. For the first time ever, scientists were able to induce bacterial cells to make human insulin by inserting human DNA into the cells. This new technology, termed genetic engineering, can be used to make products that treat the symptoms of certain genetic diseases.

To carry out genetic engineering, you need good laboratory skills. In this chapter, you'll focus on gaining practice in the use of micropipettes (instruments used to accurately transfer small volumes of liquid) and gel electrophoresis (a technique for separating and identifying biomolecules) - two critical skills for biotechnology. You can complete two practice exercises, using instruments and supplies that are identical to the ones used in biotechnology research laboratories. These practical activities are the first step in building the skills you'll need to carry out the subsequent labs and complete your challenge to create genetically modified bacteria.

The micropipette is an important tool used in genetic engineering. It is used to transfer very small and exact volumes of liquids in either millilitres (mL, 1/1,000 or one thousandth of a litre) or microlitres (µL, 1/1,000,000 or one millionth of a litre), which are the measurements of volume most often used in genetic engineering. Molecular biologists use such small volumes because of the cost of biomolecules and the difficulty purifying them from the thousands of other substances in a cell. This laboratory will give you the chance to learn how to use the micropipette to transfer very small, exact volumes of reagents and to see the relative amounts of solution measured by this very precise tool.

Gel electrophoresis is a technique used to separate a mixture of biomolecules, including DNA, by their size. The biomolecules to be separated have (or are prepared with) a negative charge, which means that they will move through a gel towards a positive electrical charge. Speed of movement through the gel varies primarily according to their weight, although molecular shape and degree of charge also influence their movement. In the genetic engineering process, gel electrophoresis is used to separate and characterise plasmids and short linear pieces of DNA.

Learning objectives

By the end of this laboratory you will be able to:

- Use a micropipette correctly
- Explain the importance of a micropipette
- Use gel electrophoresis
- Explain the purpose of gel electrophoresis

Laboratory 1.1: Using a micropipette

Aims

To use a micropipette and examine the volumes it can dispense.

Materials

EQUIPMENT & SUPPLIES

P-20 micropipette (2-20 µL)
Disposable pipette tips
Laminated practice sheet
Waste container

REAGENTS

Red dye solution (RD)

Health and safety

As good laboratory practice you should clear up any spills immediately and wash your hands well with soap after completing the practical work.

Methods

1. Check that you have all the materials
2. Review the parts of a micropipette in Figure 1.1 opposite.
3. Find the display window on the micropipette
4. Turn the plunger button on the top of the micropipette clockwise (right) to decrease the volume, or anti-clockwise (left) to increase the volume.

⚠ Never set the P-20 micropipette lower than 2.0 µL or higher than 20.0 µL or you could damage the equipment.

5. Figure 1.2 shows five micropipette volumes. Practice setting the micropipette to these volumes.

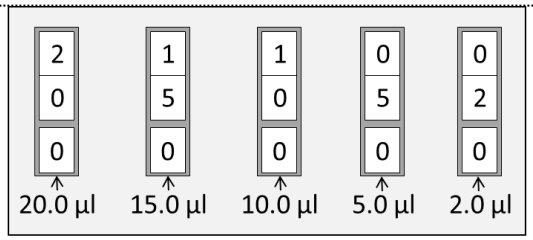


Figure 1.2 The display window for five micropipette volumes.

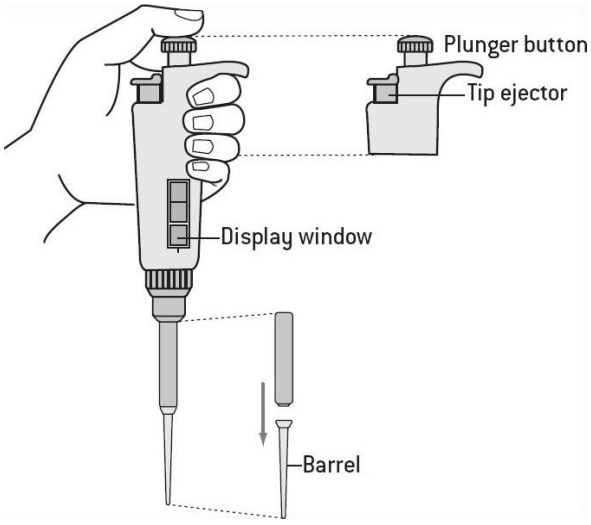


Figure 1.1 A P-20 micropipette

The display window of a micropipette shows how much fluid it will aspirate (suck up) and dispense. Five examples of displays and the corresponding amounts are shown.

6. Look at the laminated micropipette practice sheet. Each group member will pipette five drops of different volumes onto the sheet. Pipetting consists of two parts: loading the liquid into the micropipette, and dispensing the liquid from the micropipette.
7. Load the micropipette with 20.0 µL of red dye solution (RD) as follows:
   a. Set the P-20 micropipette to 20.0 µL in the display window.
   b. Open the tip box. Lower the micropipette onto a tip and press down firmly (do not touch the tip with your fingers). Close the box when done.
   c. Bring the micropipette and the RD tube to eye level.
   d. Use your thumb to press the plunger to the first stop position (figure 1.3a), which is your first point of resistance.
   e. Put your pipette tip into the RD and slowly release the plunger to draw up the solution.

⚠ When loading the micropipette, only press the plunger to the first stop or you will draw too much solution into the pipette tip.

⚠ Do not lay down a micropipette with fluid in the tip or hold it with the tip pointed upward because fluid could leak back into the pipette.

8. Dispense RD onto the laminated sheet by doing the following:
   a. Place the pipette tip over the 20.0 µL circle.
   b. Use your thumb to press the plunger to the first stop position and then press down to the second stop (figure 1.3b).

⚠ When dispensing liquid from the micropipette, press the plunger to the second stop in order to dispense all of the liquid.

   c. With the plunger still depressed, pull the pipette out of the liquid – this prevents you from accidentally pulling the liquid back into the tip.
9. Without setting down the micropipette, twist the plunger button to set it to 15.0 µL and repeat steps 7c–9, placing the pipette tip over the 15.0 µL circle when dispensing the liquid.
10. Without setting down the micropipette, twist the plunger button to set it to 10.0 µL and repeat steps 7c–9, placing the pipette tip over the 10.0 µL circle when dispensing the liquid.
11. Without setting down the micropipette, twist the plunger button to set it to 5.0 µL and repeat steps 7c–9, placing the pipette tip over the 5.0 µL circle when dispensing the liquid.
12. Without setting down the micropipette, twist the plunger button to set it to 2.0 µL and repeat steps 7c–9, placing the pipette tip over the 2.0 µL circle when dispensing the liquid.
13. Use the tip ejector to place your pipette tip into the waste container.

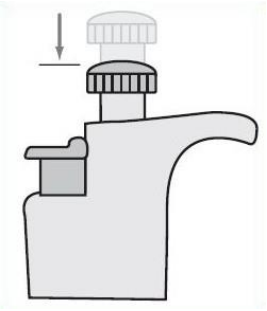


Figure 1.3a The first stop

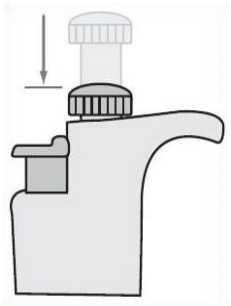


Figure 1.3b The second stop

14. Using the micropipette practice sheet, make sure that each person in your group pipettes five drops of different volumes, with each person using a new pipette tip.
15. When everyone in your group has had a chance to dispense RD onto the micropipette practice sheet, draw the approximate sizes of each drop in your notebook (or take a photograph and tape it into your notebook) and label them with the amounts.

Questions

1. When loading or dispensing a solution, why is it important to actually see the solution enter or leave the pipette tip?
2. You were instructed to avoid contact with the pipette tips—for example, you were asked to put the pipette tip on without using your hands, to avoid setting down the micropipette, to use the ejector button to remove the tip, and to keep the tip box closed. If you were working with plasmids and bacterial cells, why would these precautions be important?
3. Why do you think it is necessary to use very small and exact volumes of reagents in biotechnology?

Laboratory 1.2: Gel electrophoresis

Aims

To use gel electrophoresis and understand its purpose during the genetic engineering process.

Introduction

Gel electrophoresis involves placing an agarose gel into an electrophoresis tank of buffer, which has two electrodes either end that create an electric field across the gel when attached to a powerpack. The agarose for the gel is a type of polysaccharide (complex sugar) found in seaweed. An agarose gel looks like a lump of jelly, but it is a porous matrix (like a sponge) with lots of microscopic holes through which the solution and biomolecules flow.

DNA is negatively charged. When loaded into holes in the gel, known as wells, next to (black) negative electrodes, it will migrate towards the positive electrode (red) when an electric current is applied. As it has to move through the porous matrix of the agarose gel, smaller DNA molecules move faster and larger DNA molecules move more slowly. Figure 1.4 illustrates the separation of biomolecules by gel electrophoresis.

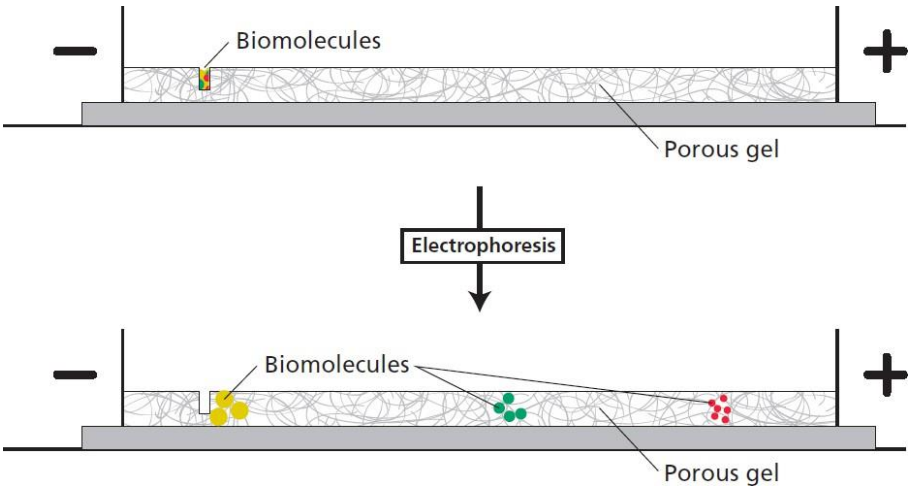


Figure 1.4 Biomolecules, including DNA, moving through the agarose gel matrix in gel electrophoresis.

Materials

EQUIPMENT & SUPPLIES

P-20 micropipette (2-20 µL)
Disposable pipette tips
Microcentrifuge
Electrophoresis tank
Gel tray containing an 0.8% agarose gel
Powerpack
Waste container
Agarose plates (optional)

REAGENTS

Microfuge tubes containing:

- Red dye solution (RD)
- Dye solution 1 (S1)
- Dye solution 2 (S2)
- Dye solution 3 (S3)

Tris-acetate-EDTA buffer (1 X TAE)

Health and safety

The dyes in solutions 1 to 3 do not require any special handling instructions, however they may stain the skin, so try to avoid contact with them as good practice would dictate. You should wear safety glasses and chemical-resistant gloves when using the electrophoresis buffer, to direct contact with the solution.

Finally, the electrophoresis chamber contains 1 X TAE buffer at 100 volts. To prevent contact with the liquid during electrophoresis the tanks are designed so that the electric supply is cut off whenever the lid is removed. Additionally, all connections are plastic coated so that there is no possibility of touching a metal contact which is live at 100 volts.

Methods

PART A: PIPETTING INTO WELLS

You may have a chance to practice pipetting red dye (RD) into the wells of an agarose plate.

1. Cover the agarose plate with water, so that the 'dimples' over the wells disappear.
2. Set the P-20 micropipette to 10.0 µL and put on a pipette tip.
3. Load the pipette with 10.0 µL of RD.
4. Dispense RD into a well in one of the practice plates by doing the following:
   a. Place your elbow on the table to steady your pipette hand. If needed, also use your other hand to support your pipette hand.
   b. Lower the pipette tip until it is under the buffer but just above the well.

⚠ Be careful not to place your pipette tip into the well or you might puncture the gel and lose your sample out the hole.

   c. Gently press the plunger to slowly dispense the sample. To avoid getting air into the buffer, do not go past the first stop. The sample will sink into the well.
5. Repeat steps 3 and 4 until all the practice plate wells have been filled. Everyone in your group should get an opportunity to practice pipetting into the wells.
6. Eject the pipette tip into the waste container.

PART B: SEPARATING DYES WITH GEL ELECTROPHORESIS

You will use gel electrophoresis to separate different dyes. First you will add dyes into wells in the agarose gel in an electrophoresis tank. Then you will turn the electrical current on to separate the negatively charged dye molecules in the gel. (You will share the electrophoresis boxes with one other group: your teacher will tell you which wells your group should use.)

1. Check your rack to make sure that you have the three dye solutions (S1, S2, and S3).

2. Check to make sure that the wells in your gel are located near the negative (black) electrode (figure 1.5).

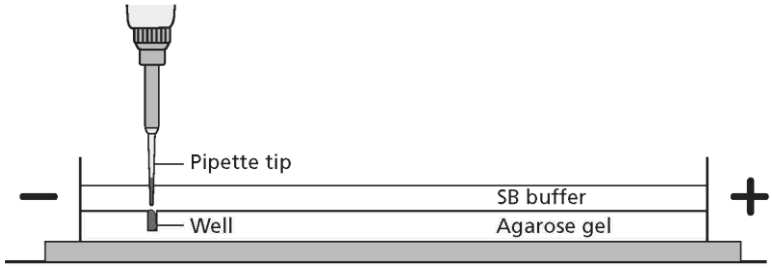


Figure 1.5 The gel electrophoresis unit

3. Fill the tank with 1x TAE buffer to a level that just covers the entire surface of the gel. If you see any "dimples" over the wells, add more buffer.
4. Use the microcentrifuge to pool the dye in the bottom of the S1, S2, and S3 tubes.

⚠ Distribute the tubes evenly in the microcentrifuge so that their weight is balanced.

5. Make a drawing in your notebook that shows the location of the wells in the electrophoresis box. Record which solution you will place in each well.
6. Set the P-20 micropipette to 10.0 µL and put on a pipette tip.
7. Load 10.0 µL of S1 into the pipette.
8. Dispense the S1 into the well you've designated for that solution by doing the following:
   a. Place your elbow on the table to steady your pipette hand. If needed, also use your other hand to support your pipette hand.
   b. Lower the pipette tip until it is under the buffer but just above the well.

⚠ Be careful not to place your pipette tip into the well or you might puncture the gel and lose your sample out the hole.

   c. Gently press the plunger to slowly dispense the sample. To avoid getting air into the buffer, do not go past the first stop. The sample will sink into the well.

⚠ Use a fresh pipette tip for each sample.

9. Repeat steps 7 and 8 for S2 and S3, using a new pipette tip to load each solution into a new well of the agarose gel.
10. When all the samples have been loaded, close the cover tightly over the electrophoresis box. Lower the cover in a horizontal motion, so that the samples don't spill.
11. Connect the electrical leads to the power supply. Connect both leads to the same channel, with negative electrode to negative

electrode (black to black) and positive electrode to positive electrode (red to red). See figure 1.6 below.

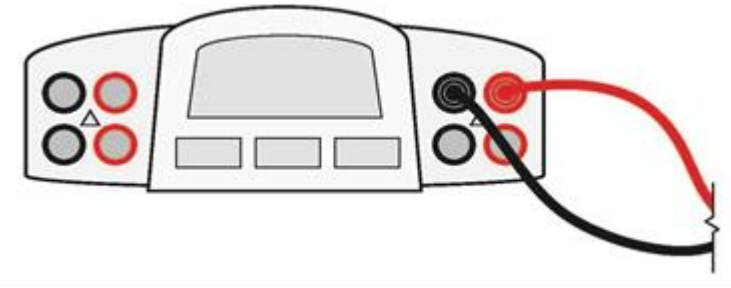


Figure 1.6 Connecting leads from the electrophoresis tank to the powerpack.

12. Turn on the power supply and set the voltage to approx.. 100 V.
13. After two or three minutes, check to see if the dyes are moving toward the positive (red) electrode. You should see the purple dye (bromophenol blue) beginning to separate from the blue dye (xylene cyanole).
14. In approximately 10 minutes, or when you can distinguish all three dyes, turn off the power switch and unplug the electrodes from the power supply. Do this by grasping the electrode at the plastic plug, NOT the cord.
15. Carefully remove the cover from the gel box and observe the dyes in the gel.
16. In your book, draw the relative location of the bands and their colours in each of the lanes containing your samples.
17. Leave the gels in the gel box.

Questions

1. When might it be important to use gel electrophoresis to separate and identify plasmids and short linear pieces of DNA?
2. What electrical charge do the dyes have? Explain how you know.
3. Study your gel electrophoresis results. Which solution sample contained a single dye: S1, S2, or S3? How do you know?
4. From your gel electrophoresis results, put the dyes into size order from largest to smallest. Explain your reasoning.
5. The molecular weights (sizes) for the dyes are 452.38 atomic units (au) for orange G, 669.98 au for bromophenol blue, and 538.62 au for xylene cyanole. How do these weights compare with your original conclusions about the sizes of the dye molecules?
6. Can you suggest any other factors that may have played a role in the separation of these dyes?

Chapter 2: Beginning to clone a gene

Introduction

Plasmids and restriction enzymes are biomolecules found in many bacteria. Their discovery was crucial to genetic engineering as they are used in the process that scientists have developed to modify the genetic material in microorganisms so that they produce useful biomolecules. The biomolecules that microorganisms produce can be medicines that save lives, such as insulin. This chapter is about plasmids, restriction enzymes and their role in cloning a gene (that is copying a gene from one location to another).

Many bacteria carry two forms of DNA: (1) a single chromosome made up of a large DNA molecule that contains all the information needed by the organism to survive and reproduce, and (2) plasmids, which are small circular DNA molecules, present in multiple copies, separate from the chromosomal DNA. This is shown in figure 2.1.

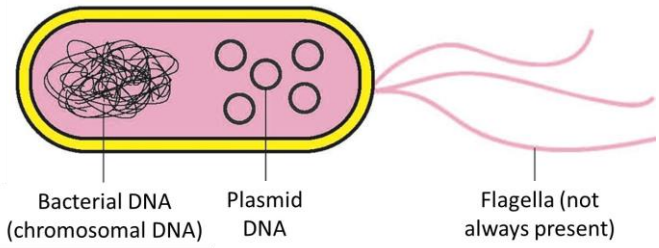


Figure 2.1. DNA in bacterial cells

Plasmids are vectors: that is vehicles for carrying DNA sequences from one organism to another. They are replicated within cells. Cells containing a plasmid can be selected by their resistance to antibiotics and if a gene of interest is inserted by the promoter sequence, the gene of interest will be expressed. So, in simple terms, if the plasmid DNA can be cut up and stuck back together to include the gene of interest, then bacteria containing the plasmid will make the gene product of interest.

The discovery of restriction enzymes in the early 1950s provided the tool for 'cutting up' the DNA sequence. Restriction enzymes are proteins that bacteria produce to recognise and destroy bacteriophage (viruses that infect bacteria) DNA without damaging the host (bacterial) DNA. Restriction enzymes from different strains of bacteria cut at specific DNA sequences, called recognition sites.

During this laboratory you will use restriction enzymes to produce the DNA fragments that will be joined to make a recombinant plasmid that can make a red fluorescent protein in bacteria. You will be supplied with 2 plasmids. Both plasmids contain the site for initiating DNA replication (*ori*). In addition, the first plasmid, pKAN-R, carries the gene that makes bacteria resistant to the antibiotic kanamycin (*kanR*), the red fluorescent protein gene (*rfp*) and a promoter sequence (*pBAD*). The second plasmid, pARA, contains the gene that makes bacteria resistant to the antibiotic ampicillin (*ampR*) and an activator (*araC*) (figure 2.2).

The kanamycin resistance gene *(kanR)* encodes a phosphotransferase enzyme that transfers a phosphate group to the kanamycin molecule destroying its antibiotic effects. The ampicillin resistance gene (*ampR*) encodes the protein beta lactamase, an enzyme that destroys the antibiotic ampicillin enabling bacteria to reproduce in the presence of ampicillin. The arabinose activator (*araC*) is a specific DNA sequence that activates a promoter when the bacteria are grown in the presence of arabinose, a five-carbon sugar that naturally occurs in various plant and bacterial carbohydrates. If arabinose is present in the bacteria, the activator causes the promoter to bind RNA polymerase, and transcription will occur. If arabinose is not present, the activator prevents the promoter from binding RNA polymerase, and transcription will not occur.

The location of these DNA sequences, restriction enzyme sites and size of the plasmids (measured in base pairs) are shown in figure 2.2. A 'base pair' is either adenine: thymine or guanine: cytosine and is the common method used to give the size of DNA molecules.

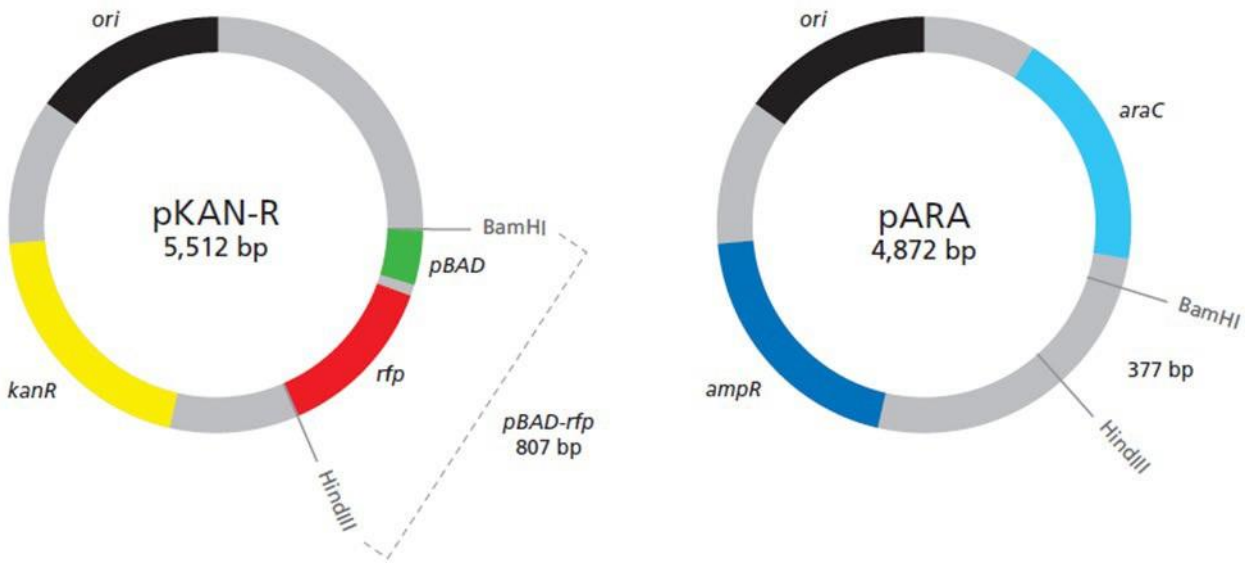


Figure 2.2 Plasmid maps showing the location of the *rfp* gene, the promoter (*pBAD*), the kanamycin resistance gene (*kanR*), the ampicillin resistance gene (*ampR*), the arabinose activator (*araC*), and the origin of replication sites (*ori*).

To clone the *rfp* gene from the plasmid pKAN-R into the expression plasmid pARA, you will use 2 restriction enzymes: *Bam*HI and *Hin*dIII. These will excise the *rfp* gene and promoter from the pKAN-R plasmid and a short 377 bp fragment from the expression plasmid pARA. Using two different restriction enzymes allows the inserted gene to be oriented in the correct position for transcribing the "sense" strand of DNA (the strand that codes for the protein), and it prevents the plasmid from reforming a circle without the inserted gene.

Learning objectives

By the end of this laboratory you will be able to:

- Describe the characteristics of plasmids
- Explain how plasmids are used in cloning a gene
- Describe the function of restriction enzymes
- Explain how to use restriction enzymes to create a recombinant plasmid

Laboratory 2: Restriction digestion of plasmids

Aims

To use restriction digestion with two enzymes (*Bam*HI and *Hin*dIII) to create linear DNA fragments containing (i) DNA sequence for gene expression (from the plasmid pARA), and (ii) the red fluorescent protein gene (from the plasmid pKAN-R).

Materials

EQUIPMENT & SUPPLIES

P-20 micropipette (2-20 µL)
Disposable pipette tips
4 x 1.5 mL microfuge tubes
Permanent marker
Floating tube rack
Waste container
Microcentrifuge (shared)
37°C water bath (shared)

REAGENTS

Microfuge tubes containing:

- 2.5 X restriction buffer (RB)
- pKAN-R plasmid (K)
- pARA plasmid (A)
- restriction enzymes *Bam*HI and *Hin*dIII (RE)
- Distilled water (dH_2O)

Health and safety

In this laboratory you will be using biological materials, albeit in tiny amounts, and they should be used in a controlled way, using techniques that mirror the aseptic techniques used in microbiology. All of the materials that come into contact with the biological materials (pipette tips and microfuge tubes) will be autoclaved to denature the DNA and enzymes before disposal. As good laboratory practice you should clear up any spills immediately and wash your hands well with soap after completing the practical work.

Methods

1. Check your rack to make sure that you have all the reagents listed.
2. Use a marker to label four clean microfuge tubes as follows: K+, K–, A+, and A–. (Also include a personal identifier on each tube.)
3. Review table 2.1, which summarises the reagents that you will add in step 4.

Step	Reagent	K+ tube	K- tube	A+ tube	A- tube
4a	Restriction buffer (RB)	5.0 µL	5.0 µL	5.0 µL	5.0 µL
4b	pKAN-R plasmid (K)	5.0 µL	5.0 µL		
4c	pARA plasmid (A)			5.0 µL	5.0 µL
4d	*Bam*HI and *Hin*dIII (RE)	2.0 µL		2.0 µL	
4e	Distilled water (dH_2O)		2.0 µL		2.0 µL

Table 2.1 Reagents to add to the K+, K-, A+ and A- tubes

⚠ In step 4, be sure to use a new micropipette tip for each reagent in each tube to avoid contamination.

4. Add the following:
   a. 5.0 µL of restriction buffer (RB) to the K+, K–, A+, and A– tubes.
   b. 5.0 µL of plasmid pKAN-R (K) to the K+ and K– tubes.
   c. 5.0 µL of plasmid pARA (A) to the A+ and A– tubes.
   d. 2.0 µL of *Bam*HI and *Hin*dIII restriction enzymes (RE) to the K+ and A+ tubes. Add the enzymes directly into the solution at the bottom of the microfuge tube. Gently pump the solution in and out of the pipette 3 times, by repeatedly moving the plunger to the first stop and back up again, to mix the reagents. Cap the tubes when done.
   e. 2.0 µL of dH_2O to the K– and A– tubes. Gently pump the solution in and out with the pipette to mix the reagents. Cap the tubes when done.
5. Spin the four microfuge tubes (K+, A+, K–, and A–) in the microcentrifuge for four seconds to pool the reagents at the bottom of each tube.

⚠ Distribute the tubes evenly in the microcentrifuge so that their weight is balanced.

6. Place all four tubes into the 37°C water bath and incubate for one hour.
7. After the incubation is complete either continue to Laboratory 3, or place all four tubes in the freezer at –20°C until you are ready to do so.

Questions

1. Using figure 2.2, work out how many linear fragments will be produced from the plasmids pKAN-R and pARA, when they are digested with *Bam*HI and *Hin*dIII. What size will these linear fragments be?
2. Draw the linear fragments, marking on the genes and important sequences. Using table 2.2 below, include the nucleotide sequence of the sticky ends.

Source	Restriction enzyme	Recognition site
Bacillus amyloliquefaciens	*Bam*HI	5′ G↓GATCC 3′ 3′ CCTAG↑G 5′
Haemophilus influenzae	*Hin*dIII	5′ A↓AGCTT 3′ 3′ TTCGA↑A 5′

Table 2.2 The restriction enzymes used in this laboratory.

Information on the bacterial strains they were isolated from and the DNA sequences that they cut. In the examples shown, the enzymes
cut asymmetrically on the strands of DNA, leaving single-stranded
overhanging sequences at the site of the cut. Cuts are shown by arrows. For example, a cut (or digestion) with *Bam*HI will leave an GATC overhang (or "sticky end") on one strand and a CTAG sticky end on the other strand.

3. In order to create a plasmid that can produce the red fluorescent protein in bacteria, what components are needed in the plasmid?
4. Bacteria can be killed by an antibiotic unless they carry a plasmid that has the gene for resistance to that antibiotic. Biotechnologists call these genes *selectable markers* because only bacteria that carry the gene will survive an antibiotic. If the uptake of DNA by bacteria is inefficient, why is a selectable marker critical in cloning a gene in bacteria?
5. What role do restriction enzymes have in nature?
6. Why might the enzymes work best at 37°C? Why should the unused enzymes then be placed in the freezer?
7. In step 4, you are asked to set up two tubes without the restriction enzymes, *Bam*HI and *Hin*dIII. What is the purpose of this step, and why is it important?

8. Using your understanding of evolution, why would bacteria retain a gene that gives them resistance to antibiotics? How is the existence of bacteria with antibiotic resistance affecting medicine today?
9. Bacteria, sea anemones, and humans seem, on the surface, to be very different organisms. Explain how a gene from humans or a sea anemone can be expressed in bacteria to make a product never before made in bacteria.
10. Due to a mishap in the lab, bacteria carrying a plasmid with a kanamycin-resistant gene and bacteria carrying a plasmid with an ampicillin-resistant gene were accidentally mixed together. Design an experiment that will allow you to sort out the two kinds of bacteria. (Hint: Make sure that you do not kill off one of the kinds of bacteria you are trying to sort out!)

Chapter 3: Building a recombinant plasmid

Introduction

Armed with ways to transfer genes between cells using plasmids as vectors and to generate fragments of DNA using restriction enzymes, the final essential tool required to make recombinant plasmids was a way to 'glue' the DNA fragments together. In the early 1960s the discovery of DNA ligase, an enzyme that catalyses the joining of DNA fragments, provided that tool for utilising in genetic engineering.

DNA ligase is one of several enzymes involved in the replication of DNA in all cells. During replication the two parent DNA strands separate at origin of replication, or *ori* sites. An enzyme called DNA polymerase adds new nucleotides complementary to the single-stranded parent DNA to form base pairs held together by hydrogen bonds. On one parent DNA strand the new, complementary daughter strand forms continuously On the other parent strand of DNA the new, complementary nucleotides are added discontinuously. It is DNA ligase that then catalyses the formation of covalent bonds between these discontinuous DNA fragments to make a complete second daughter strand of DNA. Replication produces two new DNA molecules, each consisting of one parent strand and one daughter strand that are exact replicas of the original DNA molecule.

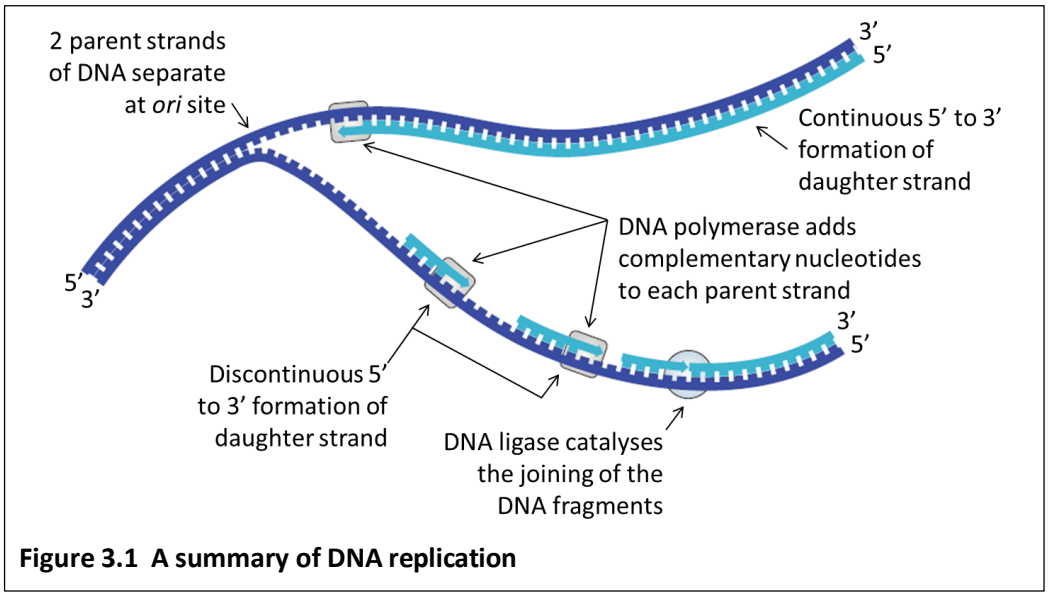


Figure 3.1 A summary of DNA replication

During the procedures used to make recombinant plasmids, DNA ligase is used to bond linear DNA fragments from restriction digestion together. Any two fragments that have ends cut with the same restriction enzyme can be ligated together. First the unpaired bases at the sticky ends form hydrogen bonds to each other, and then the ligase catalyses the formation of covalent bonds between the sugar and phosphate groups of adjacent nucleotides. If you have multiple fragments, the ligation procedure can lead to a number of possible products.

In this laboratory you will use DNA ligase to ligate the DNA fragments you produced during Laboratory 2 together, making new recombinant plasmids. These recombinant plasmids will contain the four restriction fragments from Laboratory 2 recombined in different ways to produce new sets of DNA. The ligation process will result in several different plasmids, but the plasmid that you are interested in will contain the *ori* sequence for the initiation of DNA replication, the gene for ampicillin resistance (*ampR*), the arabinose activator sequence (*araC*), a promoter sequence for initiating transcription (*pBAD*) and the red fluorescent protein (*rfp*) gene. This desired recombinant DNA plasmid is called the pARA-R plasmid and is shown in figure 3.2.

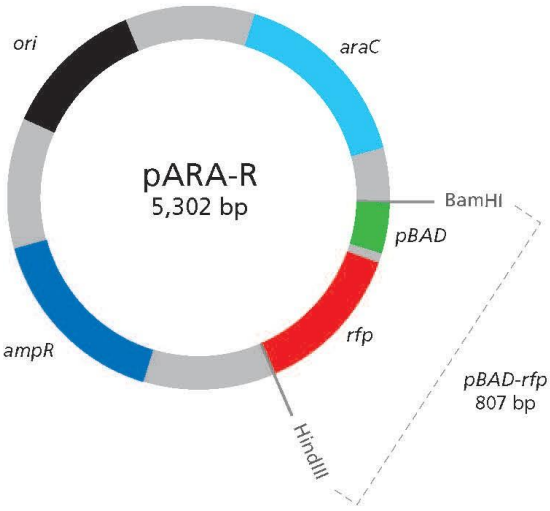


Figure 3.2 The pARA-R plasmid

Learning objectives

By the end of this laboratory you will be able to:

- Describe the role of a DNA ligase in replication
- Explain how DNA ligase is used to create a recombinant plasmid
- Describe possible recombinant plasmids that form when ligating your restriction digests

Laboratory 3: Ligating to build the pARA-R plasmid

Aims

To use the enzyme DNA ligase to catalyse covalent bond formation between the linear fragments with sticky ends formed by restriction digestion in laboratory 2, making new recombinant plasmids.

Materials

EQUIPMENT & SUPPLIES

P-20 micropipette (2-20 µL)
Disposable pipette tips
Permanent marker
Floating tube rack
Waste container
Microcentrifuge (shared)
70°C water bath (shared)

REAGENTS

Microfuge tubes containing:

- Digested pKAN-R plasmid from lab 2 (K+)
- Digested pARA plasmid from lab 2 (A+)
- Ligation buffer (LB)
- DNA ligase (LIG)
- Distilled water (dH_2O)

Health and safety

In this laboratory you will be using biological materials, albeit in tiny amounts, and they should be used in a controlled way, using techniques that mirror the aseptic techniques used in microbiology. All of the materials that come into contact with the biological materials (pipette tips and microfuge tubes) will be autoclaved to denature the DNA and enzymes before disposal. As good laboratory practice you should clear up any spills immediately and wash your hands well with soap after completing the practical work.

Methods

1. Check your rack to make sure that you have all the reagents listed.
2. Place K+ and A+ tubes from Laboratory 2 in a floating microfuge tube rack in the 70°C water bath for 30 minutes. This heat exposure will denature the restriction enzymes.
3. Label the LIG tube with your personal identifier.
4. After 30 minutes, remove the K+ and A+ tubes from the water bath and place them in your rack.
5. Add the following solutions directly into the 2.5 µL of DNA ligase solution at the bottom of the LIG tube:
   a. 6.0 µL of A+
   b. 6.0 µL of K+
   c. 4.0 µL of LB
   d. 2.0 µL of dH_2O

⚠ In steps 5a–d, be sure to use a new micropipette tip for each reagent to avoid contamination.

6. After adding the dH_2O, gently pump the solution in and out with the pipette to mix the reagents. Cap the tube when done.
7. Spin the LIG tube in the microcentrifuge for four seconds to pool the reagents at the bottom of the tube.

⚠ Distribute the tubes evenly in the microcentrifuge so that their weight is balanced.

8. Place your LIG, A+, and K+ tubes in the microfuge racks designated by your teacher. (Your LIG tube will incubate at room temperature overnight then be stored in the fridge (4 °C) until the next class. Your A+ and K+ tubes will be returned to the –20°C freezer for Laboratory 5.)

Questions

1. What is the function of enzymes in reactions?
2. Based on your understanding of DNA replication, explain the role of DNA ligase in replication.
3. Describe what happens when two DNA fragments with complementary sticky ends join, and speculate how the activity of DNA ligase ensures that the join is permanent.
4. What are the roles of hydrogen bonds and covalent bonds in the structure of DNA?
5. Using your description of the fragments that formed from the digestion of pKAN-R and pARA with *Bam*HI and *Hin*dIII draw three possible recombinant plasmids resulting from the joining of two pARA and pKAN-R fragments. For each plasmid, identify the genes, other important sequences, and the number of base pairs.
6. Why is it important to inactivate the *Bam*HI and HindIII restriction enzymes before ligating the fragments? What might happen if you did not perform this step?

Chapter 4: Verifying pARA-R is present using PCR

Introduction

During the previous laboratory, different *Bam*HI - *Hin*dIII restriction fragments were ligated together. The plasmid we are aiming to create using this procedure is the pARA-R plasmid (figure 3.2), however, other recombinant plasmids will also be created. One technique that can be used to verify the presence of a DNA fragment with size corresponding to the *rfp* gene ligated within the pARA expression plasmid is the polymerase chain reaction, or PCR.

Kary Mullis is credited with the discovery of PCR, work for which he received a Nobel prize in Chemistry in 1993. PCR uses the enzyme DNA polymerase, which is important in DNA replication (figure 3.1), to amplify (make lots of) a particular region of DNA. There are 3 different steps to a PCR, illustrated in Figure 4.1. During **denaturation** DNA becomes single stranded at temperatures high enough to break the hydrogen bonds between the bases in the centre of the DNA molecule, but not so high that they break the stronger covalent bonds between molecules in the sugar-phosphate backbone. Typically a temperature of 94 – 96°C is used. Following this, **annealing** occurs at a lower temperature, binding primers (short DNA sequences) to a complementary DNA sequence, creating a short double stranded region of DNA. Finally, the **extension** step, during which a DNA polymerase binds to the short double-stranded region and extends the primer in a 5' to 3' direction using the single stranded DNA as template. The optimum temperature for the DNA polymerase to extend the single stranded DNA varies, but tends to be in the range of 68 – 76 ^{o}C for the thermostable DNA polymerase enzymes used, which have been isolated from bacteria like *Thermus aquaticus* that live around hydrothermal vents and have enzymes adapted to tolerate high temperatures.

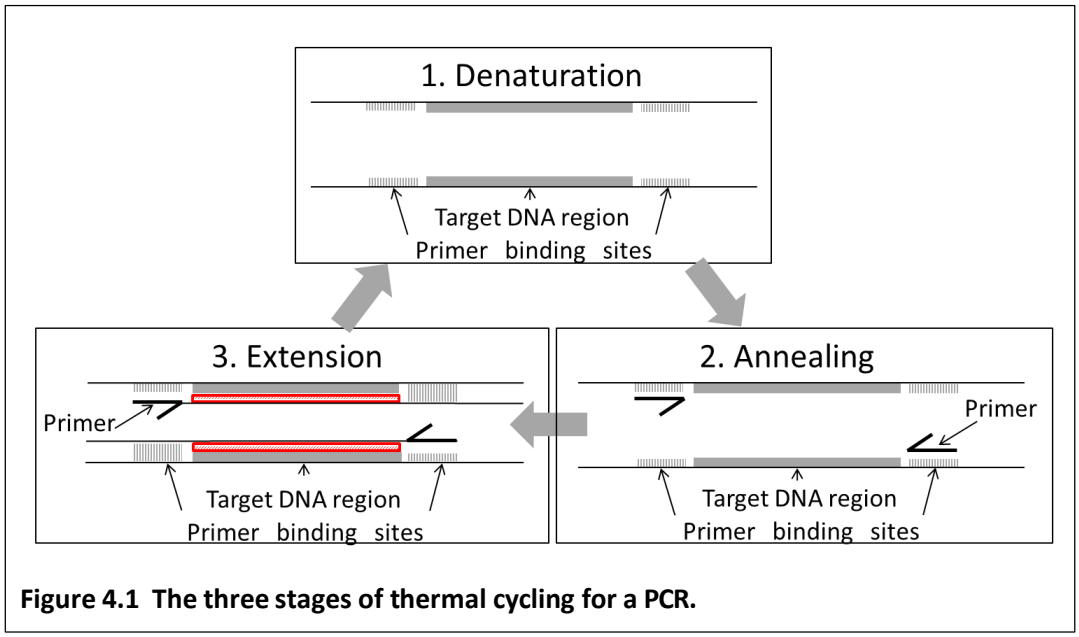


Figure 4.1 The three stages of thermal cycling for a PCR.

By using two primers complementary to the DNA sequence near the ends of the pARA restriction digest fragment, the sequence ligated within the pARA expression plasmid can be amplified. Primers that bind specifically to the DNA sequence 136 bp from the *Bam*HI restriction site and 149 bp from the *Hin*dIII restriction site will be used to amplify the region of DNA bracketing and including the *Bam*HI – *Hin*dIII region. Two restriction fragments that could be ligated into the expression plasmid are shown in figure 4.2. If the 807 bp pKAN-R restriction digest fragment carrying the *pBAD* promoter and *rfp* gene has been ligated into the pARA plasmid, creating the desired pARA-R (figure 4.2A), then DNA amplified by PCR will be 1092 bp long. If the short 377 bp restriction digest fragment has been re-ligated into the pARA plasmid (figure 4.2B), then DNA amplified by PCR will be 662 bp long.

This experiment uses two DNA templates for PCR: the pARA plasmid as a control, which is expected to produce a PCR product 662 bp long, and; the ligation reaction, which will hopefully contain the pARA-R plasmid with a PCR product of 1092 bp, among other constructs.

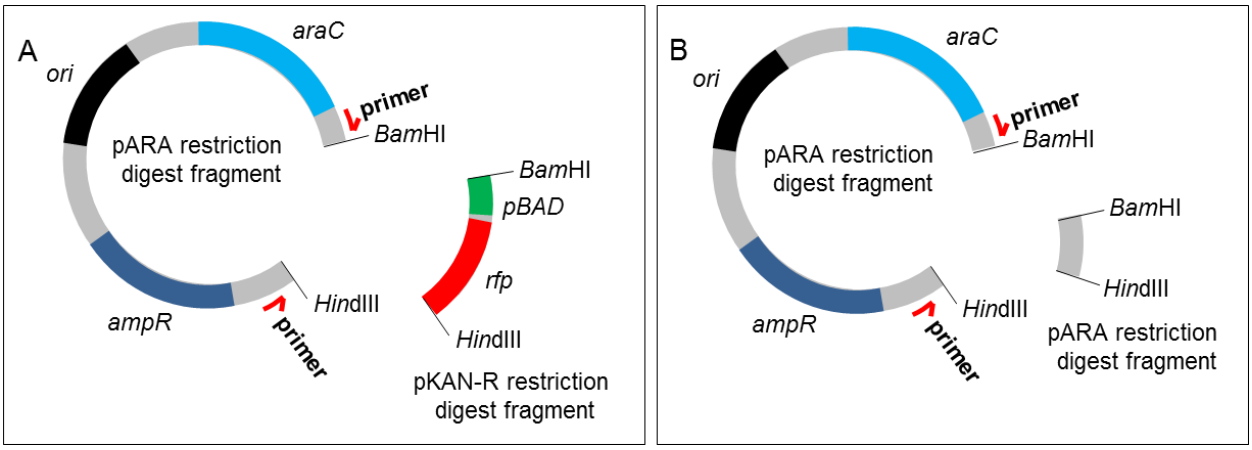


Figure 4.2 The sites where primers bind to the pARA restriction digest fragment, showing possible restriction fragments from (A) pKAN-R, and (B) pARA, which could be ligated into the plasmid.

Learning objectives

By the end of this laboratory you will be able to:

- Describe the role of a DNA polymerase in the PCR
- Explain how PCR can be used to amplify a specific DNA sequence
- Understand the role of sequence specific primers in a PCR
- Describe the size of possible PCR products from your ligation reaction

Laboratory 4: PCR of the recombinant plasmids formed by ligation

Aims

To use the enzyme DNA polymerase in a **P**olymerase **C**hain **R**eaction (PCR), catalysing the extension of sequence specific primers to discover the size of the restriction fragments ligated to the larger pARA restriction digest fragment.

Materials

EQUIPMENT & SUPPLIES

P-20 micropipette (2-20 µL)
Disposable pipette tips
Permanent marker
Polystyrene cup of ice
Waste container
Microcentrifuge (shared)
PCR machine (shared)

REAGENTS

2 PCR tubes of 2 X PCR master mix (MM)
Microfuge tubes containing:

- Combined forward and reverse primer (PRI)
- Nuclease-free water (H_2O)
- pARA plasmid (A)
- your ligation reaction (LIG) from lab 3

Health and safety

In this laboratory you will be using biological materials, albeit in tiny amounts, and they should be used in a controlled way, using techniques that mirror the aseptic techniques used in microbiology. All of the materials that come into contact with the biological materials (pipette tips, PCR tubes and microfuge tubes) will be autoclaved to denature the DNA and enzymes before disposal. As good laboratory practice you should clear up any spills immediately and wash your hands well with soap after completing the practical work.

Methods

1. Check your rack to make sure that you have all the reagents listed:
   a. A polystyrene cup, containing ice, with 2 PCR tubes (MM) in it.
   This is a mixture of the buffer, magnesium chloride, dNTPs and DNA polymerase from a thermophilic bacteria, which are required for a PCR.
   b. Microfuge tubes containing plasmid pARA (A) and the ligation reaction, returned to you from a previous class (LIG + your personal identifier).
   These tubes have the template DNA for the PCR.
   c. A microfuge tube containing forward and reverse primers (PRI).
   Primers are short stretches of DNA that bind to complementary DNA sequence during the annealing step, allowing DNA polymerase to extend a specific part of the DNA.
   d. A microfuge tube containing nuclease-free water (H_2O).
   This will be used to make up the reaction volume.

2. Use a marker to label the 2 PCR tubes containing 2 X PCR master mix (MM) on the side with your personal identifier. Label 1 tube 'pARA' and the other tube 'LIG'. Return them to the ice.

⚠ Marker pen can rub off the top of the PCR tubes in the PCR machine, so make sure you label them on the side as well.

3. Review table 4.1, which summarises the reagents that you will add in step 4.

Step	Reagent	pARA PCR	Ligation PCR
4a	2 X PCR master mix (MM)	12.5 µL	12.5 µL
4b	Nuclease-free water (H_2O)	8.5 µL	5.5 µL
4c	Combined primers (PRI)	2.0 µL	2.0 µL
4d	pARA plasmid (A)	2.0 µL	
4e	Ligation reaction (LIG)		5.0 µL

Table 4.1 Reagents to add to the pARA and LIG PCR tubes

⚠ In step 4, be sure to use a new micropipette tip for each reagent in each tube to avoid contamination.

4. a. To your labelled PCR tubes of master mix add the following:
   b. 8.5 µL of nuclease-free water (H_2O) to the pARA PCR tube, then 5.5 µL of nuclease-free water to the ligation PCR tube.
   c. 2.0 µL of combined forward and reverse primers (PRI) to each tube.
   d. 2.0 µL of pARA plasmid (A) to the pARA PCR tube.
   e. 5.0 µL of ligation reaction (LIG) to the ligation PCR tube.
5. Ensure the reagents are well mixed, by pumping gently up and down with the pipette or simply swirling with the tip. Keep your reactions on ice until the PCR machine can be started.
6. The PCR programme that you will use is given in table 4.2. It takes about 1½ hours to complete.

Reagent		Temperature (°C)	Time (sec)
Initial denaturation		95	200
30 cycles of	Denaturation	95	30
	Annealing	53	30
	Extension	68	30
Final extension		68	300
Hold		10	Indefinite

Table 4.2 The PCR programme

7. When your teacher tells you, transfer your PCR tubes from the ice into the PCR machine and start the reaction. They should have prepared a single 'control tube' to check your reagents are not contaminated.
8. After the PCR programme is complete your PCR tubes can be place in the fridge (4°C) until you are ready to use them.

Questions

1. Based on your understanding of DNA replication, explain the role of DNA polymerase in replication.
2. Why do you keep the PCR master mix, which contains DNA polymerase, on ice until the PCR is begun?
3. Why does the *Taq* DNA polymerase not denature during the denaturation step at 95°C in your PCR programme?
4. Each DNA molecule is replicated during each cycle of the PCR programme. There are 30 cycles on the PCR programme that you use. If you started with 6 DNA molecules, how many molecules would there be at the end of the PCR?
5. What is the purpose of the pARA PCR, given that the expected size of the PCR product is known to be 662 bp long?
6. What type of chemical bonds will form when the single stranded primer DNA base pairs with the complementary sequence on the recombinant plasmid?
7. The primers bind to specific DNA sequences. On the larger pARA restriction fragment one primer binding site is 136 bp from the *Bam*HI restriction site and the other is 149 bp from the *Hin*dIII restriction site. Can you suggest 3 sizes of PCR product that you may see?

Chapter 5: Checking you've created a recombinant plasmid

Introduction

As part of the gene cloning process, molecular biologists have to check that they have completed each stage successfully and created the recombinant plasmid they need: one with the gene of interest as well as all the necessary components for the protein of interest to be made. The multiple combinations of recombinant plasmids possible, as well as the sources of potential error in any procedure, make it important that the DNA products from each stage of the gene cloning process are carefully checked. Gel electrophoresis is the procedure used to check the size of DNA molecules.

The technique of gel electrophoresis involves placing DNA samples, combined with a loading dye, into the wells of an agarose gel. This gel is then submerged in electrophoresis buffer within a gel electrophoresis tank, and an electric field applied across the gel to separate the DNA. Phosphate groups in the backbone of DNA create a negative charge, so it is loaded near the negative electrode and will migrate through the porous matrix of the agarose gel towards the positive electrode. The DNA molecules are separated primarily according to their molecular size, although molecular shape and degree of charge also influence their movement. Small DNA molecules move through the agarose matrix more easily, so they migrate faster than larger DNA molecules (figure 5.1).

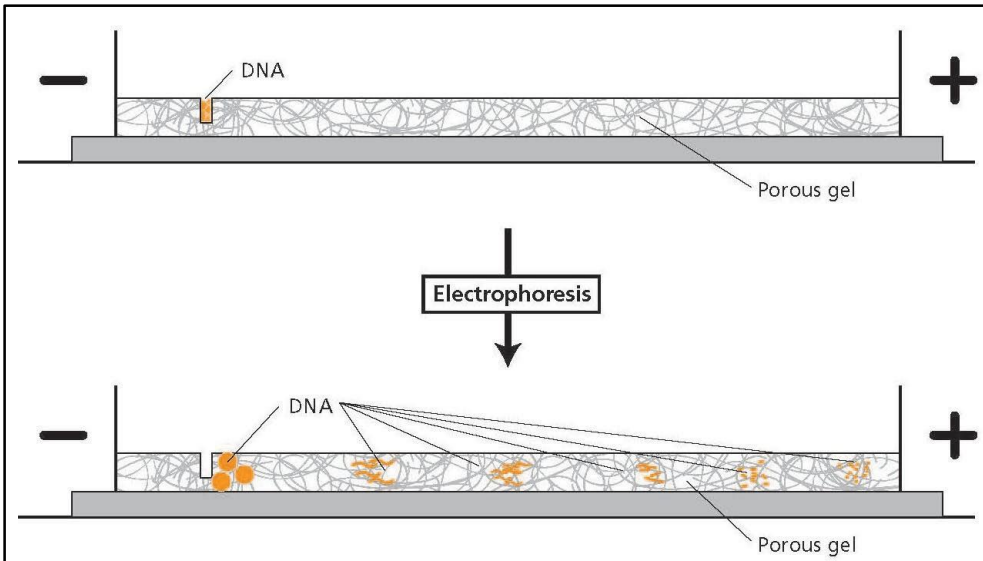


Figure 5.1 Separation of DNA by size using agarose gel electrophoresis.

Although linear pieces of DNA will separate by size as expected, the different configurations (shapes) of plasmids will alter their migration through the gel. There are three different plasmid configurations, which are shown in figure 5.2. The most common plasmid configuration is supercoiled. Supercoiling of DNA is catalysed by an enzyme in bacteria. It is the natural configuration in bacteria and is only seen in plasmids that have been replicated in bacteria. Supercoiling results in a very compact molecule, one that will move through the gel very quickly for its size. The second plasmid configuration is nicked circle. A break in one of the covalent bonds located in the sugar-phosphate backbone of one of the DNA strands along one of the two nucleotide strands means that this circular plasmid configuration will not move through the agarose gel as easily as the supercoiled configuration. The third plasmid configuration is a multimer. This configuration of two or more plasmids that are connected is only seen in plasmids that have been replicated in bacteria, because multimers form when plasmids are replicated so fast that they end up linked together. Unsurprisingly, given their size and shape multimers migrate very slowly through the gel.

Using gel electrophoresis you will examine the size of the pKAN-R and pARA plasmids and the restriction digest fragments (laboratory 2), the products from the ligation (laboratory 3) and the products from the PCRs (laboratory 4). The sizes of the DNA molecules will be determined by comparing them to a DNA ladder: a mixture of DNA fragments of known sizes. (When the DNA ladder is run on gel electrophoresis and stained, the bands that show the fragments look like the rungs of a ladder.) You will use the size of the DNA fragments to judge whether your procedures have been successful in creating the pARA-R recombinant plasmid that contains the *rfp* gene.

In order to visualise the DNA we will use a special stain that interacts with the DNA called GelRed. Then a photographic record of your gel will be made to document this vital step.

Supercoiled Nicked circle Multimer

Figure 5.2 The three different plasmid configurations

Learning objectives

By the end of this laboratory you will be able to:

- Describe why it is important to check the products at each stage of the genetic engineering process
- Predict the relative speed of DNA restriction fragments and plasmids through a gel during gel electrophoresis
- Separate and identify DNA molecules using gel electrophoresis

Laboratory 5: Visualisation of products from restriction digestion, ligation and the PCR

Aims

To check whether each of the experimental stages so far have been successful, using agarose gel electrophoresis to determine the size of the DNA products and compare them to the expected sizes.

Materials

EQUIPMENT & SUPPLIES

P-20 micropipette (2-20 µL)
Disposable pipette tips
7 x 1.5 mL microfuge tubes
Permanent marker
Waste container
Microcentrifuge (shared)
Electrophoresis tank with 0.8% agarose gel (shared)
Plastic gel staining tray
UV transilluminator
Hood
Camera

REAGENTS

Microfuge tubes containing:

- Undigested pKAN-R from lab 2 (K-)
- Digested pKAN-R from lab 2 (K+)
- Undigested pARA from lab 2 (A-)
- Digested pARA from lab 2 (A+)
- the ligation reaction from lab 3 (LIG)
- loading dye (LD)
- distilled water (dH_2O)
- DNA ladder (1kb)

PCR tubes containing:

- the PCR with pARA as template from lab 4 (MMpARA)
- the PCR with ligation reaction as template from lab 4 (MMLIG)

1 X TAE buffer

1.5 X GelRed solution

Health and safety

In this laboratory you will be using biological materials, albeit in tiny amounts, and they should be used in a controlled way, using techniques that mirror the aseptic techniques used in microbiology. All of the materials that come into contact with the biological materials (pipette tips, microfuge tubes and agarose gels) will be autoclaved to denature the DNA and enzymes before disposal.

The loading dye is low hazard, but can stain skin, so try to avoid contact with it as good practice would dictate. You should wear safety glasses and chemical-resistant gloves when using the electrophoresis buffer, to avoid contact with the solution.

The electrophoresis chamber contains 1 X TAE buffer at 100 volts. To prevent contact with the liquid during electrophoresis the tanks are designed so that the electric supply is cut off whenever the lid is removed. Additionally, all connections are plastic coated so that there is no possibility of touching a metal contact which is live at 100 volts.

GelRed is used to stain the DNA. Whilst **not** mutagenic like some stains, this is a substance that can bind to DNA (of which you have lots), therefore you will need to wear chemical resistant gloves, a lab coat and eye protection if you are to complete this part of the procedure.

As good laboratory practice you should clear up any spills immediately and wash your hands well with soap after completing the practical work.

Methods: Electrophoresis

1. Check your rack to make sure that you have all the reagents listed.
2. Use a marker to label seven clean microfuge tubes for the gel electrophoresis samples as follows: gelK-, gelK+, gelA-, gelA+, gelLIG, gelMMpARA and gelMMLIG. (Also include a personal identifier on each tube.)
3. Review table 5.1, which summarises the reagents that you will add in steps 4-8 to make up the samples for gel electrophoresis.

		Restriction digests				Ligation	PCRs	
Step	**Reagent**	**gelK- tube**	**gelK+ tube**	**gelA- tube**	**gelA+ tube**	**gelLIG tube**	**gelMMp-ARA tube**	**gelMM-LIG tube**
4	Distilled water (dH_2O)	3.0 µL	3.0 µL	3.0 µL	3.0 µL	3.0 µL	6.0 µL	6.0 µL
5	Loading dye (LD)	2.0 µL	2.0 µL	2.0 µL	2.0 µL	2.0 µL	2.0 µL	2.0 µL
6a	Undigested pKAN-R (K-)	5.0 µL						
6b	Digested pKAN-R (K+)		5.0 µL					
6c	Undigested pARA (A-)			5.0 µL				
6d	Digested pARA (A+)				5.0 µL			
7	The ligation reaction (LIG)					5.0 µL		
8a	The PCR with pARA as template (MMpARA)						2.0 µL	
8b	The PCR with ligation reaction as template (MMLIG)							2.0 µL

Table 5.1 Reagents to add to the tubes for gel electrophoresis samples

⚠ In steps 4-8, be sure to use a new micropipette tip for each reagent in each tube to avoid contamination.

4. Add the following:
   a. 3.0 µL of distilled water (dH_2O) to the gelK-, gelK+, gelA- and gelA+ tubes.
   b. 3.0 µL of distilled water (dH_2O) to the gelLIG tube.
   c. 6.0 µL of distilled water (dH_2O) to the gelMMpARA and gelMMLIG tubes.
5. Add 2.0 µL of loading dye (LD) to each of the tubes.
6. Add the following:
   a. 5.0 µL of undigested pKAN-R (K-) to gelK– tube.
   b. 5.0 µL of digested pKAN-R (K+) to gelK+ tube.
   c. 5.0 µL of undigested pARA (A-) to gelA- tube.
   d. 5.0 µL of digested pARA (A+) to gelA+ tube.
7. Add 5.0 µL of the ligation reaction (LIG) to the gelLIG tube. Return the "LIG" tube to your teacher for use in the next lab.

8. Add the following:
   a. 2.0 µL of the PCR with pARA as template (MMpARA) to the gelMMpARA tube.
   b. 2.0 µL of the PCR with ligation reaction as template (MMLIG) to the gelMMLIG tube.
9. Spin the seven microfuge tubes (gelK-, gelK+, gelA-, gelA+, gelLIG, gelMMpARA and gelMMLIG) in the microcentrifuge for four seconds to pool the reagents at the bottom of each tube.

⚠ Distribute the tubes evenly in the microcentrifuge so that their weight is balanced.

10. Make sure that the wells in your gel electrophoresis unit are located near the negative (black) electrode.
11. Fill the box with 1x TAE to a level that just covers the entire surface of the gel. If you see any "dimples" over the wells, add more buffer.

⚠ If there are "dimples," add *very small* amounts of buffer to the electrophoresis box. While the gel needs to be completely under the buffer, you don't want too much buffer in the box, as this will allow the electrical current to run through the buffer and not the gel.

12. Make a drawing in your notebook that shows the location of the wells in the electrophoresis box. **The order to load the samples into the wells is shown in figure 5.3.** If two groups are sharing a 15-well gel, there will only be space for one well containing 1 kb DNA ladder.

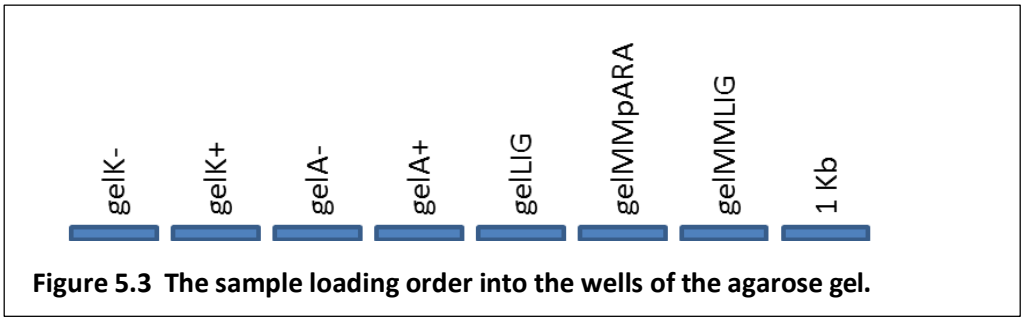


Figure 5.3 The sample loading order into the wells of the agarose gel.

13. Using a fresh pipette tip for each sample, dispense 10.0 µL of the DNA ladder (1 kb), gelK-, gelK+, gelA-, gelA+, gelLIG, gelMMpARA and gelMMLIG into their designated wells. For each sample, do the following:
   a. Place your elbow on the table to steady your pipette hand. If needed, also use your other hand to support your pipette hand.
   b. Lower the pipette tip until it is under the buffer but just above the well (see figure 1.5).

⚠ Be careful not to place your pipette tip into the well or you might puncture the gel and lose your sample out the hole.

⚠Only press the micropipette plunger to the first stop when dispensing samples to avoid getting air bubbles in the buffer. The sample will then sink into the well.

14. When all the samples have been loaded, close the cover tightly over the electrophoresis box. Lower the cover in a horizontal motion, so that the samples don't spill.
15. Connect the electrical leads to the power supply. Connect both leads to the same channel, with negative electrode to negative electrode (black to black) and positive electrode to positive electrode (red to red). See Figure 5.4 below.

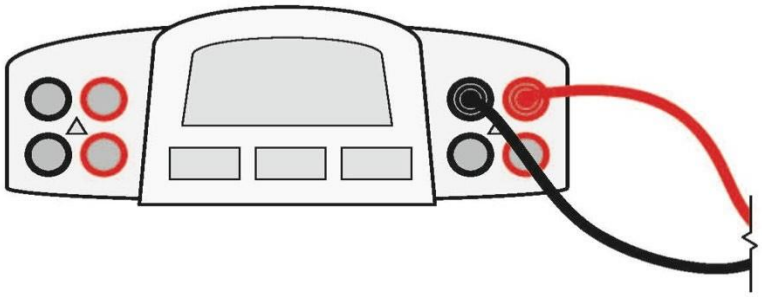


Figure 5.4 Connecting leads from the electrophoresis tank to the powerpack.

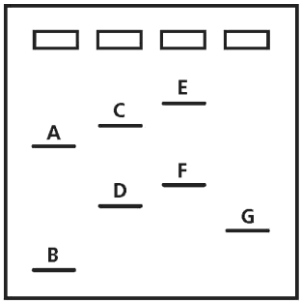
16. Turn on the power supply and set the voltage to approx. 100 V.
17. After two or three minutes, check to see if the purple loading dye (bromophenol blue) is moving toward the positive (red) electrode. If it's moving in the other direction—toward the negative (black) electrode—check the electrical leads to see whether they are plugged in to the power supply correctly.
18. Your teacher will explain what to do with your gel. You may not have sufficient time to complete the electrophoresis. The yellow loading dye will need to run just near the end of the gel. This should take less than50 minutes. After the gel has finished running, it will need to be stained to show the location of the DNA fragments and plasmids.

Methods: Visualisation

1. When electrophoresis is finished, the gel needs to be stained to allow the DNA to be visualised. This will be done using GelRed. Whilst **not** mutagenic like some stains, this is a substance that can bind inside DNA (of which you have lots), therefore you will need to wear chemical resistant gloves, a lab coat and eye protection if you are to complete this part of the procedure.
2. Turn off the Powerpack. Carefully slide the gel from the electrophoresis gel tray into the plastic gel staining tray.
3. Cover the gel with GelRed 1.5 x solution and allow the gel to stain for 30 minutes.
4. Your teacher will tell you whether (i) to pour the GelRed into a foil- covered bottle for reuse, or (ii) to dispose of the GelRed down the sink, washing it away with plenty of water.
5. Wearing chemical resistant gloves, carefully place the gel onto the glass plate of the UV transilluminator. You should be able to see the DNA stain fluorescing without using the hood and camera.
6. Use the hood and camera to take a photo of your gel for analysis.
7. Once you have obtained an image of the gel, place the gel in the box or autoclave bag on your teacher's desk for disposal.

Questions

1. Why do DNA restriction fragments and plasmids separate when analyzed by gel electrophoresis?
2. Why is it important to check each stage in the preparation of a recombinant plasmid?
3. When DNA fragments are joined with a DNA ligase an array of products are created. How does this happen?
4. After different DNA fragments and plasmids have been separated by gel electrophoresis, the gel is stained to show bands that indicate the location of each kind of fragment and plasmid. The drawing of a stained gel opposite shows a series of bands that have been labelled with letters. The locations of the wells are also shown. What is the order of the fragments, from smallest to largest?



5. The DNA is not visible as it moves through the gel. The loading dye contains the three dyes that you separated in Laboratory 1.2. Why is it useful to use the loading dye in this lab?
6. If you used gel electrophoresis to separate the same plasmid that has all three configurations, the supercoiled plasmid would move the fastest, while the multimer would move the slowest. Why do the different plasmid configurations move the way they do through the gel?
7. The pKAN-R and the pARA plasmids you digested in Laboratory 2 were replicated in a bacterial cell. What configurations—supercoiled, nicked circle, and multimer—might these two plasmids have before digestion?
8. The ligation you carried out in Laboratory 3 can result in a number of plasmids, but none of these have been replicated in a bacterial cell. What configurations—supercoiled, nicked circle, and multimer—might the ligated plasmids have?
9. What products might you expect to see from the gelK-, gelK+, gelA-, gelA+, gelLIG, gelMMpARA and gelMMLIG tubes? Create a table that shows all the possible fragments and plasmids by tube. Include the length (bp size) of each fragment or plasmid, and arrange the products found in each microfuge tube by size, from smallest to largest. Include any possible plasmid configurations, and arrange them first by size and next by speed through the gel, from fastest to slowest.

10. The DNA ladder serves as a standard because it contains a mixture of DNA molecules of known sizes. By running your samples and the DNA ladder side by side in your gel, you can estimate the actual size in base pairs of unknown DNA molecules. The DNA Ladder Diagram below shows 10 DNA bands of known sizes. Their sizes are given in kilobase (kb) pairs. 1 kb is 1,000 base pairs (bp). On the diagram predict the positions of DNA bands produced by the possible products found in the gelK-, gelK+, gelA-, gelA+, gelLIG, gelMMpARA and gelMMLIG tubes by indicating their position on the DNA Ladder Diagram (figure 5.5).

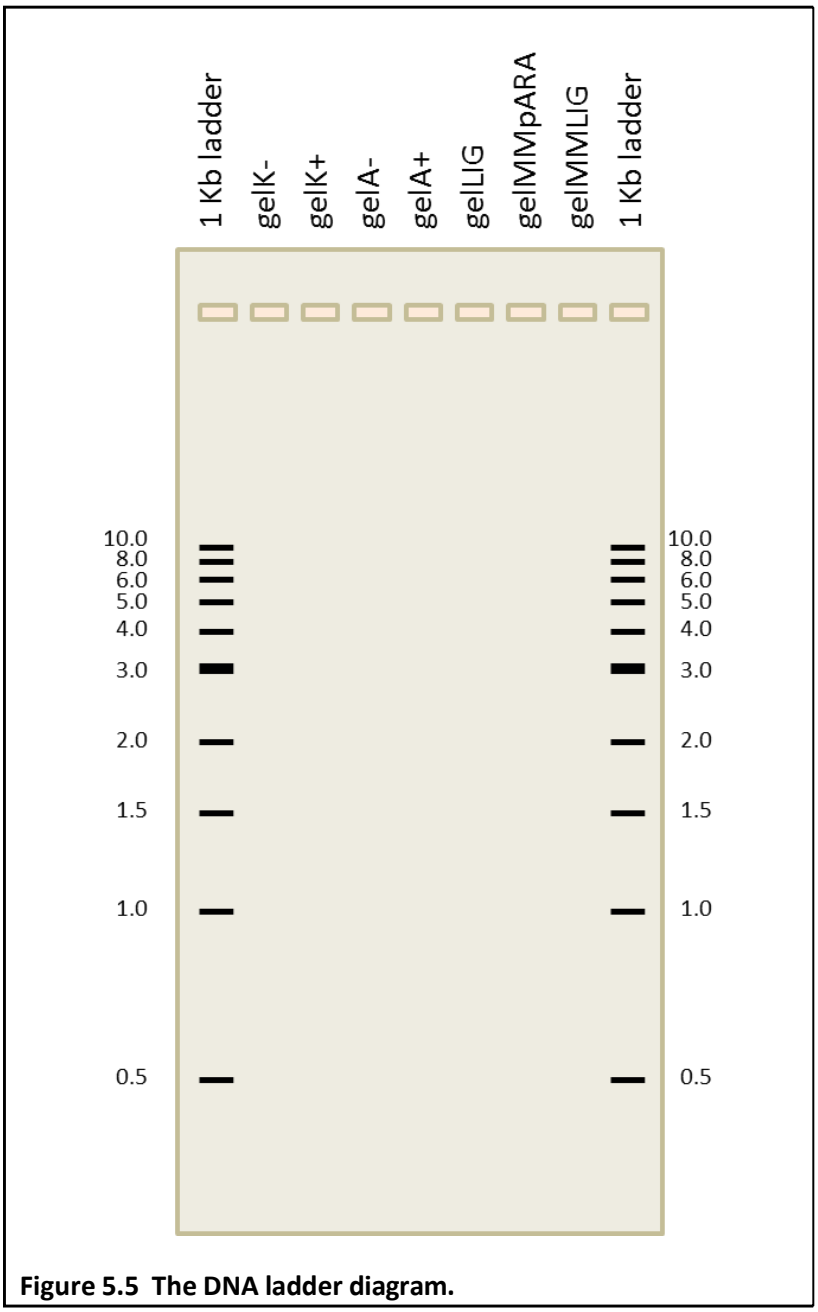


Figure 5.5 The DNA ladder diagram.

11. When you have had a chance to examine your gel photograph, analyse how your actual gel results compare to your gel predictions.
12. Do you see any bands that are not expected? What could explain the origin of these unexpected bands?
13. Does the gel show that your restriction digest and ligation procedures were successful? Describe the evidence you used to make this assessment.
14. In the gelK– and gelA– lanes, do you see evidence of multiple configurations of plasmids? Explain your answer.
15. In the gelK+ and gelA+ lanes, do you see evidence of complete digestion? Explain your answer.
16. In which lanes would you expect to find the *rfp* gene and the *ampR* gene in the gel? Are you able to locate these two genes? Explain your answer.
17. Examine the gelMMLIG lane that contains the PCR carried out using the ligation reaction as template. What length fragments have been ligated to the large pARA restriction fragment to make a recombinant plasmid? Has the small pARA restriction fragment re-inserted? Have you created the desired pARA-R by restriction digestion and ligation? Explain your answer.

Chapter 6: Inserting recombinant plasmids into bacteria

Introduction

Once a recombinant plasmid is created it needs to be replicated and the gene of interest needs to be expressed. Both of these activities can only occur inside a cell, so recombinant plasmids are inserted into a cell. *E. coli* bacterial cells are most commonly used because they replicate quickly and are easy to maintain in a laboratory. Plasmids are inserted into *E. coli* bacteria using a process that is called transformation, because it changes the DNA content of the bacteria. Figure 6.1 depicts a bacterium transformed with plasmids.

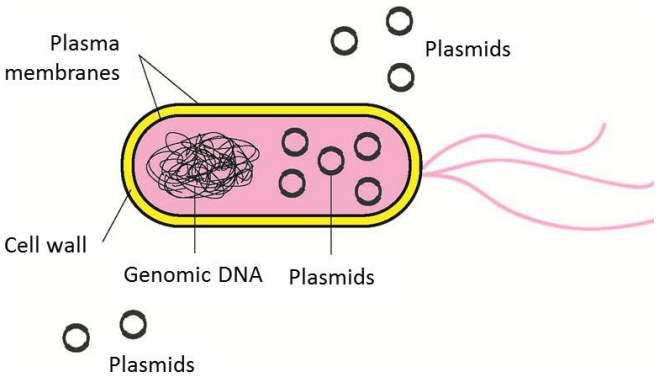


Figure 6.1 A bacterium transformed with plasmids

The uptake of DNA from the environment of a bacterial cell occurs with a very low efficiency in nature. *E. coli* bacteria have complex plasma membranes that separate the external environment from the internal environment of the cell and carefully regulate which substances can enter and exit the cell. In addition, the cell wall is negatively charged and repels negatively charged DNA molecules.

In order to increase the efficiency of DNA uptake, bacteria are treated in two ways. First, the *E. coli* bacteria are placed in a solution that contains positive calcium ions, which neutralize the negative charge on the cells' outer membranes, enabling DNA molecules to cross the membranes and enter the cell. Next, the bacteria are subjected to a heat shock, a sudden increase in temperature, which causes the pressure outside the cell to increase, relative to inside. This pressure difference enables the plasmid DNA to enter the bacterial cell from the outside.

Cells treated with calcium and heat are considered *competent* to take up DNA more efficiently, but even with this treatment only about 1 in 10,000 bacterial cells takes up a plasmid in its environment. Bacteria that have successfully been transformed with a recombinant plasmid can be selected by their resistance to an antibiotic. Only bacterial cells that are expressing the antibiotic resistance proteins will grow.

Once a recombinant plasmid has entered the bacterial cell, DNA polymerase initiates replication at the *ori* site, and multiple copies of the recombinant plasmid are made by the bacterial DNA replication enzymes. Gene expression is initiated from the promoter on the multiple plasmids. The gene of interest is transcribed and translated using the enzymes and molecules within the bacterial cell and the protein of interest is produced. The protein can then act to alter the observable traits of the organism and/or be purified for medicinal use. Medicinal proteins from other organisms can only be made in bacteria using this process of genetic engineering because the way that information is encoded in DNA is universal on Earth.

After confirming that you have created the desired recombinant pARA-R plasmid, containing the *rfp* gene that can make the red fluorescent protein, you next need to transform *E. coli* bacteria with this plasmid. You will be supplied with *E. coli* bacteria that have been pre-treated with calcium chloride, which you will divide into two groups: a control group to which no plasmid is added, and a treatment group to which you add the pARA-R plasmid. After heat-shocking both groups of cells, you will grow them: (i) on Luria Bertani agar (a medium that supplies the nutrients and support for bacterial growth), and; (ii) on Luria Bertani agar with the antibiotic ampicillin. In addition, the treatment group will be grown in the presence of Luria Bertani agar, ampicillin, and the sugar arabinose.

By examining the growth of bacteria under these conditions, you can verify that your procedure worked, and you can identify the bacteria transformed with the pARA-R plasmid. If you are successful the bacteria will have a new and highly visible trait: It will now produce red fluorescent protein, which makes the cells red or bright pink!

Learning objectives

By the end of this laboratory you will be able to:

- Describe the role of transformation in the gene cloning process
- Explain the purpose of each control in the transformation experiment
- Explain how the information encoded in a gene is expressed as a trait

Laboratory 6: Transforming bacteria with the ligation products

Aims

To insert the recombinant plasmids made by restriction and ligation into bacterial cells, using a procedure known as bacterial transformation, then to select the bacteria with the desired recombinant plasmid, by growing them under different conditions.

Materials

EQUIPMENT & SUPPLIES

P-20 micropipette (2-20 µL)
P-200 micropipette (50-200 µL)
Disposable pipette tips
Permanent marker
2 x 1.5 mL microfuge tubes
Waste container
Floating tube rack
Timer
Sticking tape
Pack of cell spreaders (shared)
Polystyrene cup of crushed ice in cold water
42°C water bath (shared)
37°C incubator (shared)
Autoclave bag for disposal (shared)

REAGENTS

Microfuge tubes containing:

- Ligated plasmid from Laboratory 3 (LIG)
- Luria Bertani broth (LB broth)

Petri dishes with agar:

- 1 of LB
- 1 of LB / amp
- 1 of LB / amp / ara

A microfuge tube of chilled competent *E. coli* cells (CC) in a cup of iced water

⚠ The competent cells MUST be kept on ice at all times.

Health and safety

Aseptic technique must be used when undertaking microbiological work. This is described in detail on pages A.1 – A.3. If good aseptic technique is used you do not need to wear gloves or eye protection. After the bacteria are spread on the agar plates the lids must be taped down in 3, evenly spaced, places around the circumference of the petri dish.

All of the materials that come into contact with the biological materials (pipette tips, microfuge tubes, cell spreaders and agar plates) will be autoclaved prior to disposal.

As good laboratory practice you should clear up any spills immediately and wash your hands well with soap after completing the practical work.

Methods

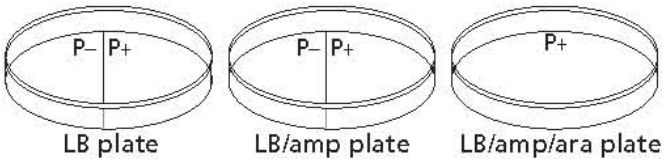
1. Check your rack to make sure that you have all the reagents listed.
2. Obtain a CC tube from the ice-filled container and place it in a polystyrene cup of ice.

⚠ The competent cells in this lab MUST be kept cold. Hold microfuge tubes by the upper rim to avoid warming the cells.

3. Label two clean microfuge tubes 'P–' and 'P+'
4. Place the P– and P+ tubes in the polystyrene cup of ice with the CC tube.
5. Using the large P-200 micropipette, add the competent cells from the CC tube to the P– and P+ tubes. To do this:
   a. Set the P-200 micropipette to 50 µL.
   b. Very carefully, re-suspend the bacterial cells in the CC tube by gently pumping the pipette two times in the solution.
   c. Add 50 µL of CC to each of the empty chilled tubes (P– and P+), holding each tube at its rim to keep it cold, and return each tube quickly to the ice.
6. Using the P-20 pipette, add 10.0 µL of your ligated products (LIG) to the tube labelled 'P+'. To do this:
   a. Set the P-20 micropipette to 10.0 µL.
   b. Hold the chilled P+ tube by the upper rim and add 10.0 uL of LIG. Mix the solutions by pumping the pipette two times in the liquids, and return the P+ tube to the ice.

⚠ To avoid contamination, be sure to use a new micropipette tip for each addition.

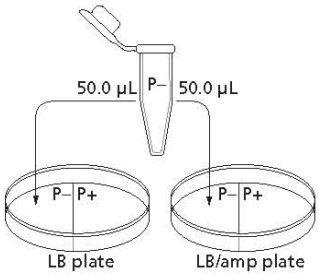
7. Keep the P– and P+ tubes on ice for 15 minutes.
8. While the cells are on ice, prepare your three agar Petri plates— one plate each of LB, LB/amp, and LB/amp/ara:
   a. Label the bottom of each plate (the part that contains the agar) in small writing at the edge of the plate with your personal identifier.
   b. With the plates closed, draw a line on the LB plate and the LB/amp plate that divides each plate in the middle. Label half of each plate 'P-', and the other half 'P+'. Label the LB/amp/ara plate 'P+'. The plates should be arranged as below:



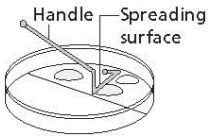
9. Following the 15-minute incubation on ice, carry the P– and P+ tubes (in the cup of ice) to the 42°C water bath. Place the two tubes in the floating microfuge tube rack in the water bath for EXACTLY 45 seconds.
10. After the 45-second heat shock, immediately place the tubes back on ice and **leave them there for at least a minute**.
11. Using the P-200 micropipette, add LB to the P– and P+ tubes, using a new tip for each addition. To do this:
    a. Set the P-200 micropipette to 150 µL.
    b. Add 150 µL of LB to the P– tube. Cap the tube and gently flick it two or three times to mix. Place in rack at room temperature.
    c. Add 150 µL of LB to the P+ tube. Cap the tube and gently flick it two or three times to mix. Place in rack at room temperature.

d. Leave to stand for 5 minutes before 'plating out' (putting onto agar plates)

12. To add cells from the P– tube onto your LB and LB/amp plates:
    a. Set the P-200 micropipette to 50 µL.
    b. Gently pump the pipette two or three times in the P– tube to suspend the cells.
    c. Open the lid of the LB plate, like a "clamshell," and add 50 µL of cells from the P– tube to the section marked 'P–'. Close the lid.
    d. Again, gently pump the pipette two or three times in the P– tube to suspend the cells.
    e. Open the lid of the LB/amp plate, like a clamshell, and add 50 µL of cells from the P– tube to the section marked 'P–'. Close the lid.



13. To spread the cells from the P– tube on your LB and LB/amp plates:
    a. Open the package of sterile cell spreaders at the end closest to the spreader handles. Remove only one spreader, and close the package to keep the others sterile.
    b. Open the lid to the LB plate, like a clamshell, and spread the cells evenly across the entire P– side of the plate by gently moving the spreader across the agar surface. Close the lid.
    c. Carefully spread the P– cells on the LB/amp plate, using the same spreader and technique.



⚠ Hold the spreader by the handle and do not allow the bent end to touch any surface, as this will contaminate the spreader. Place the used spreader in disposal for autoclaving.

14. To add cells from the P+ tube to your LB, LB/amp, and LB/amp/ara plates:
    a. Make sure that the P-200 micropipette is set to 50 µL.
    b. Gently pump the pipette two or three times in the P+ tube to suspend the cells.
    c. Open the lid of the LB plate, like a clamshell, and add 50 µL of cells from the P+ tube to the section marked 'P+'. Close the lid.
    d. Again, gently pump the pipette two or three times in the P+ tube to suspend the cells.
    e. Open the lid of the LB/amp plate, like a clamshell, and add 50 µL of cells from the P+ tube to the section marked 'P+'. Close the lid.
    f. Set the P-200 micropipette to 100 µL, gently pump the pipette two or three times in the P+ tube, and load 100 µL of the P+ cells.
    g. Open the lid of the LB/amp/ara plate, like a clamshell, and add 100 µL of P+ cells to various areas across the surface—not just a single spot. Close the lid.
15. To spread the cells from the P+ tube on LB, LB/amp, and LB/amp/ara plates:

a. Open the package of sterile cell spreaders at the end closest to the spreader handles. Remove only one spreader, and close the package to keep the others sterile.

b. Open the lid to the LB plate, like a clamshell, and evenly spread the cells on the P+ side of the plate (and only on this side) by gently moving the spreader across the agar surface. Close the lid.

⚠ Hold the spreader by the handle and do not allow the bent end to touch any surface, as this will contaminate the spreader.
Place the used spreader in disposal for autoclaving.

c. Carefully spread the P+ cells on the LB/amp plate using the same spreader and technique.

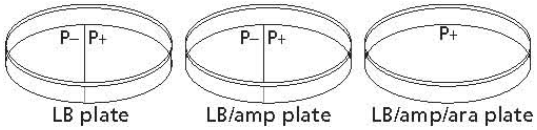
d. Carefully spread the P+ cells on the LB/amp/ara plate using the same spreader and technique. Gently rotate the plate beneath the P+ spreader so that the cells can be spread over the entire surface of this plate. Close the lid.

16. Allow all three plates to sit right side up for five minutes to allow them to dry a bit.
17. Using the sticky tape provided, secure each plate 3 times so that the lid will not come off. Make sure your plates are marked with a personal identifier.
18. Stack the 3 plates upside down to prevent condensation from dripping onto the agar and place the plates in the 37°C incubator.
19. Place all microfuge tubes, pipette tips, and cell spreaders in the autoclave bag.
20. Incubate the plates for 24–36 hours at 37°C.
21. Examine the plates and record the amount of growth on each half.
22. Place the plate on the UV transilluminator to observe the red fluorescence and use the hood and camera to make a photographic record.
23. Discard the Petri plates in the autoclave bag when directed to do so.

Questions

1. What are the steps involved in transcription and translation of a gene?
2. What is the relationship among genes, proteins, and traits (or observable characteristics)?
3. What do bacteria and humans have in common that makes it possible for a human gene to be expressed in bacteria?
4. Why is it important that the membranes of *E. coli* bacteria carefully regulate which substances can enter and exit the cell?
5. How are the P+ bacteria treated differently from the P– bacteria? What is the purpose of the P– bacteria?
6. Why is it important to use aseptic technique in this lab?
7. Why do the cells need time to recover after the heat shock?
8. Why are the cells incubated at 37°C?

9. Ampicillin is an antibiotic that kills bacterial cells by disrupting the formation of cell walls. However, the pARA-R plasmid has the ampicillin resistance gene, which produces a protein that breaks down ampicillin. What is the purpose of growing bacteria that have been transformed in the presence of ampicillin?
10. What will happen when bacterial cells that contain the pARA-R plasmid are not given arabinose?
11. You add samples of the control group P– and the treatment group P+ to plates that contain various combinations of Luria Bertani agar (LB), ampicillin, and the sugar arabinose. What are your predictions for the growth you expect on each of the plates?



12. Look at the results of your transformation. Do your actual results match your predicted results? If not, what differences do you see, and what are some explanations for these differences?
13. How many red colonies were present on your LB/amp/ara plate?
14. Why did the red colonies only appear on the LB/amp/ara plate and not the LB/amp plate?
15. Recombinant plasmids are engineered so that they can replicate in the cell independently of the chromosome replication. Why is it important to have multiple copies of a recombinant plasmid within a cell?
16. How is the information encoded in the *rfp* gene expressed as a trait? Be sure to use what you have previously learned about gene expression and the relationship between DNA, RNA, protein, and traits.
17. Why is it possible for bacteria to make a human protein, such as insulin, or a sea anemone protein, such as the red fluorescent dye?
18. The only bacteria that could produce the red fluorescent protein in Laboratory 6 were bacteria that were transformed with the pARA-R plasmid. Why?

Chapter 7: Correlating DNA fragment size (genotype) with phenotype

Introduction

After transformation of *E. coli* with the plasmids created by ligating restriction fragments of pARA and pKAN-R, you would expect to see both white and red bacterial colonies on the LB agar plate containing ampicillin and arabinose. Bacteria that are able to survive on this plate must have been successfully transformed with the restriction fragment from the pARA plasmid that contains the *ori* site and ampicillin resistance gene. However, this restriction fragment could have created circularised plasmid DNA during ligation with a variety of different other restriction fragments. The plasmid introduced during transformation may or may not have the *rfp* gene. Since the *rfp* gene from the plasmid is required for the expression of red fluorescent protein, you would expect that white colonies on the LB/amp/ara agar plate would not have the *rfp* gene and red colonies on the same plate would have the *rfp* gene.

It is possible to check whether a DNA fragment corresponding to the size of the *rfp* gene in the pARA plasmid is present within the transformed bacteria using a technique called 'colony PCR'. This allows you to correlate the phenotype (colour of the bacterial colonies) with the genotype (whether there is a fragment of DNA the correct size to be the *rfp* gene).

A colony PCR involves simply taking some of the transformed bacterial colony and using it to provide the DNA template in a PCR with sequence specific primers that bracket the region of DNA of interest. During a PCR the denaturation step (figure 4.1) heats the reaction mixture to 95°C, which disrupts the structure of the thin cell wall and cell membrane of *E. coli*. The bacterial DNA is released into the PCR mixture and becomes available for the primers to bind to during the annealing step. As in laboratory 4, the same two primers that bind specifically to the DNA sequence of the pARA plasmid 136 bp from the *Bam*HI restriction site and 149 bp from the *Hin*dIII restriction site will be used to amplify the DNA ligated to the large pARA restriction fragment (see figure 4.2). The DNA product of the PCR is then produced during the extension step.

Two recombinant plasmids that could be inserted into the *E. coli* are shown in figure 7.1. Red colonies are expected to contain the pARA-R plasmid, including the *rfp* gene, which will produce a 1092bp PCR fragment. White colonies may contain a re-ligated pARA plasmid, which will produce a 662 bp PCR fragment. White colonies may also contain other ligated plasmids.

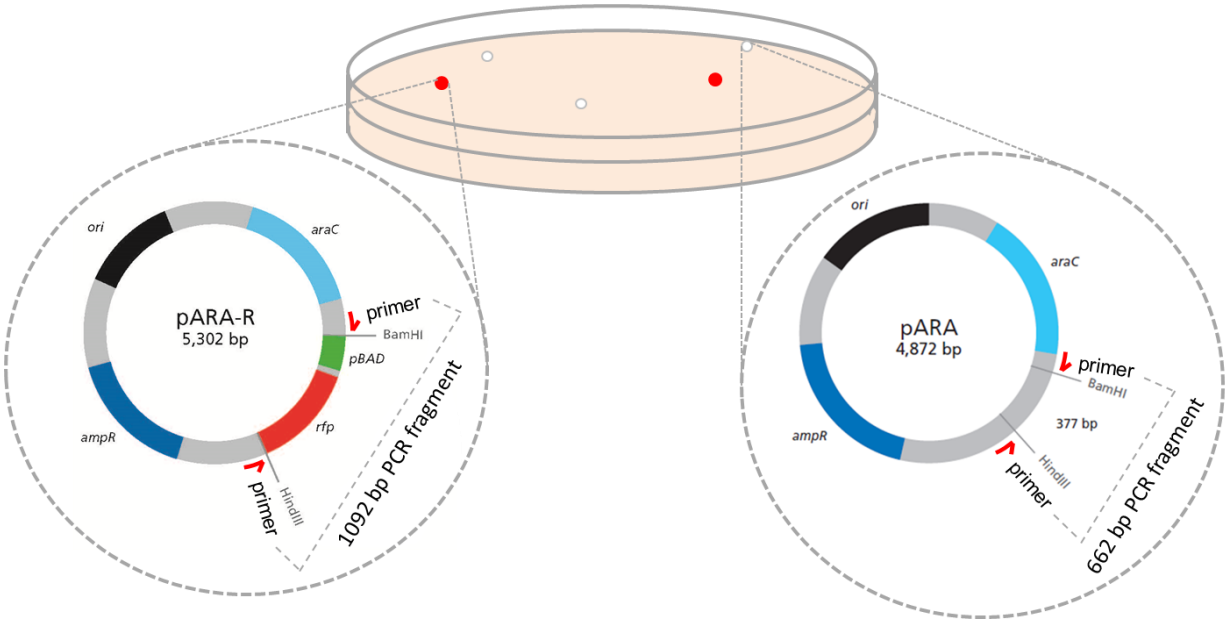


Figure 7.1 Red and white colonies on a LB/amp/ara plate, the plasmids they contain and expected size of PCR fragments.

In this lab you will select a red colony and a white colony from your transformation plate and perform colony PCR to detect whether a DNA fragment corresponding to the size of pARA-R is present or not. It is also good practice to perform the PCR with: (i) DNA template known to contain the *rfp* gene – this confirms that the PCR is working and the expected DNA size (1092 bp); (ii) DNA template known not to contain the *rfp* gene – this confirms that the PCR is working and the expected DNA size (662 bp), and (iii) no DNA template – this is a negative control, used to confirm that the reagents are not contaminated with DNA. Rather than get each pair to set up these additional reactions, your teacher will set up these 3 PCRs and run them in the PCR machine with your reactions, so that you can see the results afterwards using agarose gel electrophoresis.

Learning objectives

By the end of this laboratory you will be able to:

- Explain how PCR can be used to amplify a specific DNA sequence
- Understand the link between an organism's genotype and phenotype
- Describe the size of possible PCR products from your transformed bacteria

Laboratory 7.1: Colony PCR from the transformed bacteria

Aims

To use the enzyme DNA polymerase in a **P**olymerase **C**hain **R**eaction (PCR), catalysing the extension of sequence specific primers to discover the size of the restriction fragments ligated to the larger pARA restriction digest fragment in the plasmids of red colonies and white colonies.

Materials

EQUIPMENT & SUPPLIES

P-20 micropipette (2-20 µL)
Disposable pipette tips
Permanent marker
Polystyrene cup of ice
Waste container
Microcentrifuge (shared)
PCR machine (shared)

REAGENTS

2 PCR tubes of 2 X PCR master mix (MM)

Microfuge tubes containing:

- Combined forward and reverse primer (PRI)
- Nuclease-free water (H_2O)

Your returned LB/amp/ara plates from lab 6

Health and safety

The colony pick PCR involves opening agar plates containing the genetically modified bacteria that you have created. As it is not good practice to open agar plates after incubation that contain unknown microbes, only uncontaminated agar plates (containing only the expected red and white *E. coli* colonies) should be used and your teacher will direct you to work where lower school students will not observe and be tempted to copy this procedure. Whilst touching the pipette tip to the bacterial colony and transferring it into the PCR tube you will need to use aseptic technique. All material that comes into contact with microbiological material will be autoclaved to heat sterilise it before disposal.

In this laboratory you will also be using biological materials, albeit in tiny amounts, and they should be used in a controlled way, using techniques that mirror the aseptic techniques used in microbiology. All of the materials that come into contact with the biological materials (pipette tips, PCR tubes and microfuge tubes) will be autoclaved to denature the DNA and enzymes before disposal. As good laboratory practice you should clear up any spills immediately and wash your hands well with soap after completing the practical work.

Methods

1. Check your rack to make sure that you have all the reagents listed:
   a. A polystyrene cup, containing ice, with 2 PCR tubes (MM) in it. *This is a mixture of the buffer, magnesium chloride, dNTPs and DNA polymerase from a thermophilic bacteria, which are required for a PCR*.

   b. A LB/amp/ara plate with your transformed colonies on, returned to you from a previous class (Laboratory 6).

The bacterial colonies will provide the template DNA for the PCR.

c. A microfuge tube containing forward and reverse primers (PRI). *Primers are short stretches of DNA that bind to complementary DNA sequence during the annealing step, allowing DNA polymerase to extend a specific part of the DNA*.

d. A microfuge tube containing nuclease-free water (H_2O).
This will be used to make up the reaction volume.

2. Use a marker to label the 2 PCR tubes containing 2 X PCR master mix (MM) on the side with your personal identifier. Label one tube 'red' and the other tube 'white'. Return them to the ice.

⚠ Marker pen can rub off the top of the PCR tubes in the PCR machine, so make sure you label them on the side.

3. Review table 7.1, which summarises the reagents that you will add in steps 4-5.

Step	Reagent	Red PCR	White PCR
4a	2 X PCR master mix (MM)	12.5 µL	12.5 µL
4b	Nuclease-free water (H_2O)	9.5 µL	9.5 µL
4c	Combined primers (PRI)	2.0 µL	2.0 µL
5a	Red bacteria	~1.0 µL	
5b	White bacteria		~1.0 µL

Table 7.1 Reagents to add to the red and white PCR tubes

⚠ In step 4, be sure to use a new micropipette tip for each reagent in each tube to avoid contamination.

4. a. To your labelled PCR tube of master mix add the following:
   b. 9.5 µL of nuclease-free water (H_2O) to each PCR tube.
   c. 2.0 µL of combined forward and reverse primers (PRI) to each tube.
5. To introduce DNA from the transformed bacteria into the PCR you will use aseptic technique (see pages A.1 – A.3). Select one red colony and one white colony for use. Mark their position using permanent marker on the base of the petri dish.
   a. Place a fresh tip on a P-20 micropipette. Opening the petri dish like a 'clamshell' facing away from yourself, touch a sterile pipette tip to the circled red colony. Close the transformation plate. Taking care not to touch anything else with the micropipette tip, lower it into the 'red' PCR tube. Gently pipette the PCR mixture up and down about 10 times to transfer the bacteria into the PCR mixture. Dispose of the pipette tip in the waste container.
   b. Place a fresh tip on a P-20 micropipette. Opening the petri dish like a 'clamshell' facing away from yourself, touch a sterile pipette tip to the circled white colony. Close the transformation plate. Taking care not to touch anything else with the micropipette tip, lower it into the 'white' PCR tube. Gently pipette the PCR mixture up and down about 10 times to transfer the bacteria into the PCR mixture. Dispose of the pipette tip in the waste container.
6. Ensure the reagents are well mixed, by pumping gently up and down with the pipette or simply swirling with the tip. Keep your reactions on ice until the PCR machine can be started.

7. The PCR programme that you will use is given in table 7.2. It takes about 1½ hours to complete.

Reagent		Temperature (°C)	Time (sec)
Initial denaturation		95	200
30 cycles of	Denaturation	95	30
	Annealing	53	30
	Extension	68	30
Final extension		68	300
Hold		10	Indefinite

Table 7.2 The PCR programme

8. When your teacher tells you, transfer your PCR tubes from the ice into the PCR machine and start the reaction. You will need to include some 'control samples' they have prepared
9. After the PCR programme is complete your PCR tubes can be place in the fridge (4°C) until you are ready to use them.

Questions

1. What is the structure of a prokaryotic cell? Do prokaryotes have cell walls? What about cell membranes and nuclear membranes?
2. Why does DNA need to be extracted from animal cells for use in PCR (laboratory 8), but not from bacterial cells?
3. What is aseptic technique? Why do you need to use it when performing colony PCR? What constitutes good aseptic technique?
4. Why does the *Taq* DNA polymerase not denature during the denaturation step at 95°C in your PCR programme?
5. What plasmid constructs could be present in the transformed bacteria that produce white colonies on the LB/amp/ara plates? Can you suggest 3 possible constructs?

Laboratory 7.2: Visualisation of products from the colony PCR

Aims

To visualise the size of PCR products from red and white colonies using agarose gel electrophoresis, allowing the size of the DNA products (which is dependent on the genotype) to be correlated with the colour of the transformed bacteria (the phenotype).

Materials

EQUIPMENT & SUPPLIES

P-20 micropipette (2-20 µL)
Disposable pipette tips
2 x 1.5 mL microfuge tubes
Permanent marker
Waste container
Microcentrifuge (shared)
Electrophoresis tank with 0.8% agarose gel (shared)
Plastic gel staining tray
UV transilluminator
Hood
Camera

REAGENTS

Microfuge tubes containing:

- loading dye (LD)
- distilled water (dH_2O)
- DNA ladder (1kb)

PCR tubes containing:

- the PCR with a white colony as template from lab 7.1 (MMwhite)
- the PCR with a red colony as template from lab 7.1 (MMred)

1 X TAE buffer

1.5 X GelRed solution

Health and safety

In this laboratory you will be using biological materials, albeit in tiny amounts, and they should be used in a controlled way, using techniques that mirror the aseptic techniques used in microbiology. All of the materials that come into contact with the biological materials (pipette tips, microfuge tubes and agarose gels) will be autoclaved to denature the DNA and enzymes before disposal.

The loading dye is low hazard, but can stain skin, so try to avoid contact with it as good practice would dictate. You should wear safety glasses and chemical-resistant gloves when using the electrophoresis buffer, to avoid contact with the solution.

The electrophoresis chamber contains 1 X TAE buffer at 100 volts. To prevent contact with the liquid during electrophoresis the tanks are designed so that the electric supply is cut off whenever the lid is removed. Additionally, all connections are plastic coated so that there is no possibility of touching a metal contact which is live at 100 volts.

GelRed is used to stain the DNA. Whilst **not** mutagenic like some stains, this is a substance that can bind to DNA (of which you have lots), therefore you will need to wear chemical resistant gloves, a lab coat and eye protection if you are to complete this part of the procedure.

As good laboratory practice you should clear up any spills immediately and wash your hands well with soap after completing the practical work.

Methods: Electrophoresis

1. Check your rack to make sure that you have all the reagents listed.
2. Use a marker to label two clean microfuge tubes for the gel electrophoresis samples: gelwhite and gelred. (Also include a personal identifier on each tube.)
3. Review table 7.3, which summarises the reagents that you will add in steps 4-6 to make up the samples for gel electrophoresis.

Step	Reagent	Gelwhite tube	Gelred tube
4	Distilled water (dH_2O)	6.0 µL	6.0 µL
5	Loading dye (LD)	2.0 µL	2.0 µL
6a	The PCR with a white colony as template from lab 7.1 (MMwhite)	2.0 µL	
6b	The PCR with a red colony as template from lab 7.1 (MMred)		2.0 µL

Table 7.3 Reagents to add to the tubes for gel electrophoresis samples

⚠ In steps 4-6, be sure to use a new micropipette tip for each reagent in each tube to avoid contamination.

4. Add 6.0 µL of distilled water (dH_2O) to the gelwhite and gelred tubes.
5. Add 2.0 µL of loading dye (LD) to each of the tubes.
6. Add the following:
   a. 2.0 µL of the PCR with a white colony as template from lab 7.1 (MMwhite) to the gelwhite tube.
   b. 2.0 µL of the PCR with a red colony as template from lab 7.1 (MMred) to the gelwhite tube.
7. Spin the two microfuge tubes (gelwhite and gelred) in the microcentrifuge for four seconds to pool the reagents at the bottom of each tube.

⚠ Distribute the tubes evenly in the microcentrifuge so that their weight is balanced.

8. Make sure that the wells in your gel electrophoresis unit are located near the negative (black) electrode.
9. Fill the box with 1x TAE to a level that just covers the entire surface of the gel. If you see any "dimples" over the wells, add more buffer.

⚠ If there are "dimples," add *very small* amounts of buffer to the electrophoresis box. While the gel needs to be completely under the buffer, you don't want too much buffer in the box, as this will allow the electrical current to run through the buffer and not the gel.

10. Make a drawing in your notebook that shows the location of the samples in wells in the electrophoresis box. The order to load the samples into the wells is shown in Figure 7.2. If two groups are sharing an 8-well gel, there will be space for one well containing 1 kb DNA ladder and the three samples prepared by the teacher.

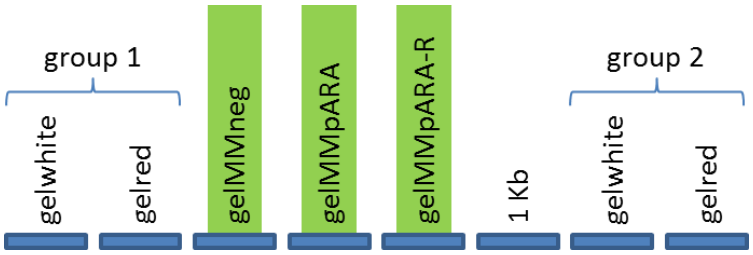


Figure 7.2 A sample loading order into the wells of the agarose gel. The samples shown in green are prepared by the teacher.

11. Using a fresh pipette tip for each sample, dispense 10.0 µL of the DNA ladder (1 kb), gelMMneg, gelMMpARA and, gelMMpARA-R into the designated wells for each gel. Dispense 10.0 µL of the gelwhite and gelred samples for each pair into their designated wells. For each sample, do the following:
    a. Place your elbow on the table to steady your pipette hand. If needed, also use your other hand to support your pipette hand.
    b. Lower the pipette tip until it is under the buffer but just above the well.

⚠ Be careful not to place your pipette tip into the well or you might puncture the gel and lose your sample out the hole.

⚠ Only press the micropipette plunger to the first stop when dispensing samples to avoid getting air bubbles in the buffer. The sample will then sink into the well.

12. When all the samples have been loaded, close the cover tightly over the electrophoresis box. Lower the cover in a horizontal motion, so that the samples don't spill.
13. Connect the electrical leads to the power supply. Connect both leads to the same channel, with negative electrode to negative electrode (black to black) and positive electrode to positive electrode (red to red). See figure 5.4.
14. Turn on the power supply and set the voltage to approx. 100 V.
15. After two or three minutes, check to see if the purple loading dye (bromophenol blue) is moving toward the positive (red) electrode. If it's moving in the other direction—toward the negative (black) electrode—check the electrical leads to see whether they are plugged in to the power supply correctly.
16. Your teacher will explain what to do with your gel. You may not have sufficient time to complete the electrophoresis. The yellow loading dye will need to run just before the end of the gel. This takes about 40 minutes. After the gel has finished running, it will need to be stained to show the location of the DNA fragments and plasmids

Methods: Visualisation

1. When electrophoresis is finished, the gel needs to be stained to allow the DNA to be visualised. This will be done using GelRed. Whilst **not** mutagenic like some stains, this is a substance that can bind inside DNA (of which you have lots), therefore you will need to wear chemical resistant gloves, a lab coat and eye protection if you are to complete this part of the procedure.
2. Turn off the Powerpack. Carefully slide the gel from the electrophoresis gel tray into the plastic gel staining tray.
3. Cover the gel with GelRed 1.5 x solution and allow the gel to stain for 30 minutes.
4. Your teacher will tell you whether (i) to pour the GelRed into a foil- covered bottle for reuse, or (ii) to dispose of the GelRed down the sink, washing it away with plenty of water.
5. Wearing chemical resistant gloves, carefully place the gel onto the glass plate of the UV transilluminator. You should be able to see the DNA stain fluorescing without using the hood and camera.
6. Use the hood and camera to take a photo of your gel for analysis.
7. Once you have obtained an image of the gel, place the gel in the box or autoclave bag on your teacher's desk for disposal.

Questions

1. What is the purpose of including a negative control PCR, without a DNA template?
2. What is the purpose of the PCRs that have pARA and pARA-R as template? What size fragments would you expect to see produced by PCR with these plasmids as template?
3. What products might you expect to see from the gelwhite and gelred tubes? Include the length (bp size) of each possible PCR product.
4. The DNA ladder serves as a standard because it contains a mixture of DNA molecules of known sizes. By running your samples and the DNA ladder side by side in your gel, you can estimate the actual size in base pairs of unknown DNA molecules. The DNA ladder diagram below shows 10 DNA bands of known sizes. Their sizes are given in kilobase (kb) pairs. 1 kb is 1,000 base pairs (bp). On the diagram predict the positions of DNA bands produced by the possible products found in the gelMMpARA, gelMMpARA-R, gelwhite and gelred tubes by indicating their position on the DNA Ladder Diagram (figure 7.3).

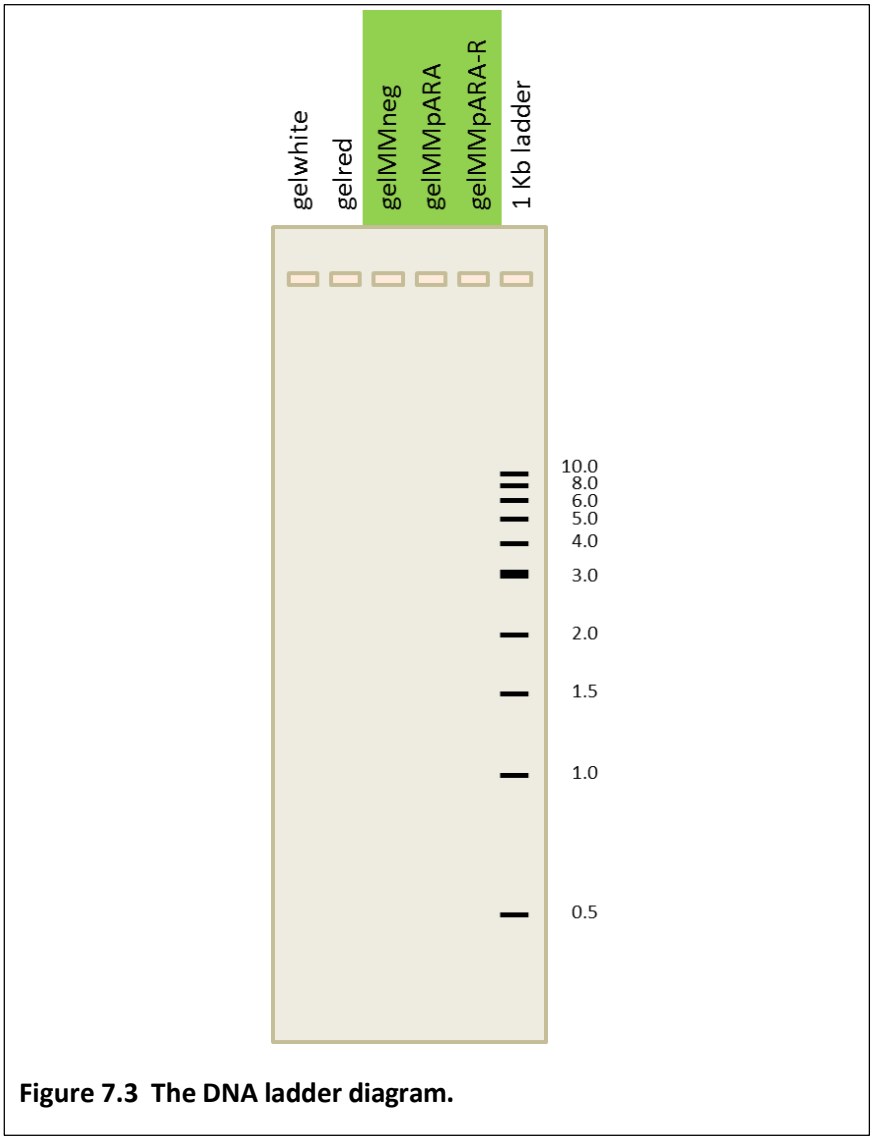


Figure 7.3 The DNA ladder diagram.

5. When you have had a chance to examine your gel photograph, analyse how your actual gel results compare to your gel predictions.
6. Do you see any bands that are not expected? What could explain the origin of these unexpected bands?
7. Can you work out what the plasmid constructs in the colonies you have analysed must be to produce the size of the DNA fragments after PCR that you observe? Does the genotype you have suggested correlate with the phenotype of the bacterial colony?
8. How does the genetic information, in the form of DNA, produce the phenotypes that you observe?

Chapter 2a: Examining the engineered plasmid pARA-R using restriction digestion

Introduction

Plasmids and restriction enzymes are biomolecules found in many bacteria. Their discovery was crucial to genetic engineering as they are used in the process that scientists have developed to modify the genetic material in microorganisms so that they produce useful biomolecules. The biomolecules that microorganisms produce can be medicines that save lives, such as insulin. This chapter is about plasmids, restriction enzymes and their role in cloning a gene (that is copying a gene from one location to another).

Many bacteria carry two forms of DNA: (1) a single chromosome made up of a large DNA molecule that contains all the information needed by the organism to survive and reproduce, and (2) plasmids, which are small circular DNA molecules, present in multiple copies, separate from the chromosomal DNA. This is shown in figure 2a.1.

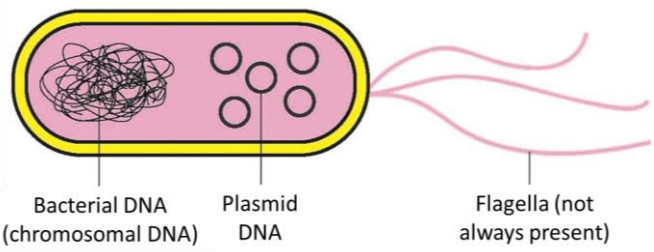


Figure 2a.1. DNA in bacterial cells

Plasmids are vectors: that is vehicles for carrying DNA sequences from one organism to another. They are replicated within cells. Cells containing a plasmid can be selected by their resistance to antibiotics and if a gene of interest is inserted by the promoter sequence, the gene of interest will be expressed. So, in simple terms, if the plasmid DNA can be cut up and stuck back together to include the gene of interest, then bacteria containing the plasmid will make the gene product of interest.

The discovery of restriction enzymes in the early 1950s provided the tool for 'cutting up' the DNA sequence. Restriction enzymes are proteins that bacteria produce to recognise and destroy bacteriophage (viruses that infect bacteria) DNA without damaging the host (bacterial) DNA. Restriction enzymes from different strains of bacteria cut at specific DNA sequences, called recognition sites.

During this laboratory you will use restriction enzymes to examine a recombinant plasmid that has been engineered to express the *rfp* gene, which produces the Red Fluorescent Protein (RFP), which causes a bacterium carrying this recombinant plasmid to fluoresce red. The recombinant plasmid is named pARA-R, and in addition to the *rfp* gene and its adjacent promoter sequence (*pBAD*), this plasmid contains the site for initiating DNA replication (*ori*), the gene that makes bacteria resistant to the antibiotic ampicillin (*ampR*) and an activator (*araC*).

The ampicillin resistance gene (*ampR*) encodes the protein beta lactamase, an enzyme that destroys the antibiotic ampicillin enabling bacteria to reproduce in the presence of ampicillin. The arabinose activator (*araC*) is a specific DNA sequence that activates a promoter when the bacteria are grown in the presence of arabinose, a five-carbon sugar that naturally occurs in various plant and bacterial carbohydrates. If arabinose is present in the bacteria, the activator causes the promoter

to bind RNA polymerase, and transcription will occur. If arabinose is not present, the activator prevents the promoter from binding RNA polymerase, and transcription will not occur.

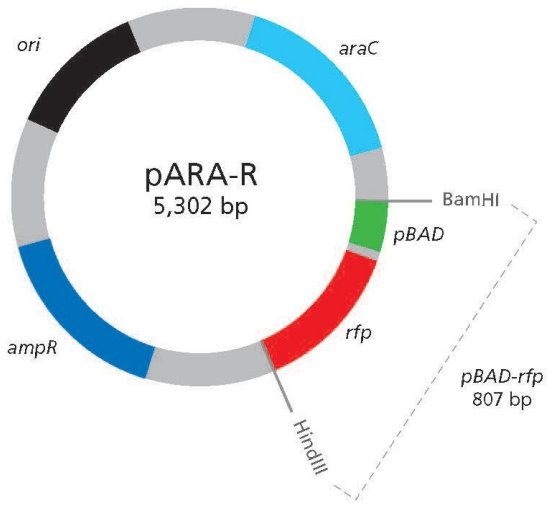


Figure 2a.2 Plasmid map of pARA-R.

The expected location of these DNA sequences, restriction enzyme sites and size of the plasmids (measured in base pairs) are shown in figure 2a.2. A 'base pair' is either adenine: thymine or guanine: cytosine and is the common method used to give the size of DNA molecules.

The purpose of this lab is to ensure that the recombinant plasmid, pARA-R, you have been given is the correct one for making the red fluorescent protein in bacteria. To do this you will use the restriction enzymes *Bam*HI and *Hin*dIII to cut the plasmid, which will generate DNA fragments of lengths characteristic of the pARA-R plasmid (see figure 2a.2). These are the same two restriction enzymes that were used to create this recombinant plasmid, following the procedure in figure I1. By using two different restriction enzymes the inserted gene can be oriented in the correct position for transcribing the "sense" strand of DNA (the strand that codes for the protein), and it prevents the plasmid from reforming a circle without the inserted gene.

Learning objectives

By the end of this laboratory you will be able to:

- Describe the characteristics of plasmids
- Explain how plasmids are used in cloning a gene
- Describe the function of restriction enzymes
- Explain how to use restriction enzymes to create a recombinant plasmid

Laboratory 2a: Restriction digestion of pARA-R

Aims

To use restriction digestion with two enzymes (*Bam*HI and *Hin*dIII) to create linear DNA fragments of known size from the engineered plasmid pARA-R.

Materials

EQUIPMENT & SUPPLIES

P-20 micropipette (2-20 µL)
Disposable pipette tips
2 x 1.5 mL microfuge tubes
Permanent marker
Floating tube rack
Waste container
Microcentrifuge (shared)
37°C water bath (shared)

REAGENTS

Microfuge tubes containing:

- 2.5 X restriction buffer (RB)
- pARA-R plasmid (R)
- restriction enzymes *Bam*HI and *Hin*dIII (RE)
- Distilled water (dH_2O)

Health and safety

In this laboratory you will be using biological materials, albeit in tiny amounts, and they should be used in a controlled way, using techniques that mirror the aseptic techniques used in microbiology. All of the materials that come into contact with the biological materials (pipette tips and microfuge tubes) will be autoclaved to denature the DNA and enzymes before disposal. As good laboratory practice you should clear up any spills immediately and wash your hands well with soap after completing the practical work.

Methods

1. Check your rack to make sure that you have all the reagents listed.
2. Use a marker to label two clean microfuge tubes R+ and R–. (Also include a personal identifier on each tube.)
3. Review table 2a.1, which summarises the reagents that you will add in step 4.

Step	Reagent	R+ tube	R- tube
4a	Restriction buffer (RB)	5.0 µL	5.0 µL
4b	pARA-R plasmid (R)	5.0 µL	5.0 µL
4c	*Bam*HI and *Hin*dIII (RE)	2.0 µL	
4d	Distilled water (dH_2O)		2.0 µL

Table 2a.1 Reagents to add to the R+ and R- tubes

⚠ In step 4, be sure to use a new micropipette tip for each reagent in each tube to avoid contamination.

4. Add the following:
   a. 5.0 µL of restriction buffer (RB) to the R+ and R– tubes.
   b. 5.0 µL of plasmid pARA-R (R) to the R+ and R– tubes.
   c. 2.0 µL of *Bam*HI and *Hin*dIII restriction enzymes (RE) to the R+ tube. Add the enzymes directly into the solution at the bottom of the microfuge tube. Gently pump the solution in and out of the pipette 3 times, by repeatedly moving the plunger to the first stop and back up again, to mix the reagents. Cap the tube when done.
   d. 2.0 µL of dH_2O to the R– tube. Gently pump the solution in and out with the pipette to mix the reagents. Cap the tube when done.
5. Spin the two microfuge tubes (R+ and R–) in the microcentrifuge for four seconds to pool the reagents at the bottom of each tube.

⚠ Distribute the tubes evenly in the microcentrifuge so that their weight is balanced.

6. Place both tubes into the 37°C water bath and incubate for one hour.
7. After the incubation is complete either continue to your next laboratory, or place all four tubes in the freezer at –20°C until you are ready to do so.

Questions

1. Using figure 2a.2, work out how many linear fragments will be produced from the plasmid pARA-R when it is digested with *Bam*HI and *Hin*dIII. What size will these linear fragments be?
2. Draw the linear fragments, marking on the genes and important sequences. Using table 2a.2 below, include the nucleotide sequence of the sticky ends.

Source	Restriction enzyme	Recognition site
Bacillus amyloliquefaciens	*Bam*HI	5′ G↓GATCC 3′ 3′ CCTAG↑G 5′
Haemophilus influenzae	*Hin*dIII	5′ A↓AGCTT 3′ 3′ TTCGA↑A 5′

Table 2a.2 The restriction enzymes used in this laboratory. Information on the bacterial strains they were isolated from and the DNA sequences that they cut. In the examples shown, the enzymes cut asymmetrically on the strands of DNA, leaving single-stranded overhanging sequences at the site of the cut. Cuts are shown by arrows. For example, a cut (or digestion) with *Bam*HI will leave an GATC overhang (or "sticky end") on one strand and a CTAG sticky end on the other strand.

3. In order to create a plasmid that can produce the red fluorescent protein in bacteria, what components are needed in the plasmid?
4. Bacteria can be killed by an antibiotic unless they carry a plasmid that has the gene for resistance to that antibiotic. Biotechnologists call these genes *selectable markers* because only bacteria that carry the gene will survive an antibiotic. If the uptake of DNA by bacteria is inefficient, why is a selectable marker critical in cloning a gene in bacteria?
5. What role do restriction enzymes have in nature?
6. Why might the enzymes work best at 37°C? Why should the unused enzymes then be placed in the freezer?
7. In step 4, you are asked to set up a tube without the restriction enzymes, *Bam*HI and *Hin*dIII. What is the purpose of this step, and why is it important?

8. Using your understanding of evolution, why would bacteria retain a gene that gives them resistance to antibiotics? How is the existence of bacteria with antibiotic resistance affecting medicine today?
9. Bacteria, sea anemones, and humans seem, on the surface, to be very different organisms. Explain how a gene from humans or a sea anemone can be expressed in bacteria to make a product never before made in bacteria.
10. Due to a mishap in the lab, bacteria carrying a plasmid with a kanamycin-resistant gene and bacteria carrying a plasmid with an ampicillin-resistant gene were accidentally mixed together. Design an experiment that will allow you to sort out the two kinds of bacteria. (Hint: Make sure that you do not kill off one of the kinds of bacteria you are trying to sort out!)

Chapter 4a: Examining the engineered plasmid pARA-R using PCR

Introduction

As in the previous laboratory, the aim is to check or verify that the engineered pARA-R plasmid contains the *rfp* gene. During the previous laboratory, a method for generating DNA fragments from the pARA-R plasmid using the restriction enzymes *Bam*HI and *Hin*dIII, in preparation for checking that they matched the expected sizes was given. The presence of a DNA band corresponding to the length of DNA between the restriction sites when the *rfp* gene is present (807 bp) provides strong evidence that the *rfp* gene is present. This laboratory uses an alternative method for checking that the expected pARA-R plasmid is present, by amplifying the same region (which is expected to contain the *rfp* gene) using the **p**olymerase **c**hain **r**eaction (PCR), before checking the PCR product is of the expected size. This is particularly useful if only limited amounts of recombinant plasmid are available, because PCR uses much less template DNA than restriction digestion.

Kary Mullis is credited with the discovery of PCR, work for which he received a Nobel prize in Chemistry in 1993. PCR uses the enzyme DNA polymerase, which is important in DNA replication (figure 3.1), to amplify (make lots of) a particular region of DNA. There are 3 different steps to a PCR, illustrated in figure 4a.1. During **denaturation** DNA becomes single stranded at temperatures high enough to break the hydrogen bonds between the bases in the centre of the DNA molecule, but not so high that they break the stronger covalent bonds between molecules in the sugar-phosphate backbone. Typically a temperature of 94 – 96°C is used. Following this, **annealing** occurs at a lower temperature, binding primers (short DNA sequences) to a complementary DNA sequence, creating a short double stranded region of DNA. Finally comes the **extension** step, during which a DNA polymerase binds to the short double-stranded region and extends the primer in a 5' to 3' direction using the single stranded DNA as

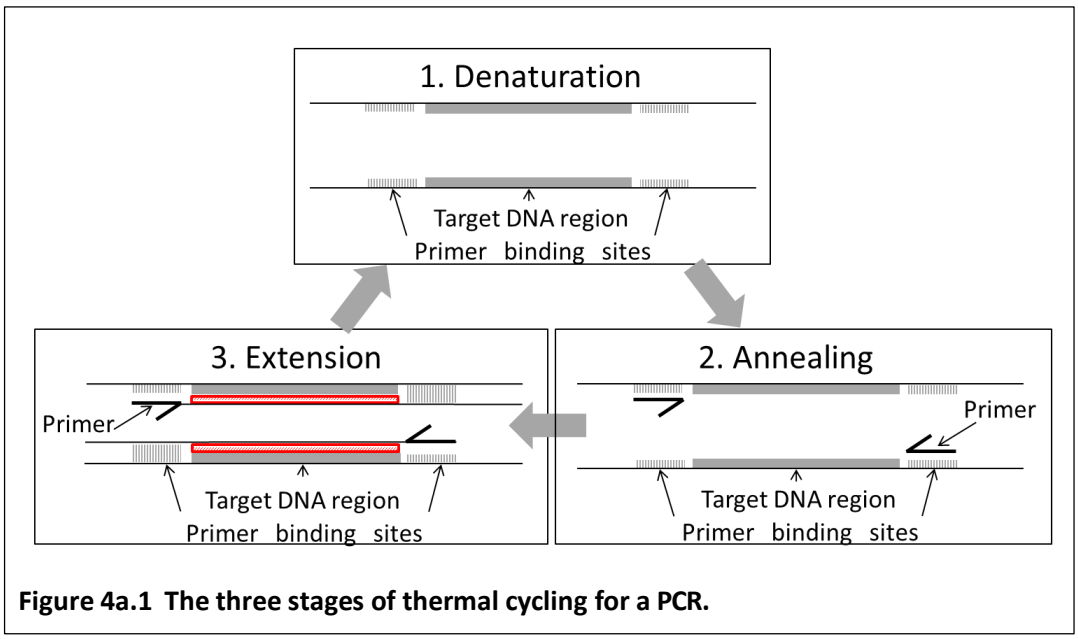


Figure 4a.1 The three stages of thermal cycling for a PCR.

template. The optimum temperature for the DNA polymerase to extend the single stranded DNA varies, but tends to be in the range of 68 – 76 °C for the thermostable DNA polymerase enzymes used, which have been isolated from bacteria like *Thermus aquaticus* that live around hydrothermal vents and therefore have enzymes adapted to tolerate high temperatures.

This experiment uses pARA-R as the DNA template for the PCR. Two primers that bind specifically to the DNA sequence 136 bp from the *Bam*HI restriction site and 149 bp from the *Hin*dIII restriction site will be used to amplify the region of DNA bracketing and including the *Bam*HI – *Hin*dIII region. A DNA band of the expected size (in this case 1092 base pairs), provides strong evidence that the *rfp* gene is present (see figure 4a.2).

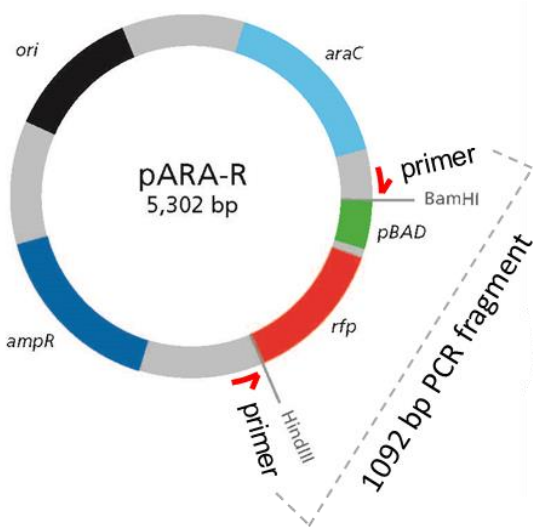


Figure 4a.2. The pARA-R plasmid with primer binding sites and expected PCR fragment size.

Learning objectives

By the end of this laboratory you will be able to:

- Describe the role of a DNA polymerase in the PCR
- Explain how PCR can be used to amplify a specific DNA sequence
- Understand the role of sequence specific primers in a PCR
- Describe the size of PCR products from your pARA-R plasmid

Laboratory 4a: The PCR with pARA-R

Aims

To use the enzyme DNA polymerase in a **P**olymerase **C**hain **R**eaction (PCR), catalysing the extension of sequence specific primers to produce a PCR fragment that should contain the *rfp* gene and be of an expected size.

Materials

EQUIPMENT & SUPPLIES

P-20 micropipette (2-20 µL)
Disposable pipette tips
Permanent marker
Polystyrene cup of ice
Waste container
Microcentrifuge (shared)
PCR machine (shared)

REAGENTS

2 PCR tubes of 2 X PCR master mix (MM)

Microfuge tubes containing:

- Combined forward and reverse primer (PRI)
- Nuclease-free water (H_2O)
- pARA-R plasmid (R)

Health and safety

In this laboratory you will be using biological materials, albeit in tiny amounts, and they should be used in a controlled way, using techniques that mirror the aseptic techniques used in microbiology. All of the materials that come into contact with the biological materials (pipette tips, PCR tubes and microfuge tubes) will be autoclaved to denature the DNA and enzymes before disposal. As good laboratory practice you should clear up any spills immediately and wash your hands well with soap after completing the practical work.

Methods

1. Check your rack to make sure that you have all the reagents listed:
   a. A polystyrene cup, containing ice, with 1 PCR tube (MM) in it. *This is a mixture of the buffer, magnesium chloride, dNTPs and DNA polymerase from a thermophilic bacteria, which are required for a PCR*.
   b. A microfuge tube containing plasmid pARA-R (R).
   This tube has the template DNA for the PCR.
   c. A microfuge tube containing forward and reverse primers (PRI). *Primers are short stretches of DNA that bind to complementary DNA sequence during the annealing step, allowing DNA polymerase to extend a specific part of the DNA*.
   d. A microfuge tube containing nuclease-free water (H_2O).
   This will be used to make up the reaction volume.

2. Use a marker to label the PCR tubes containing 2 X PCR master mix (MM) on the side. Label one ‘pARA-R’ and the other ‘NEG’. Both should have your personal identifier on. Return them to the ice.

⚠ Marker pen can rub off the top of the PCR tubes in the PCR machine, so make sure you label them on the side.

3. Review Table 4a.1, which summarises the reagents that you will add in step 4.

Step	Reagent	pARA-R PCR	NEG PCR
4a	2 X PCR master mix (MM)	12.5 µL	12.5 µL
4b	Nuclease-free water (H_2O)	8.5 µL	10.5 µL
4c	Combined primers (PRI)	2.0 µL	2.0 µL
4d	pARA-R plasmid (R)	2.0 µL	

Table 4a.1 Reagents to add to the PCR tubes

⚠ In step 4, be sure to use a new micropipette tip for each reagent in each tube to avoid contamination.

4. a. To your labelled PCR tubes containing 12.5 µL of master mix add the following:
   b. 8.5 µL of nuclease-free water (H_2O) to the MMpARA-R tube and
   10.5 µL of nuclease-free water (H_2O) to the MMNEG tube.
   c. 2.0 µL of combined forward and reverse primers (PRI) to each tube.
   d. 2.0 µL of pARA-R plasmid (R) to the MMpARA-R PCR tube.
5. Ensure the reagents are well mixed, by pumping gently up and down with the pipette or simply swirling with the tip. Keep your reactions on ice until the PCR machine can be started.
6. The PCR programme that you will use is given in table 4a.2. It takes about 1½ hours to complete.

Reagent		Temperature (°C)	Time (sec)
Initial denaturation		95	200
30 cycles of	Denaturation	95	30
	Annealing	53	30
	Extension	68	30
Final extension		68	300
Hold		10	Indefinite

Table 4.2a The PCR programme

7. When your teacher tells you, transfer your PCR tubes from the ice into the PCR machine and start the reaction.
8. After the PCR programme is complete your PCR tubes can be placed in the fridge (4°C) until you are ready to use them.

Questions

1. Based on your understanding of DNA replication, explain the role of DNA polymerase in replication.
2. Why do you keep the PCR master mix, which contains DNA polymerase, on ice until the PCR is begun?
3. Why does the *Taq* DNA polymerase not denature during the denaturation step at 95°C in your PCR programme?
4. Each DNA molecule is replicated during each cycle of the PCR programme. There are 30 cycles on the PCR programme that you use. If you started with 6 DNA molecules, how many molecules would there be at the end of the PCR?
5. What is the purpose of the pARA-R PCR, given that the expected size of the PCR product is known to be 1092 bp long?
6. What is the purpose of the NEG PCR tube?
7. What type of chemical bonds will form when the single stranded primer DNA base pairs with the complementary sequence on the recombinant plasmid?
8. How many fragments would you get if you used the restriction enzymes *Bam*HI and *Hin*dIII to digest the DNA fragment amplified from pARA-R using the PCR? What size would they be?

Chapter 5a: Verifying the engineered plasmid pARA-R

Introduction

As part of the gene cloning process, molecular biologists have to check that they have completed each stage successfully and created the recombinant plasmid they need: one with the gene of interest as well as all the necessary components for the protein of interest to be made. The difficulties of engineering recombinant plasmids, as well as the sources of potential error in any procedure, make it important that the DNA products from each stage of the gene cloning process are carefully checked. Gel electrophoresis is the procedure used to check the size of DNA molecules.

The technique of gel electrophoresis involves placing DNA samples, combined with a loading dye, into the wells of an agarose gel, submerged in electrophoresis buffer within a gel electrophoresis tank, then using an electric field through the gel to separate the DNA. Phosphate groups in the backbone of DNA create a negative charge, so it is loaded near the negative electrode and will migrate through the porous matrix of the agarose gel towards the positive electrode. The DNA molecules are separated primarily according to their molecular size, although molecular shape and degree of charge also influence their movement. Small DNA molecules move through the agarose matrix more easily, so they migrate faster than larger DNA molecules (figure 5a.1).

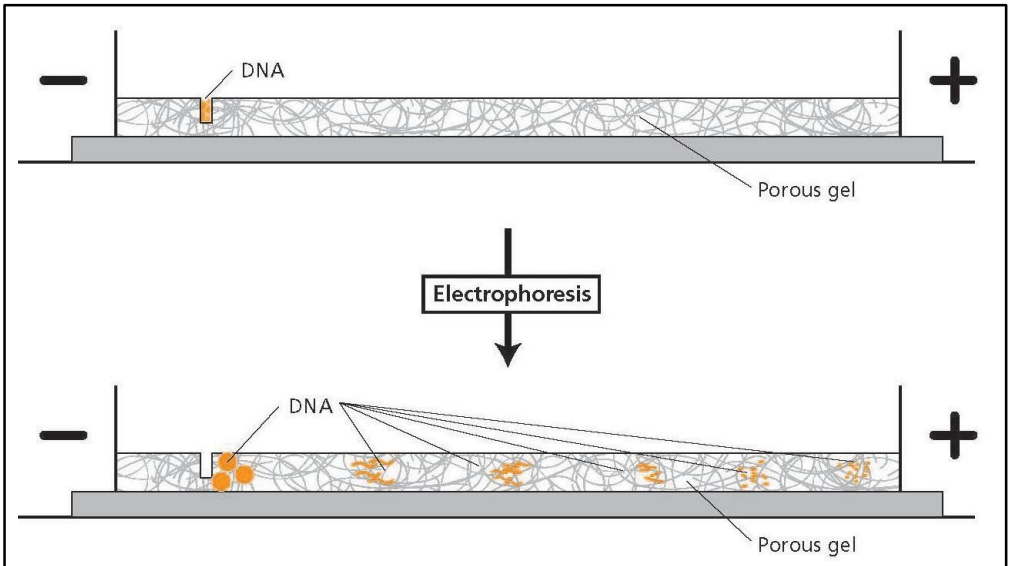


Figure 5a.1 Separation of DNA by size using agarose gel electrophoresis.

Although linear pieces of DNA will separate by size as expected, the different configurations (shapes) of plasmids will alter their migration through the gel. There are three different plasmid configurations, which are shown in figure 5.2. The most common plasmid configuration is supercoiled. Supercoiling of DNA is catalysed by an enzyme in bacteria. It is the natural configuration in bacteria and is only seen in plasmids that have been replicated in bacteria. Supercoiling results in a very compact molecule, one that will move through the gel very quickly for its size. The second plasmid configuration is nicked circle. A break in one of the covalent bonds located in the sugar-phosphate backbone of one of the DNA strands along one of the two nucleotide strands means that this circular plasmid configuration will not move through the agarose gel as easily as the supercoiled configuration. The third plasmid configuration is a multimer. This configuration of two or more plasmids that are connected is only seen in plasmids that have been replicated in bacteria, because multimers form when plasmids are replicated so fast that they end up linked together. Unsurprisingly, given their size and shape multimers migrate very slowly through the gel.

Using gel electrophoresis you will examine the size of the pARA-R plasmid and the restriction digest fragments (laboratory 2a) and / or the products from the PCR (laboratory 4a). The sizes of the DNA molecules will be determined by comparing them to a DNA ladder: a mixture of DNA fragments of known sizes. (When the DNA ladder is run on gel electrophoresis and stained, the bands that show the fragments look like the rungs of a ladder.) You will use the size of the DNA fragments to judge whether you do have the recombinant plasmid pARA-R that contains the *rfp* gene.

In order to visualise the DNA we will use a special stain that interacts with the DNA called GelRed. Then a photographic record of your gel will be made to document this vital step.

Supercoiled Nicked circle Multimer

Figure 5.2 The three different plasmid configurations

Learning objectives

By the end of this laboratory you will be able to:

- Describe why it is important to check the products at each stage of the genetic engineering process
- Predict the relative speed of DNA restriction fragments and plasmids through a gel during gel electrophoresis
- Separate and identify DNA molecules using gel electrophoresis

Laboratory 5a: Visualisation of products from restriction digestion and the PCR

Aims

To check whether the DNA fragment sizes produced by restriction digestion and /or PCR are consistent with the recombinant plasmid pARA-R, using agarose gel electrophoresis.

Materials

EQUIPMENT & SUPPLIES

P-20 micropipette (2-20 µL)
Disposable pipette tips
4 x 1.5 mL microfuge tubes
Permanent marker
Waste container
Microcentrifuge (shared)
Electrophoresis tank with 0.8% agarose gel (shared)
Plastic gel staining tray
UV transilluminator
Hood
Camera

REAGENTS

Microfuge tubes containing:

- undigested pARA-R from lab 2a (R-)
- digested pARA-R from lab 2a (R+)
- loading dye (LD)
- distilled water (dH_2O)
- DNA ladder (1kb)

PCR tubes containing:

- the pARA-R PCR from lab 4a (MMpARA-R)
- the NEG PCR from lab 4a (MMNEG)

1 X TAE buffer

1.5 X GelRed solution

Health and safety

In this laboratory you will be using biological materials, albeit in tiny amounts, and they should be used in a controlled way, using techniques that mirror the aseptic techniques used in microbiology. All of the materials that come into contact with the biological materials (pipette tips, microfuge tubes and agarose gels) will be autoclaved to denature the DNA and enzymes before disposal.

The loading dye is low hazard, but can stain skin, so try to avoid contact with it as good practice would dictate. You should wear safety glasses and chemical-resistant gloves when using the electrophoresis buffer, to avoid contact with the solution.

The electrophoresis chamber contains 1 X TAE buffer at 100 volts. To prevent contact with the liquid during electrophoresis the tanks are designed so that the electric supply is cut off whenever the lid is removed. Additionally, all connections are plastic coated so that there is no possibility of touching a metal contact which is live at 100 volts.

GelRed is used to stain the DNA. Whilst **not** mutagenic like some stains, this is a substance that can bind to DNA (of which you have lots), therefore you will need to wear chemical resistant gloves, a lab coat and eye protection if you are to complete this part of the procedure.

As good laboratory practice you should clear up any spills immediately and wash your hands well with soap after completing the practical work.

Methods: Electrophoresis

1. Check your rack to make sure that you have all the reagents listed.
2. Use a marker to label four clean microfuge tubes for the gel electrophoresis samples as follows: gelR-, gelR+, gelMM-R and gelMMNEG. (Also include a personal identifier on each tube.)
3. Review table 5a.1, which summarises the reagents that you will add in steps 4-7 to make up the samples for gel electrophoresis.

		Restriction digestion		**PCRS**	
Step	**Reagent**	**gelR- tube**	**gelR+ tube**	**gelMM-R tube**	**gelMMNEG tube**
4	Distilled water (dH_2O)	4.0 µL	4.0 µL	6.0 µL	6.0 µL
5	Loading dye (LD)	2.0 µL	2.0 µL	2.0 µL	2.0 µL
6a	Undigested pARA-R (R-)	4.0 µL			
6b	Digested pARA-R (R+)		4.0 µL		
7a	The pARA-R PCR (MMpARA-R)			2.0 µL	
7b	The NEG PCR (MMNEG)				2.0 µL

Table 5a.1 Reagents to add to the tubes for gel electrophoresis samples

⚠ In steps 4-7, be sure to use a new micropipette tip for each reagent in each tube to avoid contamination.

4. Add the following:
   a. 4.0 µL of distilled water (dH_2O) to the gelR- and gelR+ tubes.
   b. 6.0 µL of distilled water (dH_2O) to the gelMM-R and gelMMNEG tubes.
5. Add 2.0 µL of loading dye (LD) to each of the tubes.
6. Add the following:
   a. 4.0 µL of undigested pARA-R (R-) to gelR– tube.
   b. 4.0 µL of digested pARA-R (R+) to gelR+ tube.
7. Add the following:
   a. 2.0 µL of the PCR with pARA-R as template (MMpARA-R) to the gelMM-R tube.
   b. 2.0 µL of the PCR with no template (MMNEG) to the gelMMNEG tube.
8. Spin the four microfuge tubes (gelR-, gelR+, gelMM-R and gelMMNEG) in the microcentrifuge for four seconds to pool the reagents at the bottom of each tube.

⚠ Distribute the tubes evenly in the microcentrifuge so that their weight is balanced.

9. Make sure that the wells in your gel electrophoresis unit are located near the negative (black) electrode.
10. Fill the box with 1x TAE to a level that just covers the entire surface of the gel. If you see any "dimples" over the wells, add more buffer.

⚠ If there are "dimples," add *very small* amounts of buffer to the electrophoresis box. While the gel needs to be completely under the buffer, too much buffer in the box will allow the electrical current to run through the buffer and not the gel.

11. Make a drawing in your notebook that shows the location of the wells in the electrophoresis box. The order to load the samples into the wells is shown in figure 5a.3. Two or three groups can share an 15-well gel.

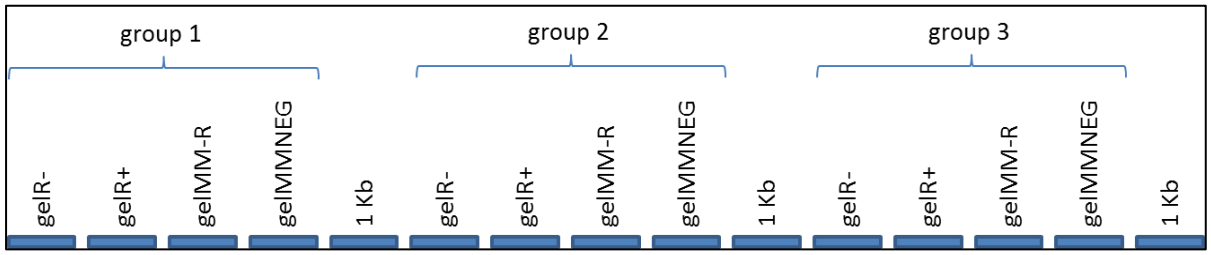


Figure 5a.3 The sample loading order into the wells of the agarose gel.

12. Using a fresh pipette tip for each sample, dispense 10.0 µL of the gelR-, gelR+, gelMM-R, gelMMNEG and DNA ladder (1 kb) into their designated wells. For each sample, do the following:
    a. Place your elbow on the table to steady your pipette hand. If needed, also use your other hand to support your pipette hand.
    b. Lower the pipette tip until it is under the buffer but just above the well.

⚠ Be careful not to place your pipette tip into the well or you might puncture the gel and lose your sample out the hole.

⚠ Only press the micropipette plunger to the first stop when dispensing samples to avoid getting air bubbles in the buffer. The sample will then sink into the well.

13. When all the samples have been loaded, close the cover tightly over the electrophoresis box. Lower the cover in a horizontal motion, so that the samples don't spill.
14. Connect the electrical leads to the power supply. Connect both leads to the same channel, with negative electrode to negative electrode (black to black) and positive electrode to positive electrode (red to red). See figure 5a.4 below.

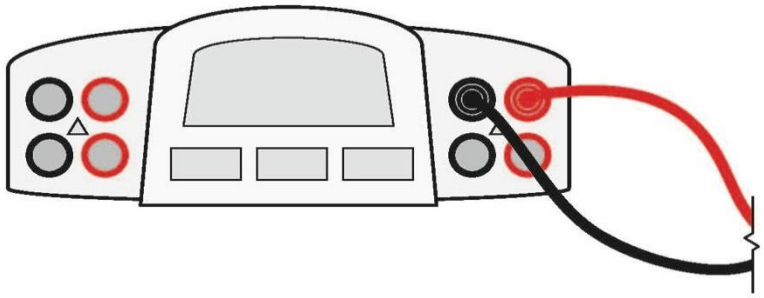


Figure 5a.4 Connecting leads from the electrophoresis tank to the powerpack.

15. Turn on the power supply and set the voltage to approx. 100 V.

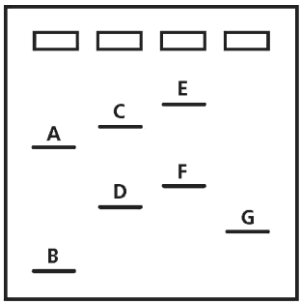
16. After two or three minutes, check to see if the purple loading dye (bromophenol blue) is moving toward the positive (red) electrode. If it's moving in the other direction—toward the negative (black) electrode—check the electrical leads to see whether they are plugged in to the power supply correctly.
17. Your teacher will explain what to do with your gel. You may not have sufficient time to complete the electrophoresis. The yellow loading dye will need to run just near the end of the gel. This takes about 50 minutes. After the gel has finished running, it will need to be stained to show the location of the DNA fragments and plasmids.

Methods: Visualisation

1. When electrophoresis is finished, the gel needs to be stained to allow the DNA to be visualised. This will be done using GelRed. Whilst **not** mutagenic like some stains, this is a substance that can bind inside DNA (of which you have lots), therefore you will need to wear chemical resistant gloves, a lab coat and eye protection if you are to complete this part of the procedure.
2. Turn off the Powerpack. Carefully slide the gel from the electrophoresis gel tray into the plastic gel staining tray.
3. Cover the gel with GelRed 1.5 x solution and allow the gel to stain for 30 minutes.
4. Your teacher will tell you whether (i) to pour the GelRed into a foil- covered bottle for reuse, or (ii) to dispose of the GelRed down the sink, washing it away with plenty of water.
5. Wearing chemical resistant gloves, carefully place the gel onto the glass plate of the UV transilluminator. You should be able to see the DNA stain fluorescing without using the hood and camera.
6. Use the hood and camera to take a photo of your gel for analysis.
7. Once you have obtained an image of the gel, place the gel in the box or autoclave bag on your teacher's desk for disposal.

Questions

1. Why do DNA restriction fragments and plasmids separate when analyzed by gel electrophoresis?
2. Why is it important to check that the restriction fragment and PCR product sizes from a recombinant plasmid match the expected sizes?
3. After different DNA fragments and plasmids have been separated by gel electrophoresis, the gel is stained to show bands that indicate the location of each kind of fragment and plasmid. The drawing of a stained gel opposite shows a series of bands that have been labelled with letters. The locations of the wells are also shown. What is the order of the fragments, from smallest to largest?



4. The DNA is not visible as it moves through the gel. The loading dye contains the three dyes that you separated in laboratory 1.2. Why is it useful to use the loading dye in this lab?
5. If you used gel electrophoresis to separate the same plasmid that has all three configurations, the supercoiled plasmid would move the fastest, while the multimer would move the slowest. Why do the different plasmid configurations move the way they do through the gel?
6. The pARA-R plasmid you digested in laboratory 2a was replicated in a bacterial cell. What configurations—supercoiled, nicked circle, and multimer—might this plasmid have before digestion?
7. What products might you expect to see from the gelR-, gelR+, gelMM-R and gelMMNEG tubes? Create a table that shows the possible fragments and plasmids by tube. Include the length (bp size) of each fragment or plasmid, and arrange the products found in each microfuge tube by size, from smallest to largest. Include any possible plasmid configurations and arrange them first by size and next by speed through the gel, from fastest to slowest.
8. The DNA ladder serves as a standard because it contains a mixture of DNA molecules of known sizes. By running your samples and the DNA ladder side by side in your gel, you can estimate the actual size in base pairs of unknown DNA molecules. The DNA Ladder Diagram below shows 10 DNA bands of known sizes. Their sizes are given in kilobase (kb) pairs. 1 kb is 1,000 base pairs (bp). Predict the positions of DNA bands produced by the possible products found in the gelR-, gelR+, gelMM-R and gelMMNEG tubes by drawing them on the DNA Ladder Diagram (figure 5a.5).
9. When you have had a chance to examine your gel photograph, analyse how your actual gel results compare to your gel predictions.
10. Do you see any bands that are not expected? What could explain the origin of these unexpected bands?

11. Does the gel show that your restriction digest and PCR procedures were successful? Describe the evidence you used to make this assessment.
12. In the gelR– lane, do you see evidence of multiple configurations of plasmids? Explain your answer.
13. In the gelR+ lane, do you see evidence of complete digestion? Explain your answer.
14. Does the gel show that you are using the correct recombinant plasmid, pARA-R? Describe the evidence that you used to make this assessment.

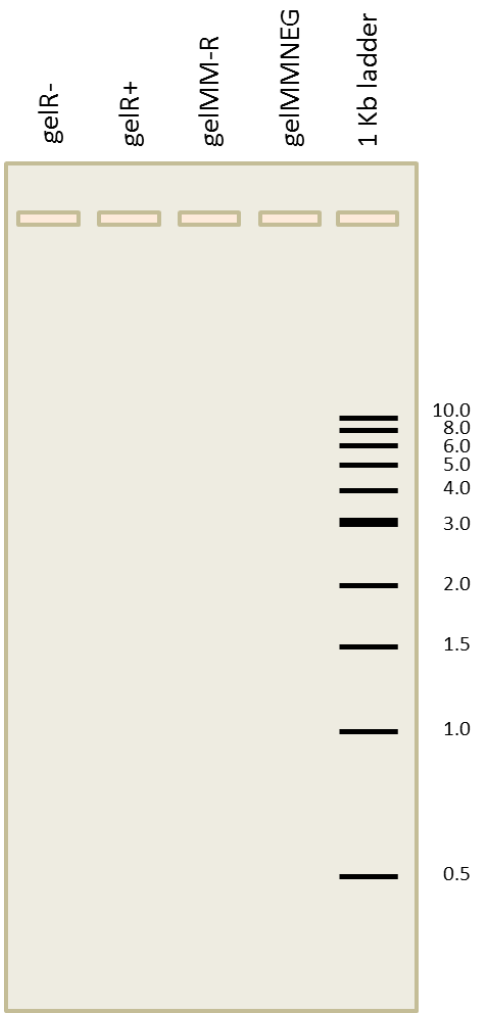


Figure 5a.5 The DNA ladder diagram.

Chapter 6a: Inserting recombinant plasmids into bacteria

Introduction

Once a recombinant plasmid is created it needs to be replicated and the gene of interest needs to be expressed. Both of these activities can only occur inside a cell, so recombinant plasmids are inserted into a cell. *E. coli* bacterial cells are most commonly used because they replicate quickly and are easy to maintain in a laboratory. Plasmids are inserted into *E. coli* bacteria using a process that is called transformation, because it changes the DNA content of the bacteria. Figure 6a.1 depicts a bacterium transformed with plasmids.

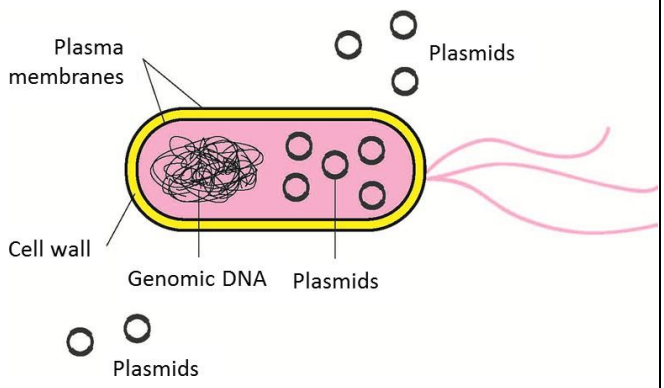


Figure 6a.1 A bacterium transformed with plasmids

The uptake of DNA from the environment of a bacterial cell occurs with a very low efficiency in nature. *E. coli* bacteria have complex plasma membranes that separate the external environment from the internal environment of the cell and carefully regulate which substances can enter and exit the cell. In addition, the cell wall is negatively charged and repels negatively charged DNA molecules.

In order to increase the efficiency of DNA uptake, bacteria are treated in two ways. First, the *E. coli* bacteria are placed in a solution that contains positive calcium ions, which neutralize the negative charge on the cells' outer membranes, enabling DNA molecules to cross the membranes and enter the cell. Next, the bacteria are subjected to a heat shock, a sudden increase in temperature, which causes the pressure outside the cell to increase, relative to inside. This pressure difference enables the plasmid DNA to enter the bacterial cell from the outside.

Cells treated with calcium and heat are considered *competent* to take up DNA more efficiently, but even with this treatment only about 1 in 10,000 bacterial cells takes up a plasmid in its environment. Bacteria that have successfully been transformed with a recombinant plasmid can be selected by their resistance to an antibiotic. Only bacterial cells that are expressing the antibiotic resistance proteins will grow.

Once a recombinant plasmid has entered the bacterial cell, DNA polymerase initiates replication at the *ori* site, and multiple copies of the recombinant plasmid are made by the bacterial DNA replication enzymes. Gene expression is initiated from the promoter on the multiple plasmids. The gene of interest is transcribed and translated using the enzymes and molecules within the bacterial cell and the protein of interest is produced. The protein can then act to alter the observable traits of the organism and/or be purified for medicinal use.

Medicinal proteins from other organisms can only be made in bacteria using this process of genetic engineering because the way that information is encoded in DNA is universal on Earth.

After confirming that you have got the desired recombinant pARA-R plasmid, containing the *rfp* gene that can make the red fluorescent protein, you next need to transform *E. coli* bacteria with this plasmid. You will be supplied with *E. coli* bacteria that have been pre-treated with calcium chloride, which you will divide into two groups: a control group to which no plasmid is added, and a treatment group to which you add the pARA-R plasmid. After heat-shocking both groups of cells, you will grow them: (i) on Luria Bertani agar (a medium that supplies the nutrients and support for bacterial growth), and; (ii) on Luria Bertani agar with the antibiotic ampicillin. In addition, the treatment group will be grown in the presence of Luria Bertani agar, ampicillin, and the sugar arabinose.

By examining the growth of bacteria under these conditions, you can verify that your transformation procedure worked, and you can identify the bacteria transformed with the pARA-R plasmid. If you are successful the bacteria will have a new and highly visible trait: they will now produce red fluorescent protein, which makes the cells red or bright pink!

Learning objectives

By the end of this laboratory you will be able to:

- Describe the role of transformation in the gene cloning process
- Explain the purpose of each control in the transformation experiment
- Explain how the information encoded in a gene is expressed as a trait

Laboratory 6a: Transforming bacteria with the recombinant plasmid pARA-R

Aims

To insert the recombinant plasmid pARA-R into bacterial cells, using a procedure known as bacterial transformation, then to select the bacteria containing pARA-R by growing them under different conditions.

Materials

EQUIPMENT & SUPPLIES

P-20 micropipette (2-20 µL)
P-200 micropipette (50-200 µL)
Disposable pipette tips
Permanent marker
2x 1.5 mL microfuge tubes
Waste container
Floating tube rack
Timer
Sticking tape
Pack of cell spreaders (shared)
Polystyrene cup of crushed ice in cold water
42°C water bath (shared)
37°C incubator (shared)
Autoclave bag for disposal (shared)

REAGENTS

Microfuge tubes containing:

- pARA-R plasmid (R)
- Luria Bertani broth (LB broth)

Petri dishes with agar:

- 1 of LB
- 1 of LB / amp
- 1 of LB / amp / ara

A microfuge tube of chilled competent *E. coli* cells (CC) in a cup of iced water

⚠ The competent cells MUST be kept on ice at all times.

Health and safety

Aseptic technique must be used when undertaking microbiological work. This is described in detail on pages A.1 – A.3. If good aseptic technique is used you do not need to wear gloves or eye protection. After the bacteria are spread on the agar plates the lids must be taped down in 3, evenly spaced places around the circumference of the petri dish.

All of the materials that come into contact with the biological materials (pipette tips, microfuge tubes, cell spreaders and agar plates) will be autoclaved prior to disposal.

As good laboratory practice you should clear up any spills immediately and wash your hands well with soap after completing the practical work.

Methods

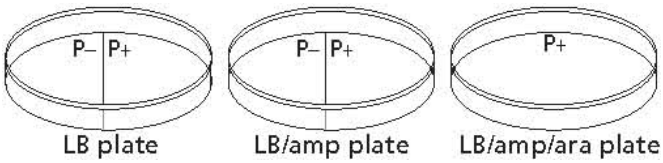
1. Check your rack to make sure that you have all the reagents listed.
2. Obtain a CC tube from the ice-filled container and place it in a polystyrene cup of ice.

⚠ The competent cells in this lab MUST be kept cold. Hold microfuge tubes by the upper rim to avoid warming the cells.

3. Label two clean microfuge tubes 'P–' and 'P+'
4. Place the P– and P+ tubes in the polystyrene cup of ice with the CC tube.
5. Using the large P-200 micropipette, add the competent cells from the CC tube to the P– and P+ tubes. To do this:
   a. Set the P-200 micropipette to 50 µL.
   b. Very carefully, re-suspend the bacterial cells in the CC tube by gently pumping the pipette two times in the solution.
   c. Add 50 µL of CC to each of the empty chilled tubes (P– and P+), holding each tube at its rim to keep it cold, and return each tube quickly to the ice.
6. Using the P-20 pipette, add 10.0 µL of pARA-R plasmid (R) to the tube labelled 'P+'. To do this:
   a. Set the P-20 micropipette to 10.0 µL.
   b. Hold the chilled P+ tube by the upper rim and add 10.0 uL of pARA-R plasmid (R). Mix the solutions by pumping the pipette two times in the liquids, and return the P+ tube to the ice.

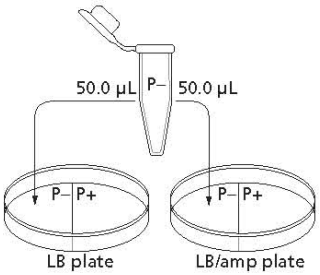
⚠ To avoid contamination, be sure to use a new micropipette tip for each addition.

7. Keep the P– and P+ tubes on ice for 15 minutes.
8. While the cells are on ice, prepare your three agar Petri plates— one plate each of LB, LB/amp, and LB/amp/ara. To do this:
   a. Label the bottom of each plate (the part that contains the agar) in small writing at the edge of the plate with your personal identifier.
   b. With the plates closed, draw a line on the base of the LB plate and the LB/amp plate that divides each plate in the middle. Label half of each plate 'P-', and the other half 'P+'. Label the LB/amp/ara plate 'P+'. The plates should be arranged as below:

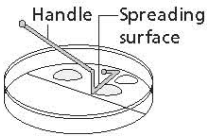


9. Following the 15-minute incubation on ice, carry the P– and P+ tubes (in the cup of ice) to the 42°C water bath. Place the two tubes in the floating microfuge tube rack in the water bath for EXACTLY 45 seconds.
10. After the 45-second heat shock, immediately place the tubes back on ice and **leave them there for at least a minute**.
11. Using the P-200 micropipette, add LB to the P– and P+ tubes, using a new tip for each addition. To do this:
    a. Set the P-200 micropipette to 150 µL.
    b. Add 150 µL of LB to the P– tube. Cap the tube and gently flick it two or three times to mix. Place in rack at room temperature.
    c. Add 150 µL of LB to the P+ tube. Cap the tube and gently flick it two or three times to mix. Place in rack at room temperature.
    d. Leave to stand for 5 minutes before 'plating out' (putting onto agar plates)

12. To add cells from the P– tube onto your LB and LB/amp plates:
    a. Set the P-200 micropipette to 50 µL.
    b. Gently pump the pipette two or three times in the P– tube to suspend the cells.
    c. Open the lid of the LB plate, like a "clamshell," and add 50 µL of cells from the P– tube to the section marked 'P–'. Close the lid.
    d. Again, gently pump the pipette two or three times in the P– tube to suspend the cells.
    e. Open the lid of the LB/amp plate, like a clamshell, and add 50 µL of cells from the P– tube to the section marked 'P–'. Close the lid.



13. To spread the cells from the P– tube on your LB and LB/amp plates:
    a. Open the package of sterile cell spreaders at the end closest to the spreader handles. Remove only one spreader, and close the package to keep the others sterile.
    b. Open the lid to the LB plate, like a clamshell, and spread the cells evenly across the entire P– side of the plate by gently moving the spreader across the agar surface. Close the lid.
    c. Carefully spread the P– cells on the LB/amp plate, using the same spreader and technique.



⚠ Hold the spreader by the handle and do not allow the bent end to touch any surface, as this will contaminate the spreader. Place the used spreader in disposal for autoclaving.

14. To add cells from the P+ tube to your LB, LB/amp, and LB/amp/ara plates:
    a. Make sure that the P-200 micropipette is set to 50 µL.
    b. Gently pump the pipette two or three times in the P+ tube to suspend the cells.
    c. Open the lid of the LB plate, like a clamshell, and add 50 µL of cells from the P+ tube to the section marked 'P+'. Close the lid.
    d. Again, gently pump the pipette two or three times in the P+ tube to suspend the cells.
    e. Open the lid of the LB/amp plate, like a clamshell, and add 50 µL of cells from the P+ tube to the section marked 'P+'. Close the lid.
    f. Set the P-200 micropipette to 100 µL, gently pump the pipette two or three times in the P+ tube, and load 100 µL of the P+ cells.
    g. Open the lid of the LB/amp/ara plate, like a clamshell, and add 100 µL of P+ cells to various areas across the surface—not just a single spot. Close the lid.
15. To spread the cells from the P+ tube on LB, LB/amp, and LB/amp/ara plates:

a. Open the package of sterile cell spreaders at the end closest to the spreader handles. Remove only one spreader, and close the package to keep the others sterile.

b. Open the lid to the LB plate, like a clamshell, and evenly spread the cells on the P+ side of the plate (and only on this side) by gently moving the spreader across the agar surface. Close the lid.

⚠ Hold the spreader by the handle and do not allow the bent end to touch any surface, as this will contaminate the spreader.
Place the used spreader in disposal for autoclaving.

c. Carefully spread the P+ cells on the LB/amp plate using the same spreader and technique.

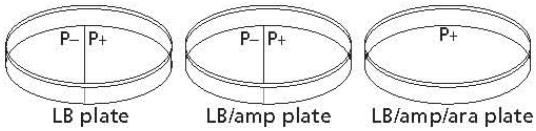
d. Carefully spread the P+ cells on the LB/amp/ara plate using the same spreader and technique. Gently rotate the plate beneath the P+ spreader so that the cells can be spread over the entire surface of this plate. Close the lid.

16. Allow all three plates to sit right side up for five minutes to allow them to dry a bit.
17. Using the sticky tape provided, secure each plate 3 times so that the lid will not come off. Make sure your plates are marked with a personal identifier.
18. Stack the 3 plates upside down to prevent condensation from dripping onto the agar and place the plates in the 37°C incubator.
19. Place all microfuge tubes, pipette tips, and cell spreaders in the autoclave bag.
20. Incubate the plates for 24–36 hours at 37°C.
21. Examine the plates and record the amount of growth on each half.
22. Place the plates on the UV transilluminator to observe the red fluorescence and use the hood and camera to make a photographic record.
23. Discard the Petri plates in the autoclave bag when directed to do so.

Questions

1. What are the steps involved in transcription and translation of a gene?
2. What is the relationship among genes, proteins, and traits (or observable characteristics)?
3. What do bacteria and humans have in common that makes it possible for a human gene to be expressed in bacteria?
4. Why is it important that the membranes of *E. coli* bacteria carefully regulate which substances can enter and exit the cell?
5. How are the P+ bacteria treated differently from the P– bacteria? What is the purpose of the P– bacteria?

6. Why is it important to use aseptic technique in this lab?
7. Why do the cells need time to recover after the heat shock?
8. Why are the cells incubated at 37°C?
9. Ampicillin is an antibiotic that kills bacterial cells by disrupting the formation of cell walls. However, the pARA-R plasmid has the ampicillin resistance gene, which produces a protein that breaks down ampicillin. What is the purpose of growing bacteria that have been transformed in the presence of ampicillin?
10. What will happen when bacterial cells that contain the pARA-R plasmid are not given arabinose?
11. You add samples of the control group P– and the treatment group P+ to plates that contain various combinations of Luria Bertani agar (LB), ampicillin, and the sugar arabinose. What are your predictions for the growth you expect on each of the plates?



12. Look at the results of your transformation. Do your actual results match your predicted results? If not, what differences do you see, and what are some explanations for these differences?
13. How many red colonies were present on your LB/amp/ara plate?
14. Why did the red colonies only appear on the LB/amp/ara plate and not the LB/amp plate?
15. Recombinant plasmids are engineered so that they can replicate in the cell independently of the chromosome replication. Why is it important to have multiple copies of a recombinant plasmid within a cell?
16. How is the information encoded in the *rfp* gene expressed as a trait? Be sure to use what you have previously learned about gene expression and the relationship between DNA, RNA, protein, and traits.
17. Why is it possible for bacteria to make a human protein, such as insulin, or a sea anemone protein, such as the red fluorescent dye?

Chapter 7a: Correlating DNA fragment size (genotype) with phenotype

Introduction

After transformation, all bacterial colonies that turn red on the LB agar plate containing ampicillin and arabinose are expected to have been successfully transformed with the pARA-R plasmid. The ampicillin resistance gene carried

by the plasmid is required for the bacteria to survive in the presence of ampicillin, and the *rfp* gene from the plasmid is required for the expression of red fluorescent protein. It is however possible to check that a DNA fragment corresponding to the size of the *rfp* gene in the pARA-R plasmid is present within the transformed bacteria using a technique called 'colony PCR'. This allows you to correlate the phenotype (colour of the bacterial colonies) with the genotype (whether there is a fragment of DNA the correct size to be the *rfp* gene).

A colony PCR involves simply taking some of the transformed bacterial colony and using it to provide the DNA template in a PCR with sequence specific primers that bracket the region of DNA of interest. During a PCR the denaturation step (figure 4a.1) heats the reaction mixture to 95°C, which disrupts the structure of the thin cell wall and cell membrane of *E. coli*. The bacterial DNA is released into the PCR mixture and becomes available for the primers to bind to during the annealing step. As in laboratory 4a, two primers that bind specifically to the DNA sequence of the pARA plasmid 136 bp from the *Bam*HI restriction site and 149 bp from the *Hin*dIII restriction site will be used to amplify the DNA bracketing and including the *rfp* gene in the pARA-R plasmid. The DNA product of the PCR is then produced during the extension step. For this particular PCR, the expected size of the DNA product from plasmid pARA-R is 1092 base pairs (see figure 7a.1). Presence of a DNA fragment of this size provides strong evidence that the *rfp* gene in the pARA-R plasmid is present, as predicted.

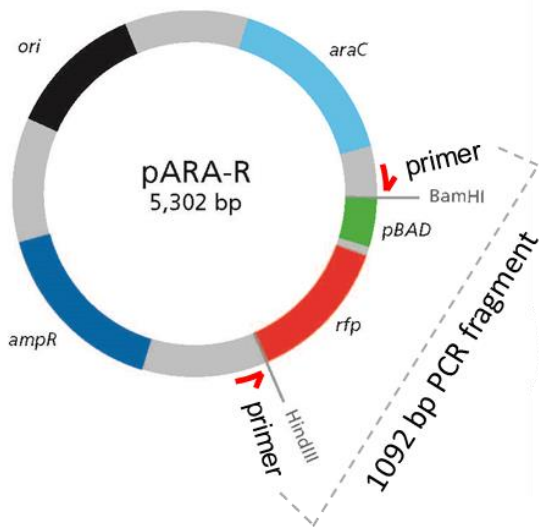


Figure 7a.1. The pARA-R plasmid with primer binding sites and expected PCR fragment size.

In this lab you will select a red colony from your LB/amp/ara transformation plate and perform colony PCR to detect whether a DNA fragment corresponding to the size of pARA-R is present or not. It is also good practice to perform the PCR with: (i) DNA template known to contain the *rfp* gene (pARA-R) – this confirms that the PCR is working and the expected DNA size (1092 bp); and (ii) no DNA template – this is a negative control, used to confirm that the reagents are not contaminated with DNA. Rather than get each pair to set up these additional reactions, your teacher will set up these 2 PCRs and run them in the PCR machine with your reactions, so that you can see the results afterwards using agarose gel electrophoresis.

Learning objectives

By the end of this laboratory you will be able to:

- Explain how PCR can be used to amplify a specific DNA sequence
- Understand the link between an organism's genotype and phenotype
- Describe the size of possible PCR products from your transformed bacteria

Laboratory 7a.1: Colony PCR from the transformed bacteria

Aims

To use the enzyme DNA polymerase in a **P**olymerase **C**hain **R**eaction (PCR), catalysing the extension of sequence specific primers to confirm the presence of a DNA fragment corresponding to the size of the *rfp* gene in a red bacterial colony.

Materials

EQUIPMENT & SUPPLIES

P-20 micropipette (2-20 µL)
Disposable pipette tips
Permanent marker
Polystyrene cup of ice
Waste container
Microcentrifuge (shared)
PCR machine (shared)

REAGENTS

1 PCR tube of 2 X PCR master mix (MM)

Microfuge tubes containing:

- Combined forward and reverse primer (PRI)
- Nuclease-free water (H_2O)

Your returned LB/amp/ara plates from lab 6a

Health and safety

The colony pick PCR involves opening agar plates containing the genetically modified bacteria that you have created. As it is not good practice to open agar plates after incubation that contain unknown microbes, only uncontaminated agar plates (containing only the expected red and white *E. coli* colonies) should be used and your teacher will direct you to work where lower school students will not observe and be tempted to copy this procedure. Whilst touching the pipette tip to the bacterial colony and transferring it into the PCR tube you will need to use aseptic technique. All material that comes into contact with microbiological material will be autoclaved to heat sterilise it before disposal.

In this laboratory you will also be using biological materials, albeit in tiny amounts, and they should be used in a controlled way, using techniques that mirror the aseptic techniques used in microbiology. All of the materials that come into contact with the biological materials (pipette tips, PCR tubes and microfuge tubes) will be autoclaved to denature the DNA and enzymes before disposal. As good laboratory practice you should clear up any spills immediately and wash your hands well with soap after completing the practical work.

Methods

1. Check your rack to make sure that you have all the reagents listed:
   a. A polystyrene cup, containing ice, with 1 PCR tube (MM) in it. *This is a mixture of the buffer, magnesium chloride, dNTPs and DNA polymerase from a thermophilic bacteria, which are required for a PCR*.
   b. A LB/amp/ara plate with your transformed colonies on returned to you from a previous class (Laboratory 6a).
   The bacterial colonies will provide the template DNA for the PCR.

c. A microfuge tube containing forward and reverse primers (PRI). *Primers are short stretches of DNA that bind to complementary DNA sequence during the annealing step, allowing DNA polymerase to extend a specific part of the DNA*.

d. A microfuge tube containing nuclease-free water (H_2O).
This will be used to make up the reaction volume.

2. Use a marker to label the PCR tube containing 2 X PCR master mix (MM) on the side with your personal identifier and the word 'red'. Return it to the ice.

⚠ Marker pen can rub off the top of the PCR tubes in the PCR machine, so make sure you label them on the side.

3. Review table 7a.1, which summarises the reagents that you will add in steps 4-5.

Step	**Reagent**	**Red PCR**
4a	2 X PCR master mix (MM)	12.5 µL
4b	Nuclease-free water (H_2O)	9.5 µL
4c	Combined primers (PRI)	2.0 µL
5	Red bacteria	~1.0 µL

Table 7a.1 Reagents to add to the red PCR tubes

⚠ In steps 4-5, be sure to use a new micropipette tip for each reagent to avoid contamination.

4. a. To your labelled PCR tube of master mix add the following:
   b. 9.5 µL of nuclease-free water (H_2O).
   c. 2.0 µL of combined forward and reverse primers (PRI).
5. To introduce DNA from the transformed bacteria into the PCR you will use aseptic technique (see pages A.1 – A.3). Select one red colony for use. Mark its position using permanent marker on the base of the petri dish.
   a. Place a fresh tip on a P-20 micropipette.
   b. Opening the petri dish like a 'clamshell' facing away from yourself, touch a sterile pipette tip to the circled red colony. Close the transformation plate.
   c. Taking care not to touch anything else with the micropipette tip, lower it into the 'red' PCR tube.
   d. Gently pipette the PCR mixture up and down about 10 times to transfer the bacteria into the PCR mixture.
   e. Dispose of the pipette tip in the waste container.
6. Ensure the reagents are well mixed, by pumping gently up and down with the pipette or simply swirling with the tip. Keep your reactions on ice until the PCR machine can be started.
7. The PCR programme that you will use is given in table 7a.2. It take about 1½ hours to complete.

Reagent		**Temperature (°C)**	**Time (sec)**
Initial denaturation		95	200
cycles of	Denaturation 30	95	30
	Annealing	53	30
	Extension	68	30
Final extension		68	300
Hold		10	Indefinite

Table 7a.2 The PCR programme.

8. When your teacher tells you, transfer your PCR tube from the ice into the PCR machine and start the reaction.
9. After the PCR programme is complete your PCR tube can be place in the fridge (4°C) until you are ready to use it.

Questions

1. What is the structure of a prokaryotic cell? Do prokaryotes have cell walls? What about cell membranes and nuclear membranes?
2. Why does DNA need to be extracted from animal cells for use in PCR (laboratory 8), but not from bacterial cells?
3. What is aseptic technique? Why do you need to use it when performing colony PCR? What constitutes good aseptic technique?
4. Why does the *Taq* DNA polymerase not denature during the denaturation step at 95°C in your PCR programme?
5. What size of DNA fragment do you expect to be amplified by PCR from the transformed bacteria that produce red colonies on the LB/amp/ara plates?

Laboratory 7a.2: Visualisation of products from the colony PCR

Aims

To visualise the size of PCR product from a red bacterial colony using agarose gel electrophoresis, allowing the size of the DNA product (which is dependent on the genotype) to be correlated with the colour of the transformed bacteria (the phenotype).

Materials

EQUIPMENT & SUPPLIES

P-20 micropipette (2-20 µL)
Disposable pipette tips
1 x 1.5 mL microfuge tubes
Permanent marker
Waste container
Microcentrifuge (shared)
Electrophoresis tank with 0.8% agarose gel (shared)
Plastic gel staining tray
UV transilluminator
Hood
Camera

REAGENTS

Microfuge tubes containing:

- loading dye (LD)
- distilled water (dH_2O)
- DNA ladder (1kb)

PCR tubes containing:

- the PCR with a red colony as template from lab 7a.1 (MMred)

1.5 X GelRed solution

1 x TAE buffer

Health and safety

In this laboratory you will be using biological materials, albeit in tiny amounts, and they should be used in a controlled way, using techniques that mirror the aseptic techniques used in microbiology. All of the materials that come into contact with the biological materials (pipette tips, microfuge tubes and agarose gels) will be autoclaved to denature the DNA and enzymes before disposal.

The loading dye is low hazard, but can stain skin, so try to avoid contact with it as good practice would dictate. You should wear safety glasses and chemical-resistant gloves when using the electrophoresis buffer, to avoid contact with the solution.

The electrophoresis chamber contains 1 X TAE buffer at 100 volts. To prevent contact with the liquid during electrophoresis the tanks are designed so that the electric supply is cut off whenever the lid is removed. Additionally, all connections are plastic coated so that there is no possibility of touching a metal contact which is live at 100 volts.

GelRed is used to stain the DNA. Whilst **not** mutagenic like some stains, this is a substance that can bind to DNA (of which you have lots), therefore you will need to wear chemical resistant gloves, a lab coat and eye protection if you are to complete this part of the procedure.

As good laboratory practice you should clear up any spills immediately and wash your hands well with soap after completing the practical work.

Methods: Electrophoresis

1. Check your rack to make sure that you have all the reagents listed.
2. Use a marker to label a clean microfuge tube 'gelred' for the gel electrophoresis sample. (Also include a personal identifier.)
3. Review table 7a.3, which summarises the reagents that you will add in steps 4-6 to make up the samples for gel electrophoresis.

Step	Reagent	Gelred tube
4	Distilled water (dH_2O)	6.0 µL
5	Loading dye (LD)	2.0 µL
6	The PCR with a red colony as template from lab 7a.1 (MMred)	2.0 µL

Table 7a.3 Reagents to add to the tubes for gel electrophoresis samples

⚠ In steps 4-6, be sure to use a new micropipette tip for each reagent in each tube to avoid contamination.

4. Add 6.0 µL of distilled water (dH_2O) to the gelred tube.
5. Add 2.0 µL of loading dye (LD) to the gelred tube.
6. Add 2.0 µL of the red colony PCR from lab 7a.1 (MMred) to the gelred tube.
7. Spin the microfuge tube in the microcentrifuge for four seconds to pool the reagents at the bottom.

⚠ Distribute the tubes evenly in the microcentrifuge so that their weight is balanced.

8. Make sure that the wells in your gel electrophoresis unit are located near the negative (black) electrode.
9. Fill the box with 1x TAE to a level that just covers the entire surface of the gel. If you see any "dimples" over the wells, add more buffer.

⚠ If there are "dimples," add *very small* amounts of buffer to the electrophoresis box. While the gel needs to be completely under the buffer, you don't want too much buffer in the box, as this will allow the electrical current to run through the buffer and not the gel.

10. Make a drawing in your notebook that shows the location of the samples in wells in the electrophoresis box. A suggested order for loading the samples into the wells is shown in Figure 7a.2. If four groups share an 8-well gel, there will be space for two wells containing 1 kb DNA ladder and the two samples prepared by the teacher.

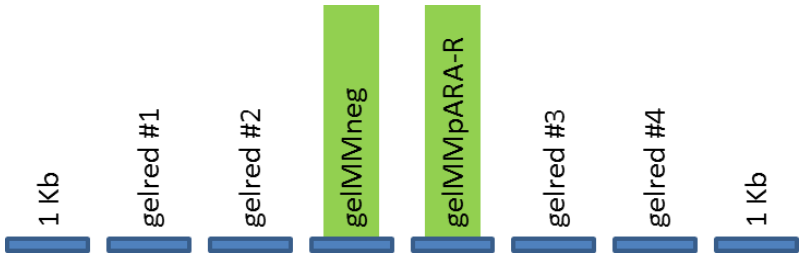


Figure 7a.2 A sample loading order into the wells of the agarose gel. The samples shown in green are prepared by the teacher.

11. Using a fresh pipette tip for each sample, dispense 10.0 µL of the DNA ladder (1 kb), gelMMneg and gelMMpARA-R into the designated wells for each gel. Dispense 10.0 µL of the gelred sample for each group into their designated wells. For each sample, do the following:
    a. Place your elbow on the table to steady your pipette hand. If needed, also use your other hand to support your pipette hand.
    b. Lower the pipette tip until it is under the buffer but just above the well.

⚠ Be careful not to place your pipette tip into the well or you might puncture the gel and lose your sample out the hole.

⚠ Only press the micropipette plunger to the first stop when dispensing samples to avoid getting air bubbles in the buffer. The sample will then sink into the well.

12. When all the samples have been loaded, close the cover tightly over the electrophoresis box. Lower the cover in a horizontal motion, so that the samples don't spill.
13. Connect the electrical leads to the power supply. Connect both leads to the same channel, with negative electrode to negative electrode (black to black) and positive electrode to positive electrode (red to red). See figure 5a.4.
14. Turn on the power supply and set the voltage to approx. 100 V.
15. After two or three minutes, check to see if the purple loading dye (bromophenol blue) is moving toward the positive (red) electrode. If it's moving in the other direction—toward the negative (black) electrode—check the electrical leads to see whether they are plugged in to the power supply correctly.
16. Your teacher will explain what to do with your gel. You may not have sufficient time to complete the electrophoresis. The yellow loading dye will need to run just before the end of the gel. This takes about 40 minutes. After the gel has finished running, it will need to be stained to show the location of the DNA fragments and plasmids.

Methods: Visualisation

1. When electrophoresis is finished, the gel needs to be stained to allow the DNA to be visualised. This will be done using GelRed. Whilst **not** mutagenic like some stains, this is a substance that can bind inside DNA (of which you have lots), therefore you will need to wear chemical resistant gloves, a lab coat and eye protection if you are to complete this part of the procedure.
2. Turn off the Powerpack. Carefully slide the gel from the electrophoresis gel tray into the plastic gel staining tray.
3. Cover the gel with GelRed 1.5 x solution and allow the gel to stain for 30 minutes.
4. Your teacher will tell you whether (i) to pour the GelRed into a foil- covered bottle for reuse, or (ii) to dispose of the GelRed down the sink, washing it away with plenty of water.
5. Wearing chemical resistant gloves, carefully place the gel onto the glass plate of the UV transilluminator. You should be able to see the DNA stain fluorescing without using the hood and camera.
6. Use the hood and camera to take a photo of your gel for analysis.
7. Once you have obtained an image of the gel, place the gel in the box or autoclave bag on your teacher's desk for disposal.

Questions

1. What is the purpose of including a negative control PCR, without a DNA template?
2. What is the purpose of the PCR that has pARA-R as template? What size fragment would you expect to see produced by PCR with this plasmid as template?
3. What product would you expect to see from the red colony PCR? Include the length (bp size) of the PCR product.
4. The DNA ladder serves as a standard because it contains a mixture of DNA molecules of known sizes. By running your samples and the DNA ladder side by side in your gel, you can estimate the actual size in base pairs of unknown DNA molecules. The DNA ladder diagram below shows 10 DNA bands of known sizes. Their sizes are given in kilobase (kb) pairs. 1 kb is 1,000 base pairs (bp). On the diagram predict the positions of DNA bands produced from the gelMMpARA-R and gelred tubes by drawing them on the DNA Ladder Diagram (figure7a.3).
5. When you have had a chance to examine your gel photograph, analyse how your actual gel results compare to your gel predictions.
6. Do you see any bands that are not expected? What could explain the origin of these unexpected bands?
7. Can you work out what the plasmid constructs in the colonies you have analysed must be to produce the size of the DNA fragments after PCR that you observe? Does the genotype you have suggested correlate with the phenotype of the bacterial colony?
8. How does the genetic information, in the form of DNA, produce the phenotype that you observe?

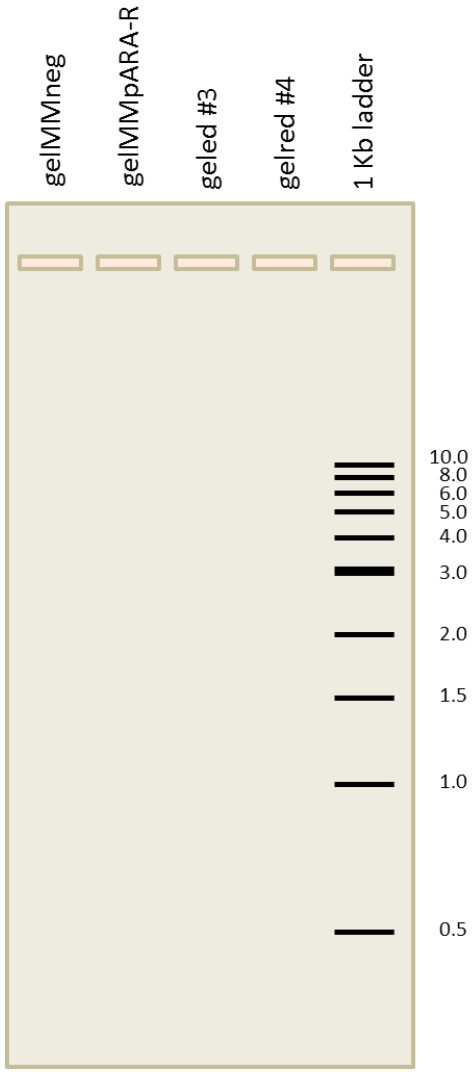


Figure 7a.3 The DNA ladder diagram.

Chapter 9: DNA profiling

Introduction

In 1984 a scientist called Alec Jeffreys, working at the University of Leicester, reported a DNA profiling technique. This technique, also known as DNA fingerprinting or genetic fingerprinting, used the fact that although humans share the vast majority of their DNA sequence (>99.9%), there are regions of DNA sequence that are highly variable. These DNA regions are known as microsatellites, or **v**ariable **n**umber **t**andem **r**epeat (VNTR) sequences. VNTR sequences appear in the same place in the DNA of different individuals, but are so different in length that they allow individuals to be distinguished.

DNA profiling determines the length of these VNTR sequences. By looking at several VNTR sequences in an individual, a unique DNA profile is created. Within each diploid cell, an individual has two copies of each chromosome, one inherited from their mother and one inherited from their father, and therefore two different lengths of each VNTR region, one inherited from their mother and one inherited from their father. This is why closely related individuals share more VNTR sequences of the same length than unrelated individuals. It means that DNA profiling can be used:

- to monitor the spread of animal or plant populations for conservation purposes,
- to ensure that zoo animals do not become inbred, and
- to test for paternity.

DNA profiling techniques are also used in criminal investigations.

In the DNA profiling process, research biologists would normally use a technique called Southern blotting. This is a lengthy process, shown in figure 9.1, and you will use a simplified version. Southern blotting usually involves cutting up an individual's genomic DNA (using restriction enzymes), separating the DNA

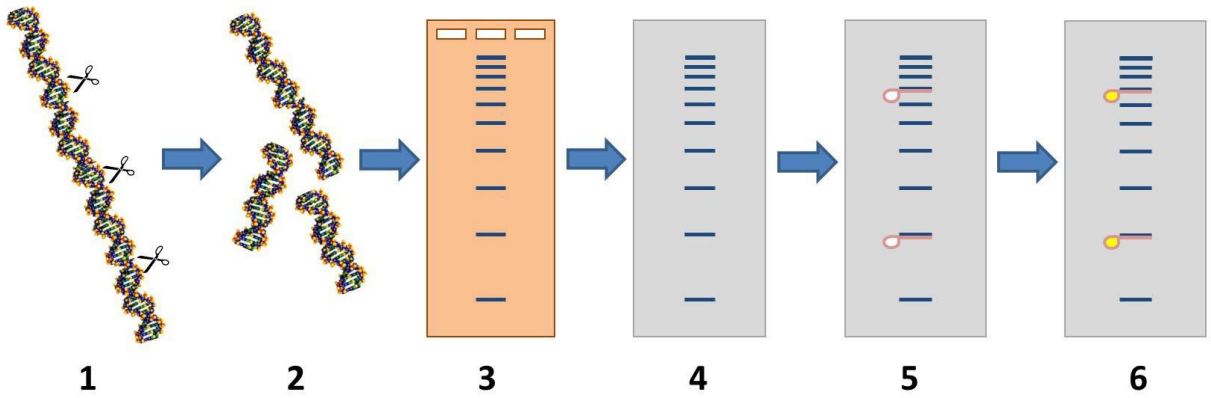


Figure 9.1 Southern blotting. A length of DNA is cut (**1**), using proteins called restriction enzymes. The resulting fragments of DNA (**2**) are loaded into the well of an agarose gel. The wells are shown as white rectangles (**3**). When an electric current is passed through the agarose gel the negatively charged DNA (shown as blue bands) moves to the positive electrode. Smaller fragments of DNA move faster through the agarose gel matrix, getting further from the wells, and the DNA becomes separated by size (**3**). The DNA can **not** be seen at this point. The size-separated DNA is transferred onto a special membrane (**4**) and treated so that it becomes single-stranded. A probe of labelled, single-stranded DNA with a VNTR sequence is washed over the membrane and forms hydrogen-bonds with complementary base pairs (A with T, C with G) (**5**). Finally, the label is detected, DNA is seen for the first time, and the size of the VNTR sequence is determined (**6**).

by length (using agarose gel electrophoresis), transferring the DNA onto a membrane, sticking labelled DNA (of the sequence of the VNTR being studied) onto the DNA on the membrane, then detecting the labelled DNA and size of the VNTR (figure 9.1).

To produce a DNA profile you will examine the different banding patterns produced when DNA from a group of individuals is cut with restriction enzymes and separated using agarose gel electrophoresis. This follows the start of the Southern blotting procedure. Then instead of blotting you will visualise a DNA profile in the agarose gel using a special stain that binds to DNA. You will be testing samples of DNA from 5 individuals and trying to match 1 of the samples to a reference sample of DNA given to you as part of a scenario. Carry out the laboratory work correctly, and you will be able to conclude the investigation.

Learning objectives

By the end of this laboratory you will be able to:

- Explain how the DNA profile of an individual can be generated.
- Describe 3 different uses of DNA profiling.
- Understand what a VNTR is and the role it plays in DNA profiling.
- Explain the function of restriction enzymes.
- Describe how agarose gel electrophoresis can be used to separate DNA.

Laboratory 9.1: Cutting DNA by restriction digestion

Aims

To use restriction digests to cut up the 6 DNA samples that you have been given.

Introduction

Restriction enzymes were discovered in the early 1950s, in bacteria. They are proteins that recognise and destroy the DNA of viruses that infect bacteria, without damaging the bacterial DNA – like a primitive immune system. Restriction enzymes always cut at the same, specific DNA sequence, called a recognition site.

Since the Human Genome Project has completed the sequencing of human DNA, recognition sites in highly conserved areas of DNA sequence either side of VNTRs can be identified. The same can be done in other organisms for which the genome sequence is known. Restriction enzymes can then be used to 'cut out' the VNTR, before agarose gel electrophoresis is used to determine its size. During this laboratory you will use restriction enzymes to produce DNA fragments containing VNTRs.

Materials

EQUIPMENT & SUPPLIES

P-20 micropipette (2-20 µL)
Disposable pipette tips
Permanent marker
Waste container
Floating tube rack
Stopwatch
Polystyrene cup of ice
Microcentrifuge (shared)
37°C water bath (shared)

REAGENTS

Microfuge tubes containing:

- Reference sample DNA (DNAref)
- DNA sample 1 (DNA1)
- DNA sample 2 (DNA2)
- DNA sample 3 (DNA3)
- DNA sample 4 (DNA4)
- DNA sample 5 (DNA5)
- 2.5 X restriction buffer (BF)
- Restriction enzyme (ENZ)

Health and safety

In this laboratory you will be using biological materials, albeit in tiny amounts, and they should be used in a controlled way, using techniques that mirror the aseptic techniques used in microbiology. All of the materials that come into contact with the biological materials (pipette tip and microfuge tubes) will be autoclaved to denature the DNA and enzymes before disposal.
As good laboratory practice you should clear up any spills immediately and wash your hands well with soap after completing the practical work

Methods

1. Check your rack to make sure that you have all the reagents listed.
2. Use a marker to label the six microfuge tubes containing the DNA samples (DNAref, DNA1, DNA2, DNA3, DNA4 and DNA5) with your personal identifier. You will set up the restriction digests in these tubes.
3. Review table 9.1, which summarises the reagents that you will add in step 4.

Step	Reagent	DNAref	DNA1	DNA2	DNA3	DNA4	DNA5
4a	DNA sample	2.0 µL	2.0 µL	2.0 µL	2.0 µL	2.0 µL	2.0 µL
4b	Restriction buffer (BF)	4.0 µL	4.0 µL	4.0 µL	4.0 µL	4.0 µL	4.0 µL
4c	Distilled water (dH_2O)	2.0 µL	2.0 µL	2.0 µL	2.0 µL	2.0 µL	2.0 µL
4d	Restriction enzyme (ENZ)	2.0 µL	2.0 µL	2.0 µL	2.0 µL	2.0 µL	2.0 µL

Table 9.1 Reagents in the restriction digest reactions

⚠ In step 4, be sure to use a new micropipette tip for each reagent in each tube to avoid contamination.

4. To set up the restriction digest reactions:
   a. 2.0 µL of DNA sample has already been prepared for you in labelled tubes (DNAref, DNA1, DNA2, DNA3, DNA4 and DNA5) as there is often a limited amount of DNA sample available.
   b. Add 4.0 µL of restriction buffer (BF) to each tube.
   c. Add 2.0 µL of Distilled water (dH_2O) to each tube.
   d. Add 2.0 µL of restriction enzyme (ENZ) to each tube.
5. Spin the six microfuge tubes (DNAref, DNA1, DNA2, DNA3, DNA4 and DNA5) in the microcentrifuge for four seconds to pool the reagents at the bottom of each tube.

⚠ Distribute the tubes evenly in the microcentrifuge so that their weight is balanced.

6. Place all six tubes into the 37°C water bath and incubate for one hour (minimum).

After the incubation is complete either continue to laboratory 9.2, or place all four tubes in the freezer (–20°C) until you are ready to do so.

Questions

1. Which organisms produce restriction enzymes?
2. What do restriction enzymes do?
3. Why is important to cut DNA in conserved sequences either side of a VNTR during DNA profiling?
4. What is a VNTR?

Laboratory 9.2: DNA separation by agarose gel electrophoresis

Aims

To separate the DNA fragments containing the VNTRs by size using agarose gel electrophoresis. Then to compare the relative sizes of the DNA fragments containing VNTRs and to determine which matches the reference sample.

Introduction

Agarose gel electrophoresis is the technique used to check the size of DNA molecules. DNA samples are mixed with loading dye, which helps to show where the sample is and to make it sink. The DNA samples, combined with loading dye, are placed into the wells of an agarose gel. The agarose gel is submerged in electrophoresis buffer within a gel electrophoresis tank. An electric field is then put through the gel to separate the DNA. DNA is negatively charged, because of the phosphate groups in the backbone, so it is loaded near the negative electrode and will move through the porous matrix of the agarose gel towards the positive electrode. The DNA molecules are separated primarily by size. Small DNA molecules move through the agarose matrix more easily, going further than larger DNA molecules (figure 9.2).

Using gel electrophoresis and staining you will examine the size of the DNA fragments containing VNTRs. The exact size of DNA fragments can be determined by comparison to a DNA ladder (that is a mixture of DNA fragments of known sizes, which look like the rungs of a ladder after gel electrophoresis and staining). **For DNA profiling, however, it is more important to compare the banding patterns produced for each sample, known as a 'DNA profiles'**. By matching the DNA profile of the reference DNA sample to one of the unknown DNA samples you will be able to resolve a DNA profiling scenario.

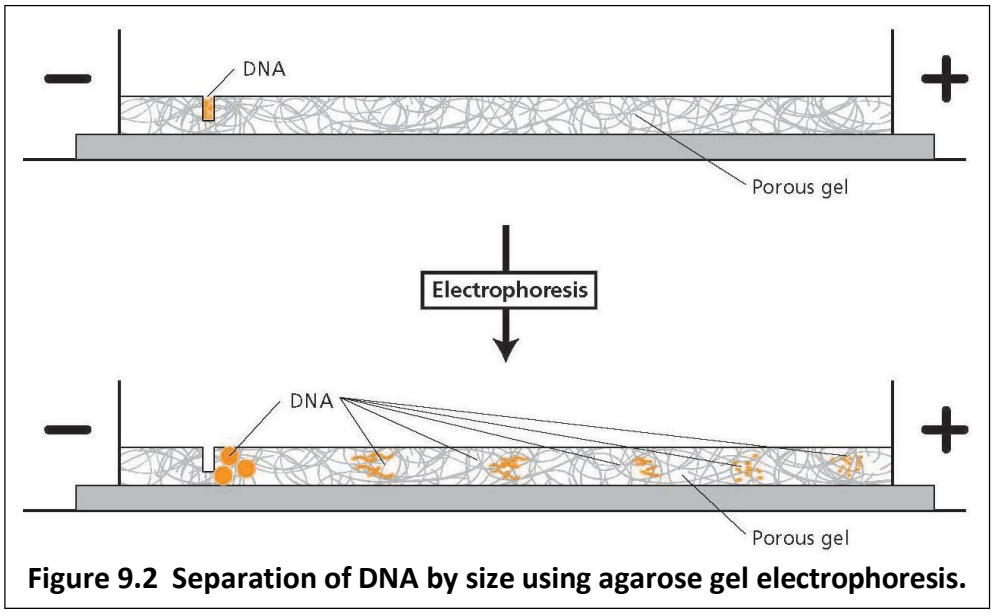


Figure 9.2 Separation of DNA by size using agarose gel electrophoresis.

Materials

EQUIPMENT & SUPPLIES

P-20 micropipette (2-20 µL)
Disposable pipette tips
Permanent marker
Waste container
Microcentrifuge (shared)
Electrophoresis tank with 0.8% agarose gel (shared)
Plastic gel staining tray
UV transilluminator
Hood
Camera

REAGENTS

Microfuge tubes containing:

- Digested DNAref from lab 9.1
- Digested DNA1 from lab 9.1
- Digested DNA2 from lab 9.1
- Digested DNA3 from lab 9.1
- Digested DNA4 from lab 9.1
- Digested DNA5 from lab 9.1
- loading dye (LD)
- DNA ladder (1kb)

1 X TAE buffer
1.5 X GelRed solution

Health and Safety

In this laboratory you will be using biological materials, albeit in tiny amounts, and they should be used in a controlled way, using techniques that mirror the aseptic techniques used in microbiology. All of the materials that come into contact with the biological materials (pipette tips, microfuge tubes and agarose gels) will be autoclaved to denature the DNA and enzymes before disposal.

The loading dye is low hazard, but can stain skin, so try to avoid contact with it as good practice would dictate. You should wear safety glasses and chemical-resistant gloves when using the electrophoresis buffer, to avoid contact with the solution.

The electrophoresis chamber contains 1 X TAE buffer at 100 volts. To prevent contact with the liquid during electrophoresis the tanks are designed so that the electric supply is cut off whenever the lid is removed. Additionally, all connections are plastic coated so that there is no possibility of touching a metal contact which is live at 100 volts.

GelRed is used to stain the DNA. Whilst **not** mutagenic like some stains, this is a substance that can bind to DNA (of which you have lots), therefore you will need to wear chemical resistant gloves, a lab coat and eye protection if you are to complete this part of the procedure.

As good laboratory practice you should clear up any spills immediately and wash your hands well with soap after completing the practical work.

Methods

1. Check your rack to make sure that you have all the reagents listed.
2. Use the microcentrifuge to spin the 6 microfuge tubes containing your DNA samples (DNAref, DNA1, DNA2, DNA3, DNA4 and DNA5) for four seconds. This will pool any condensation resulting from incubation in the water bath.

⚠ Distribute the tubes evenly in the microcentrifuge so that their weight is balanced.

3. Review table 9.2, which summarises the reagents that you will add in step 4 to make up the samples for gel electrophoresis.

Step	Reagent	Gel tube
4a	Digested DNA sample from lab 9.1	10.0 µL
4b	Loading dye (LD)	2.5 µL

Table 9.2 Reagents to add to prepare the samples for gel electrophoresis.

⚠ In step 4, be sure to use a new micropipette tip for each reagent in each tube to avoid contamination.

4. To prepare your samples to load onto the gel:
   a. Take your tube containing 10.0 µL of digested DNA sample.
   b. Add 2.5 µL of loading dye (LD).
   c. Gently pump up and down 5 times with the micropipette to mix.
5. Spin the microfuge tube in the microcentrifuge for four seconds to pool the reagents at the bottom of each tube.

⚠ Distribute the tubes evenly in the microcentrifuge so that their weight is balanced.

6. Make sure that the wells in your gel electrophoresis unit are located near the negative (black) electrode.
7. Fill the box with 1x TAE to a level that just covers the entire surface of the gel. If you see any "dimples" over the wells, add more buffer.

⚠ If there are "dimples," add *very small* amounts of buffer to the electrophoresis box. While the gel needs to be completely under the buffer, you don't want too much buffer in the box, as this will allow the electrical current to run through the buffer and not the gel.

8. Make a drawing in your notebook that shows the location of the samples in wells in the electrophoresis box. A suggested order for loading the samples into the wells is shown in figure 9.3. Using a 15-well gel, 2 sets of DNA profiling samples can be loaded with 1 spare well.

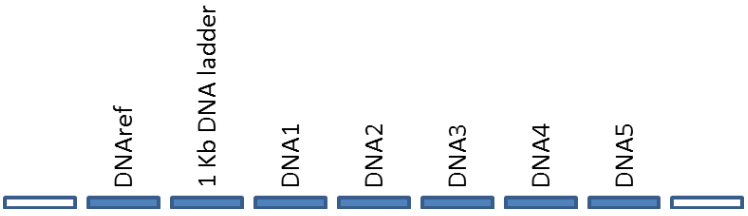


Figure 9.3 Sample loading order into the wells of the agarose gel.

9. Using a fresh pipette tip for each sample, dispense into the designated wells:
   a. 10.0 µL of the DNA ladder (1 kb).
   b. 12.5 µL of the digested DNA samples.

   For each sample, place your elbow on the table to steady your

pipette hand. If needed, also use your other hand to support your pipette hand. Lower the pipette tip until it is under the buffer but just above the well to dispense.

⚠ Be careful not to place your pipette tip into the well or you might puncture the gel and lose your sample out the hole.

⚠ Only press the micropipette plunger to the first stop when dispensing samples to avoid getting air bubbles in the buffer. The sample will then sink into the well.

10. When all the samples have been loaded, close the cover tightly over the electrophoresis box. Lower the cover in a horizontal motion, so that the samples don't spill.
11. Connect the electrical leads to the power supply. Connect both leads to the same channel, with negative electrode to negative electrode (black to black) and positive electrode to positive electrode (red to red). See figure 9.4 below.

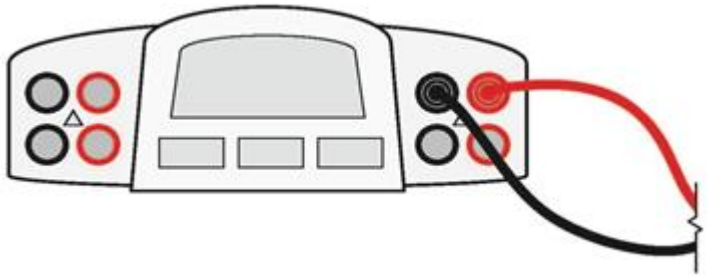


Figure 9.4 Connecting leads from the electrophoresis tank to the powerpack.

12. Turn on the power supply and set the voltage to 90 V.
13. After two or three minutes, check to see if the purple of the loading dye (bromophenol blue) is moving toward the positive (red) electrode. If it's moving in the other direction—toward the negative (black) electrode—check the electrical leads to see whether they are plugged in to the power supply correctly.
14. Your teacher will explain what to do with your gel. You may not have sufficient time to complete the electrophoresis. The orange loading dye will need to run to about 1cm from the bottom of the gel. This takes about 1 hour. After the gel has finished running, it will need to be stained to show the location of the DNA fragments and plasmids.

Methods: Visualisation

1. When electrophoresis is finished, the gel needs to be stained to allow the DNA to be visualised. This will be done using GelRed. Whilst **not** mutagenic like some stains, this is a substance that can bind inside DNA (of which you have lots), therefore you will need to wear chemical resistant gloves, a lab coat and eye protection if you are to complete this part of the procedure.

2. Turn off the Powerpack. Carefully slide the gel from the electrophoresis gel tray into the plastic gel staining tray.
3. Cover the gel with GelRed 1.5 x solution and allow the gel to stain for 30 minutes.
4. Your teacher will tell you whether (i) to pour the GelRed into a foil- covered bottle for reuse, or (ii) to dispose of the GelRed down the sink, washing it away with plenty of water.
5. Wearing chemical resistant gloves, carefully place the gel onto the glass plate of the UV transilluminator. You should be able to see the DNA stain fluorescing without using the hood and camera.
6. Use the hood and camera to take a photo of your gel for analysis.
7. Once you have obtained an image of the gel, place the gel in the box or autoclave bag on your teacher's desk for disposal.

Questions

1. Why was a loading dye mixed with the samples before they were loaded onto the agarose gel?
2. Why was the DNA loaded by the negative electrode on the agarose gel?
3. How does agarose gel electrophoresis separate DNA molecules by size?
4. How many copies of each VNTR does an individual have in a somatic cell? How many copies of a VNTR would an individual have in a gamete?
5. VNTRs are always found in the same location in the DNA sequence, but vary in length. How does this help when we want to generate a DNA profile?
6. DNA profiling is frequently used in crime scene investigations. Can you describe 2 or more other applications for DNA profiling?
7. When you have had a chance to examine your gel photograph, analyse whether the restriction digests were successful. A successfully digested sample of DNA will have discrete bands visible, an unsuccessful digest will still have most of the DNA in one large band near the wells in the gel. If your digest did not work, can you identify what the problem was?
8. Compare the 5 DNA samples to the reference DNA sample. Can you identify the banding pattern, or DNA profile', that matches? Use this information to write a short conclusion about the outcome of your investigation.
9. How could a fluorescently-labelled DNA probe be used to show the presence of a specific DNA sequence?
10. DNA profiles are stored in National DNA databases. There are many controversial issues about these: Who is responsible? Are they secure? Whose DNA profile is held? How long for? Who can access the information? You may explore these issues further.

Chapter 10: Serial dilutions

Introduction

Serial dilution is a series of repeated dilutions performed on the same chemical to decrease its concentration. To achieve this a sample is mixed with another substance, usually water, to make it more dilute. The dilution factor is found by dividing the volume of sample by the final volume of sample and diluent (so if 1 ml of sample was added to 9 ml of water the dilution factor would be 1 in 10).

Serial dilutions are used when a solution is too concentrated either to be used, or for determination of sample concentration by a piece of equipment. For biological purposes serial dilutions are frequently used if the number of cells or micro-organisms in a liquid need to determined. Additionally, when analysing blood samples if the results are outside the range most accurately found by a machine, the blood is diluted, retested and the results multiplied by the dilution factor.

An example of this is in the determination of blood glucose level for diabetics. Blood glucose levels can be high in people with diabetes, as insulin is unavailable or present at reduced levels, meaning that glucose cannot be transported through the cell membrane into the cell. After ingestion of carbohydrates the blood glucose level can exceed the optimum range of glucose detection in the analysis machines. In this case, serial dilution of the sample to obtain a measurement in the range the machine is capable of, then multiplying this result by the dilution factor will give a more accurate measurement.

Learning objectives

By the end of this laboratory you will be able to:

- Use a micropipette correctly
- Perform serial dilutions
- Calculate concentrations and interpret data from serial dilutions

Laboratory 10.1: Performing serial dilutions

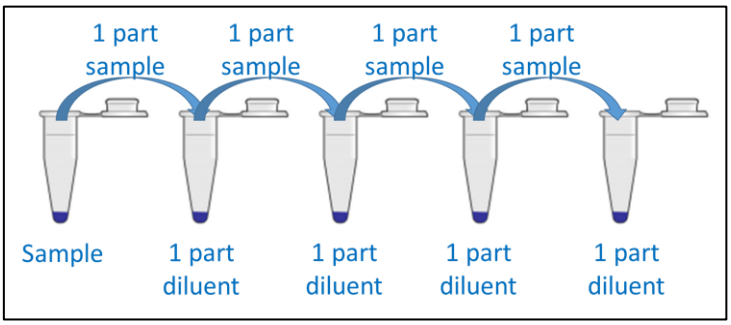
Aims

To use serial dilutions to determine the concentration of an unknown solution.

Introduction

Serial dilutions are used in many laboratory procedures to quantitate samples or concentrations that would otherwise be difficult to enumerate. For example, serial dilutions are frequently used with cultures of cells to achieve a concentration of cells that is possible to count under the microscope or with blood samples to determine the concentration of glucose.

The serial dilutions can be made to achieve different concentrations and ultimate dilution factors. Doubling dilutions involve making a series of 1 in 2 dilutions. Each successive tube has half the amount of the solution being diluted. The process of making doubling dilutions is shown below.



So if a sample is diluted 1 in 2 the resulting solution will be half the concentration of the original sample. After a second 1 in 2 dilution the solution produced will one quarter the concentration of the original sample. In successive steps the solutions produce will be one eighth then one sixteenth the original concentration. This can be written as a dilution factor. For the above example the dilution factors would be written as 1/2 dilution, 1/4 dilution, 1/8 dilution and 1/16 dilution.

In this lab you will practise making a series of doubling dilutions.

Materials

EQUIPMENT & SUPPLIES

P-200 micropipette (50-200 µL)
Disposable pipette tips
Microfuge tubes
Waterproof marker pens
Pasteur pipettes
Piece of tin foil (about 20cm x 20 cm)
Microfuge tube rack
Waste container
Vortex
Boiling water bath
Dimple tiles

REAGENTS

Distilled water (**dH_2O**)

1 M Glucose solution (labelled **G**)

Benedict's Reagent

Mystery tube (labelled **M**)

Health and safety

Glucose solution does not require any special handling instructions, however good laboratory practice should be observed. According to CLEAPSS advice, Benedict's reagent is Low Hazard. It contains slightly alkaline copper sulphate solution (Student Safety sheet 40), but at concentration only categorised as low hazard. There is some risk of spitting when heating test-tubes. It is advisable to take the precaution of wearing goggles when working with Benedict's reagent.

Using a boiling water bath with the samples confers a risk of burns. Care should be taken when using the boiling water bath to avoid scalds or burns.

Methods

PART A: MAKING THE DOUBLING DILUTIONS

You will be using your skills in micropipetting to make 4 consecutive doubling dilutions.

1. Take 6 microfuge tubes and label the top of them 1 to 6 in waterproof marker pen.
2. Use the micropipette to put 200 µl of water into the microfuge tubes labelled 2 to 5.
3. Put a fresh tip on the micropipette, then use the micropipette to put 200 µl of the 1 M glucose solution into the microfuge tube labelled 1.
4. Micropipette 200 µl of the 1 M glucose solution into the microfuge tube labelled 2.
5. Push the top of the micropipette up and down gently 5 times to mix the sample.
6. Aspirate 200 µl of the resulting solution and dispense it into the microfuge tube labelled 3. Gently pump the micropipette to mix the sample.
7. Aspirate 200 µl of the resulting solution and dispense it into the microfuge tube labelled 4. Gently pump the micropipette to mix the sample.
8. Aspirate 200 µl of the resulting solution and dispense it into the microfuge tube labelled 5. Gently pump the micropipette to mix the sample.
9. Aspirate 200 µl of the resulting solution and dispense it into the microfuge tube labelled 6.

PART B: PREPARING THE SAMPLES TO DETERMINE GLUCOSE CONCENTRATION

1. Use a Pasteur pipette to add 1 ml of Benedict's reagent to your microfuge tubes labelled 1-5, and to the mystery tube, labelled **M**.
2. Mix briefly using the vortex.
3. Place the microfuge tubes into holes in a folded layer of tin foil and place into a boiling water bath.
4. Incubate the microfuge tubes in the water bath for 5 minutes.
5. Mix briefly using the vortex.
6. Use 6 clean Pasteur pipettes to transfer each sample into one well of a dimple tray. MAKE SURE that you remember which sample is which.

PART C: PLOTTING AND USING A CALIBRATION CURVE

1. Using the "*ColorMeter Free app*" from an Android phone or "*Colorimeter app*" from an Apple device focus on the solution in each well, one after the other. Record the Red / Green / Blue intensities into a table, like the one shown.

Glucose solution dilution factor	Glucose solution concentration (M)	Red intensity	Green intensity	Blue intensity
1	1			
1:2	0.5			
1:4	0.25			
1:8	0.125			
1:16	0.0625			
M	Mystery			

2. Plot the data onto a graph. The x axis should show the 'glucose solution concentration (moles)'. The y axis should show the 'colour intensity (arbitrary units)'. You will need to use a key to show the data for the 3 different colours on one graph.
3. Now use your graph to determine the glucose concentration in the mystery sample.
4. To determine how reliable your data is, compare your results for the serial dilutions and mystery sample with other groups.

Questions

1. If you had a glucose solution with an initial concentration of 8 M, what would be the dilution factor after each of 6 doubling dilutions? What would be the concentration of the solutions after each of 6 doubling dilutions?
2. As the concentration of glucose increased what happened to the intensities of each of the colours (red, green and blue)?
3. How reliable was your graph for determining the concentration of the mystery sample?
4. What factors could affect the reliability of your results?

Further questions

1. What is the dilution factor from adding a 0.1 ml aliquot of a specimen to 9.9 ml of diluent?
2. If a 1/8 dilution of the stock solution is made followed by a 1/6 dilution what is the final dilution?
3. You have a microfuge tube containing 1 ml of a solution with 4.3 x 10^4 cells/ml and you are to produce a solution that contains 43 cells/ml. How many 1/10 dilutions must you perform?
4. You have decided to determine how many microbes are living on the lettuce in the salad bar at your favourite restaurant. You place 1 gram of lettuce and 99 ml of water in a blender and blend the mixture. This is sample A. You then transfer 1 ml of this dilution into to another that contains 9 ml of water. This becomes sample
   B. You next transfer 1 ml of sample B into a separate container that contains 9 ml of water. This is sample C. Next you transfer 1 ml each from samples B & C onto separate nutrient rich agar plates, swirl, let harden and incubate at 37°C. When you examine the plates after 48 hours you find 110 colonies growing on plate C. (For your information 1 gram lettuce = 1 ml).

(a) What was the bacterial count in SAMPLE A?

(b) Would you expect to find more colonies growing on the plate that was inoculated from sample B or sample C?

(c) How many microbes were living on that 1 gram of lettuce?

5. During examination of Chinese Hamster Ovary (CHO) cells kept in culture, the technician discovers that the flask of cells has signs of contamination. Given that the CHO cell line is relatively hardy, she decides to use antibiotics to try and salvage the cells. The stock solution of the antibiotic is 0.1 g/ml, and it needs to be used at a working concentration of 10 µg/ml.

(a) How many 10-fold serial dilutions will need to be performed to reach the working concentration of the antibiotic in the cell culture medium? (A 10-fold dilution is 1/10^{th} the concentration of the previous dilution. Each subsequent dilution is made from a previous dilution, so a 10-fold serial dilution is a series of 10-fold dilutions.)

(b) The working concentration of the antibiotic is how many orders of magnitude less than the stock concentration?

Aseptic technique

It is very important in microbiology to keep cultures of bacteria pure and uncontaminated. Unfortunately, microorganisms are all around us, even carried on dust particles in air, which makes this difficult. Aseptic technique is the name of procedures that are used to stop pure cultures from becoming contaminated.

Top tips for aseptic technique

1. **Be neat, tidy and never do it unprotected!**
   Make sure that bags and coats are stowed away from the area where you will be working with microbes. Organise your work area, to avoid spillages and confusion. Wear lab coats and eye protection if appropriate; these protect you from what you're working with and protect what you're working with from you!
2. **Use your Bunsen burner**
   The flame can be used to sterilise glass equipment and create an updraft around the base that leaves a 'sterile zone' (as hot air rises taking airborne contaminants with it).
3. **Keep equipment clean**
   All equipment used for microbiology should be sterile. When removing plasticware from packaging try to touch only the items you intend to use to keep the remaining items from contamination. Keep your tip box closed when not in use for the same reason.
4. **Safe disposal**
   Make sure that any equipment that comes into contact with bacteria is placed in the pots of disinfectant on your bench or in an autoclave bag for decontamination & safe disposal.

When performing laboratory 6, 6a, 7 or 7a you need to practice aseptic techniques to prevent microbes from the air or non-sterile surfaces from entering your cultures of pure bacteria. This is important when: (i) adding plasmid and LB broth to your tube of competent cells during the transformation procedure; (ii) spreading the transformed bacteria onto your agar plates, and (iii) performing the colony pick PCR. Before starting it is good practice to wipe benches with disinfectant. The methods for good sterile technique during the procedures you may do are described below.

Transferring liquids into the microfuge tube of competent bacterial cells.

1. Before opening any tubes make sure that you have read the instructions and labelled all tubes.
2. Make sure that you are wearing a lab coat.
3. Place tubes between which liquids will be transferred close to each other.
4. Put a tip on the end of the pipette (shutting the pipette tip box afterwards).
5. Making sure that the pipette tip doesn't touch any non-sterile surfaces, open up the microfuge tube containing the liquid to be transferred and gently aspirate the correct amount into the pipette tip. Close the lid of the microfuge tube and return it to the microfuge tube rack.
6. Still making sure that the pipette tip doesn't touch any non-sterile surfaces, carefully open up the microfuge tube to receive the sample and dispense the liquid. Close the lid of the microfuge tube and return it to the microfuge tube rack.
7. Eject the pipette tip, which will have bacteria on it, into the disposal pot of disinfectant or autoclave bag provided by your teacher.

Spreading bacteria onto an agar plate.

1. Before spreading bacteria onto agar plates make sure that you have read the instructions and labelled all the agar plates carefully in small but clear writing.
2. Make sure that you are wearing a lab coat.
3. Place the agar plates and microfuge tube containing the bacteria near to the Bunsen burner and light the Bunsen burner. The heat of the Bunsen burner flame moves the air so that there is a zone around the bottom of the Bunsen burner, where airborne microbes are less likely to land on your agar plates.
4. Put a tip on the end of the pipette (shutting the pipette tip box afterwards).
5. Making sure that the pipette tip doesn't touch any non-sterile surfaces, open up the microfuge tube containing the liquid to be transferred and gently aspirate the correct amount into the pipette tip. Close the lid of the microfuge tube and return to the microfuge tube rack.
6. Still making sure that the pipette tip doesn't touch any non-sterile surfaces, carefully open up the agar plate like a 'clamshell', close to the bottom of the Bunsen burner (but taking care not to get burnt, close is good enough!). Dispense the liquid onto the agar plate and close the lid.
7. Eject the pipette tip, which will have bacteria on it, into the disposal pot of disinfectant or autoclave bag provided by your teacher.
8. Remove a sterile spreader from the packet, taking care only to touch the one you will use and only to touch the handle end.
9. Still making sure that the spreader doesn't touch any non-sterile surfaces, carefully open up the agar plate like a 'clamshell', close to the bottom of the Bunsen burner and use the flat surface of the spreader to spread the liquid around. Close the lid of the agar plate.
10. Dispose of the spreader, which will have bacteria on it, into the disposal pot of disinfectant or autoclave bag provided by your teacher.
11. At no time should the spreader or agar plate or its lid, have touched any non-disinfected surface (including your hands!).

Performing the colony pick PCR.

1. Before starting, ensure that you have selected the colonies you will use in your PCR from your transformation plates and circled them on the plastic base of the plastic petri dish.
2. Make sure that you are wearing a lab coat.
3. Place the transformation plates and PCR tube near to the Bunsen burner and light the Bunsen burner. The heat of the Bunsen burner flame moves the air so that there is a zone around the bottom of the Bunsen burner, where airbourne microbes are less likely to land on your agar plates.
4. Put a tip on the end of the pipette (shutting the pipette tip box afterwards).
5. Making sure that the pipette tip doesn't touch any non-sterile surfaces, carefully open up the agar plate like a 'clamshell', close to the bottom of the Bunsen burner (but taking care not to get burnt, close is good enough!). Touch the end of the pipette tip to the bacterial colony, then withdraw it and close the lid on the petri dish.
6. Taking extra care that the pipette tip doesn't touch any non-sterile surfaces, carefully open up the PCR tube and lower the pipette tip in. Pipette the liquid up and down 5 times, using the plunger to the first stop to transfer the bacteria into the PCR mix. Depress the plunger to the second stop to make sure all liquid is dispensed, then close the PCR tube lid.
7. Eject the pipette tip, which will have bacteria on it, into the disposal pot of disinfectant or autoclave bag provided by your teacher.
8. At no time should the pipette tip, agar plate or its lid, have touched any non-disinfected surface (including your hands!).

For your safety and that of others who use your laboratory, you should disinfect the surfaces where you have been doing microbiology at the end of the procedure. Your teacher will ensure that all plastics that have been in contact with bacteria are decontaminated (using an autoclave, which uses high heat and pressure to kill all bacteria, so that laboratory equipment can be safely disposed of).

Common causes of contamination

- Putting a sterile implement on the lab bench, then picking it up and using it
- Allowing sterile implements to brush against clothing, arms, hair or hands and the using them
- Touching the inside of a sterile tube or agar plate with hands
- Leaving tubes or agar plates open to the air for long periods of time (Unless the air is heavily contaminated with microorganisms, it generally requires several minutes of exposure to air to cause contamination.)

Glossary

Activator A protein that increases gene(s) transcription. Often a DNA-binding protein affecting activity at a promoter.

Adenine (A) A nitrogen-containing base, one member of the base pair AT (adenine-thymine). Adenine is found in both DNA and RNA.

Agar A polysaccharide extracted from the cell walls of some seaweed or red algae species. Frequently used as a culture medium in microbiology**.**

Agarose A highly purified polysaccharide (complex carbohydrate) extracted from seaweed. Commonly used to create a gel matrix for electrophoresis.

Aliquot A sample of some part of a whole (eg., a sample of plasmid or a sample of cells).

Allele An alternative form of a genetic locus; a single allele for each locus is inherited from each parent.

***Alu* element** A 300 base pair piece of DNA that has been randomly inserted throughout the genome. It no longer appears to have any function in the human genome. This specific DNA fragment is called the '*Alu*' element because it carries a recognition sequence for the *Alu* restriction enzyme.

Amino acid Any of a class of 20+ molecules that are combined to form proteins. The sequence of amino acids in a protein and hence protein function are determined by the genetic code.

Ampicillin A commonly used antibiotic of the penicillin family. Ampicillin prevents new cell wall material from linking properly in bacteria. This weakened cell wall will prevent the growth of new bacteria.

Amplification An increase in the number of copies of a specific DNA fragment.

ampR Symbol used to designate the gene for ampicillin resistance. This gene is located in the pARA plasmid.

Annealing Hydrogen bonding between complementary base pairs of two single stranded DNA molecules (usually of a primer and template during PCR).

Antibiotic A substance that kills or prevents the growth of cells.

Arabinose A five carbon sugar that occurs naturally in some plants and bacteria.

AraC protein A protein that is required for expression of mutant fluorescent protein in cells transformed with pARA-R.

Aspirate To draw in or suck in.

Autosome A chromosome that occurs in homologous pairs in both males and females and that does not bear the genes determining sex.

ß-lactamase An enzyme encoded by the *ampR* gene that destroys ampicillin.

Base One of the nitrogen-containing molecules that distinguish one nucleotide from another. In DNA, the bases are adenine, guanine, cytosine and thymine.

Base pair Two nitrogenous bases (adenine and thymine or guanine and cytosine) held together by hydrogen bonds. Two strands of DNA are held together in the shape of a double helix by the bonds between base pairs. The sum of the base pairs in a DNA molecule is frequently used to express the size of the molecule.

Base sequence The order of nucleotide bases in a DNA molecule; determines structure of proteins encoded by that DNA.

Biotechnology A set of biological techniques developed through basic research and now applied to research and product development. In particular, biotechnology refers to the use by industry of recombinant DNA, cell fusion and new bioprocessing techniques.

Buffer A solution used to maintain an optimal physical chemical environment for a chemical reaction. Buffers are used in restriction digests, ligations and for PCR.

Cell The basic unit of any living organism that carries on the biochemical processes of life.

Cell membrane A selectively permeable membrane that regulates movement of substances in and out of a cell.

Cell wall A structure that provides cells with physical support; surrounds some bacterial cells.

Chromosome In eukaryotes, a linear strand composed of DNA and protein, located in the nucleus of a cell, that contains the genes; in prokaryotes, a circular strand composed solely of DNA. In humans, there are 23 pairs of chromosomes in body cells.

Cloning Using specialized DNA technology to produce multiple, exact copies of a single gene or other segment of DNA. A second type of cloning exploits the natural process of cell division to make many copies of an entire cell. The genetic make-up of these cloned cells, called a *cell line*, is identical to the original cell. A third type of cloning produces complete, genetically identical animals or plants.

Cloning vector DNA molecule originating from a virus, a plasmid or the cell of a higher organism into which another DNA fragment of appropriate size can be integrated without loss of the vector's capacity for self-replication; vectors are engineered to introduce foreign DNA into host cells, where the DNA can be reproduced in large quantities. In the Amgen Labs, pKAN is used to clone many copies of the *rfp* gene.

Co-factor A non-protein compound that is needed for a protein's biological function. Often the co-factor binds to an enzyme assisting in its biochemical activity.

Codon Group of three mRNA bases that encodes a single amino acid.

Competent Cells capable of taking up plasmid DNA.

Complementary base pair Nitrogen-containing bases that are found opposite each other in a double-stranded DNA molecule. Complementarity is the result of size (a large base must be opposite a small base) and number of hydrogen bonds between the adjacent bases in the pair (A and T form two hydrogen bonds, G and C form three). Adenine is complementary to thymine and guanine is complementary to cytosine.

Covalent chemical bond One of the forces that holds atoms together in a molecule. It is considered to be a strong bond and forms from the sharing of electrons between two atoms.

Cytosine (C) One of the nitrogen-containing bases found in DNA and RNA. It is complementary to guanine.

Degrade To lower in quality; to convert into a more simple compound; to decompose.

Denaturation The melting of DNA at high temperatures into single nucleotide strands; changing the three-dimensional shape of a protein molecule.

Deoxyribose A type of five-carbon sugar found in DNA.

Dimorphic Having two forms.

Diploid A double set of chromosomes present in the somatic cells of plants and animals. Humans have a diploid number of 46.

Directed evolution An experimental procedure to evolve a desired trait. It involves using PCR to produce a library of variants, screening the library for organisms expressing the desired trait, then replicating lots of the desired variant.

DNA (deoxyribonucleic acid) The molecule that encodes genetic information. DNA is a double stranded molecule held together by hydrogen bonds between base pairs of nucleotides. The material of heredity.

DNA ligase An enzyme that joins DNA strands by forming covalent chemical bonds in the sugar-phosphate backbone.

DNA polymerase An enzyme used to replicate DNA molecules. PCR uses a DNA polymerase from the bacterium *Thermus aquaticus* and is called *Taq* polymerase.

DNA replication The use of existing DNA as a template for the synthesis of new DNA strands.

Double helix Structure in which two strands of DNA are twisted spirally around each other.

Electrophoresis Movement of charged molecules toward an electrode of the opposite charge; used to separate nucleic acids and proteins. When used to separate DNA fragments, agarose gel electrophoresis will separate the fragments by size, with smaller fragments moving faster than smaller fragments.

Enzyme A protein that acts to speed-up chemical reactions.

Escherichia coli Common bacterium used in numerous molecular biology protocols. The strain of *E. coli* used in the Amgen protocols is relatively harmless and does not grow well outside the laboratory environment.

Eukaryote An organism that shelters its genes inside a nucleus and has several linear chromosomes.

Exon Segment of a gene that encodes regions of a protein.

Express To make a protein.

Expression vector A plasmid genetically engineered specifically to express genes (e.g., pARA).

Extension The third stage in the PCR thermal cycling during which DNA polymerase catalyse the addition of nucleotides to the primer.

Fluorescence The production of light by a molecule (e.g., red fluorescent protein will release red light when exposed to ultraviolet light). The protein used in the Amgen Labs can been seen with ambient light.

Forensics Specializing in the application of scientific knowledge to legal matters. DNA is frequently used as evidence in legal matters.

Gamete Mature male or female reproductive cell.

Gene The fundamental physical and functional unit of heredity; an ordered sequence of nucleotides located in a locus that encodes a specific functional product.

Gene expression The process by which a gene's coded information is converted into the structures present and operating in the cell. Expressed genes include those that are transcribed into mRNA and then translated into protein.

Genetic code The sequence of nucleotides, coded in triplets (codons) along the mRNA, that determines the sequence of amino acids in protein synthesis.

Genetic engineering Altering the genetic material of cells or organisms to enable them to make new substances or perform new functions.

Genetic polymorphism Difference in DNA sequence among individuals, groups or populations (e.g., alleles for the *tPA* locus amplified by PCR, or height in pea plants-tall and short).

Genetics The study of inheritance.

Genome All the genetic material in the chromosomes of a particular organism; its size is generally expressed as its total number of base pairs.

Genotype The combination of alleles carried by an individual for a specific trait.

Green fluorescent protein A protein produced by the marine jellyfish, *Aequoria victoria*; protein encoded by the *gfp* gene.

Guanine (G) One of the nitrogen-containing bases found in DNA and RNA. It is complementary to cytosine.

Haploid A single set of chromosomes present in the gametes of plants and animals. Humans have a haploid number of 23.

Heterozygous The two different copies, or alleles, of the same gene.

Homologous chromosomes A chromosome containing the same linear gene sequence as another, each derived from one parent. While the gene sequence is (generally) the same, the precise alleles may differ between chromosomes.

Homozygous Having two identical copies, or alleles, of the same gene.

Hydrogen bond A weak force resulting from the attraction of a positive hydrogen atom to negatively charged regions of other atoms. This is the force that holds the two strands of nucleotides together in the DNA molecule. The GC base pairs form three H-bonds while AT pairs for two.

Hydrophilic Water loving; dissolves in water; polar. Some examples are sugar and salt.

Hydrophobic Water fearing; does not dissolve in water; non-polar. Some examples are oil, wax and mutant fluorescent protein.

In situ Whilst staying in the same place.

In vitro 'In glass'; experimental work performed in equipment, not living organisms.

In vivo 'In life'; experimental work performed in living organisms, not equipment.

Intron Segment of a gene that does not code for a protein. Introns are transcribed into mRNA but are removed before being translated into a protein.

Kanamycin An antibiotic that kills non-resistant cells by inhibiting proteins synthesis.

kanR Symbol for the kanamycin resistant gene found in the plasmid pKAN; encodes an enzyme called phosphotransferase that inactivates kanamycin.

Kilobase (kb) Unit of length for DNA fragments equal to 1,000 nucleotide pairs.

Kilobase ladder A set of standard DNA fragments with lengths differing by one kilobase; used as a size standard in electrophoresis.

Ligase The enzyme required to covalently join two fragments of DNA.

Ligation The reaction that chemically joins two fragments of DNA resulting in a recombinant DNA molecule.

Locus A place or location on a chromosome, it may be a gene or just any site with variations which can be measured. (e.g., Alu+, Alu- in the *tPA* intron).

Lyse To break open.

Marker See: *kilobase ladder.*

Messenger RNA (mRNA) The nucleic acid molecule that carries genetic information from the genes to the rest of the cell for protein synthesis.

Nitrogen-containing base A molecular component of DNA and RNA nucleotides. In DNA there are four nitrogen-containing bases: A (adenine), G (guanine), T (thymine), C (cytosine).

Nuclease A family of enzymes that will degrade nucleic acids.

Nucleic acid A large molecule composed of nucleotide subunits; a polymer of nucleotides.

Nucleotide A subunit or monomer, of DNA and RNA. Each nucleotide consists of a nitrogen containing base, a five-carbon sugar and a phosphate group.

Operon A cluster of genes transcribed together to give a single molecule of mRNA. The arabinose operon is used in the pARA expression vector to transcribe the *rfp* gene.

Phage A virus for which the natural host is a bacterial cell.

Phenotype Characteristic due to the expression of our genes; usually refers to visible properties but may refer to characteristics revealed by laboratory tests.

Plasma membrane Another name for the cell membrane.

Plasmid Circular molecule of DNA which replicates independently of the host's genomic DNA (e.g., pKAN, pARA).

Polymer A large molecule made of similar or identical subunits linked together (e.g., DNA, RNA, proteins).

Polymerase Chain Reaction (PCR) A chemical procedure used to amplify a DNA sequence by repeated cycles of replication and denaturation.

Polymorphism Difference in DNA sequence at a particular genetic locus. The differences may or may not result in a different phenotype.

See also: *locus, phenotype.*

Primer Short, pre-existing polynucleotide chain to which new nucleotides can be added by DNA polymerase. Two different primers complementary to the DNA sequence bracketing the region to be amplified are used in PCR.

Prokaryote A cell or organism with a single chromosome and no nuclear membrane; bacteria.

Promoter Region of DNA in front of a gene that binds RNA polymerase and so promotes gene expression; in the *ara* operon, the region of pARA that binds the AraC protein-arabinose complex and RNA polymerase prior to *rfp* expression.

Protein A large polymer of amino acids. Examples are enzymes, mutant fluorescent protein and some hormones.

Reagent A substance or compound that is added to a reaction to assist in that reaction.

Recognition sequence (recognition site) Specific nucleotide base sequence recognized by a restriction enzyme. The enzyme will cut the DNA within this nucleotide sequence.

Recombinant DNA molecule A combination of DNA molecules of different origin that are joined using recombinant DNA techniques.

Restriction enzyme An enzyme that binds and cuts DNA at a specific base sequence (e.g., *Bam*H I and *Hin*d III).

Restriction fragment A piece of DNA that results from the cutting of a DNA molecule with a restriction enzyme. Fragments are often separated on a gel using electrophoresis.

Restriction map Diagram of pieces of DNA, such as a plasmid, showing the restriction sites for restriction enzymes.

Ribose Five-carbon sugar found in RNA nucleotides.

Ribosome Site within the cell that assembles amino acids into protein molecules.

RNA polymerase An enzyme that catalyses the synthesis of RNA, using DNA as a template. Involved in transcription of genes.

SB buffer Sodium borate solution used as an electrophoresis buffer. It is a solution that conducts an electric current and maintains a relatively constant pH.

Somatic cells Cells making up the body which are not sex cells or gametes. These cells are usually diploid having two sets of chromosomes.

Sticky ends Ends of a DNA molecule cut with certain restriction enzymes. These ends have unpaired bases.

Supercoiling Higher level of twisting of DNA often found in plasmids.

Supernatant The liquid on top of a pellet deposited by centrifugation.

***Taq* polymerase** A heat stable enzyme commonly used in PCR; polymerase found in the bacterium *Thermus aquaticus.*

TAE (Tris-Acetic acid-EDTA) A solution used for gel electrophoresis and in the preparation of agarose gels. The solution helps conduct an electric current while maintaining a constant pH. An alternative to SB buffer.

Thermophilic 'Heat-loving'.

Thymine (T) One of the bases found in DNA; the base that is complimentary to adenine.

Transcription A chemical process that converts a DNA nucleotide sequence into an mRNA nucleotide sequence; process that uses RNA polymerase to convert a DNA template into a mRNA strand.

Transformation A process that places foreign DNA, like a plasmid, into a cell.

Translation A chemical process that converts an mRNA nucleotide sequence into an amino acid sequence; site of this reaction is the ribosome.

Vector A DNA molecule used as a vehicle to artificially carry foreign genetic material into another cell, where it can be replicated and expressed.

Wild type The original or naturally occurring version of a gene or protein.