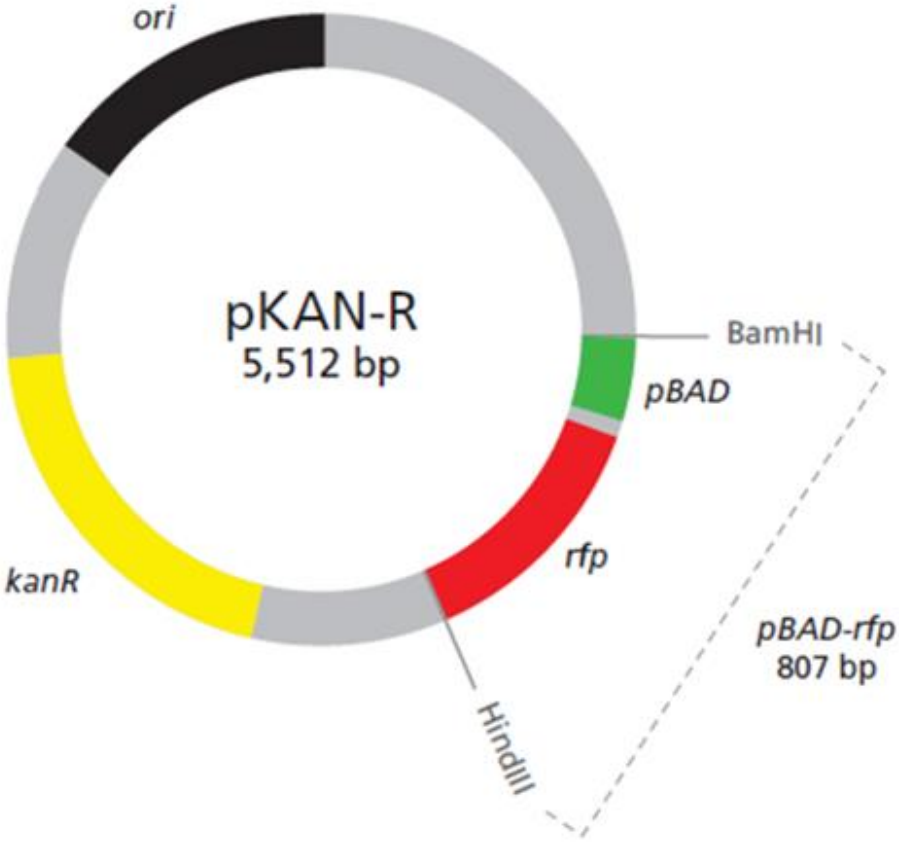
AMGEN® Biotech Experience

Scientific Discovery for the Classroom

Short Teacher Guide



30 YEARS

TSN

TEACHER SCIENTIST NETWORK

History of the Amgen Biotech Experience

From the Education Development Center

Pioneers lay the roads for those who follow to walk on.

- unknown

Though the Amgen Biotech Experience now reaches more than 50,000 students and hundreds of teachers each year, the program had humble beginnings. It all started with a group of scientists and teachers who had a passion for sharing their knowledge with students.

In 1989, molecular biologist Bruce Wallace, Scientific Executive Director Steve Elliot (now retired), and others at Amgen believed that the company could be instrumental in providing professional development to area high school teachers, toward a goal of improving science education for students. They put out a call for biology teachers interested in a summer intern program.

Intrigued, Hugh Nelson, a high school teacher in Thousand Oaks, California, responded to their invitation. Nelson set about learning the procedures Amgen uses to develop biologics and worked with an Amgen scientist to fine-tune a series of labs for high school students. Amgen agreed to provide equipment and chemicals to teach the lab procedures in local high schools.

Two years later, Amgen launched the official school program, with Nelson training teachers in Ventura County, California, schools. Within two years of the launch, 1,300 students from 12 local schools participated in the program.

“The labs put students in touch with the reality of modern science,” says Nelson. “It takes money to do experiments, and Amgen provided the funding for this important, transformative program. I’m no less in awe of the program [now] than I was in 1989.”

In 1999, Amgen enlisted Marty Ikkanda, professor of biological sciences at Pierce College in Woodland Hills, California, to revise the program’s curriculum to resemble his college classes, including giving students a chance to work with protein expression and purification. The revised program was rolled out to 20 schools the next school year, and the number reached 30 by the school year’s end.

“Teachers tell us they don’t have attendance problems when they’re doing the Amgen biotechnology labs,” says Ikkanda, who retired from the program in 2013. “It’s a fantastic way to interest students in science.”

To honour one of the program’s founders who has since passed away, the program was named the Amgen-Bruce Wallace Biotechnology Lab Program in 2003. Wallace wanted all students to experience the joy of discovery and the excitement of having science at their fingertips.

In 2005, with interest building from biology teachers in other communities, the Amgen Foundation, the main philanthropic arm of Amgen, partnered with Professor Ikkanda to expand training outside of Ventura and Los Angeles Counties in Southern California. Over several years, schools in San Diego, Seattle, San Francisco, Rhode Island, Colorado, Puerto Rico, Massachusetts, and the United Kingdom were added.

In 2013, the program was renamed the Amgen Biotech Experience (ABE) to reflect its evolution. Additionally, the Amgen Foundation has joined forces with Education Development Center, Inc., a global non-profit organization with deep experience and expertise in science education, to establish a Program Office to support and strengthen the program.

A collaboration that began 25 years ago inspired the ongoing commitment of scientists and teachers to share their knowledge of and passion for science. The Amgen Foundation is proud to continue its support of a program that is stronger than ever—and poised to bring real-world biotechnology to a new generation of teachers and students. “That pioneering spirit distinguishes Amgen,” says Jean Lim Terra, president of the Amgen Foundation. “We’re forever grateful to those early collaborators for the roots of this powerful program.”

Visit the ABE website at www.amgenbiotechexperience.com for more information.

Acknowledgements

The Amgen Foundation and Education Development Center, Inc., are particularly grateful to the following Amgen Biotech Experience (ABE) Program staff, teacher reviewers, and pilot teachers who provided invaluable suggestions and feedback:

Tracy Bailey-Gates, Ann Cortina, Anu Deshpande, Carol Fujita, Anne Gundry, Martin J. Ikkanda, Wendie Johnston, Grace Montgomery, Todd Ryan, Christina Schramm, Lisa Sequeira, Karin Steinhauer, Karen Stephens, Sherry Tsai, David Upegui, Amy Welch, Wendy Wooten, and Jo Wu.

We appreciate the assistance provided by Tara Bennett and Alia Qatarneh of the ABE site in Massachusetts and by David Vito of the ABE site in Rhode Island.

We are also grateful to ScienceBridge at UCSD, which provided the *rfp* gene used in this program. Finally, we are grateful to Amgen staff past and present, whose commitment and valuable input has kept the program relevant and aligned with current industry practices.

Contents

Chapter, section	Page

Introduction

From the Education Development Center

Integrating ABE into your classroom is a unique and important opportunity for you and your students. To support you in developing students' scientific content knowledge and practices, as well as providing a real-world application, the material in this section covers the *what, why, and how* of the curriculum: the science content addressed, the teaching and learning principles and practices employed, and practical considerations for implementing the course. Reading this section *before* you begin will enhance your teaching and the benefits that students derive from it.

The ABE labs parallel some of the important steps that the pharmaceutical biotechnology industry uses to develop drugs (therapeutic proteins) to treat a variety of diseases. Biotechnology provides the tools and techniques for modern pharmaceutical research and drug development, and it is critical that future citizens are knowledgeable about this field. Common biotechnology techniques are used to develop a wide range of products, from life-saving pharmaceuticals to an enzyme that increases the vitamin content of rice to a human vaccine expressed in a plant. The labs focus on these techniques so that your students will better understand the role of biotechnology and the potential impact of this industry on our future. In addition, by engaging in this program, students may be more motivated to understand the underlying science concepts and perhaps even pursue careers in science.

Laboratory experiences such as those provided by this program are extremely important in science education, as they offer opportunities to make science "real" and relevant to students and allow them to use the professional tools and techniques for investigating scientific questions. Effective laboratory experiences can do the following:

- Enhance students' understanding of fundamental science content and concepts
- Help students develop scientific skills and reasoning practices
- Increase students' understanding of the complexity and ambiguity of scientific research
- Allow students to develop important academic, interpersonal, and intrapersonal skills that are necessary for their future success, including problem-solving, critical thinking, and teamwork

The full sequence of these labs has students producing a recombinant DNA molecule that is a chimera of prokaryotic and eukaryotic genetic elements. The prokaryotic component consists of an engineered bacterial plasmid and its control elements—origin of replication, ampicillin-resistant gene, and the arabinose operon—while the eukaryotic element is a gene from the sea anemone, *Discosoma* sp. The gene encoding this protein is referred to as the red fluorescent protein (*rfp*) gene and encodes the red fluorescent protein, RFP, a molecule that is used extensively in research.

Once students have produced the recombinant DNA plasmid, they can beyond Year 1, use it to transform *Escherichia coli* (*E. coli*). The transformed cells are spread on the surface of an agar plate containing ampicillin and *arabinose*, a five-carbon monosaccharide required for *rfp* gene expression. Bacterial colonies expressing the *rfp* gene will appear red (or bright pink) in colour. Cells from one of these red colonies are used in a PCR to demonstrate that the phenotype (red fluorescence) is linked to a particular genotype (presence of a PCR product of the correct size to be the *rfp* gene).

By completing the full ABE laboratory sequence, your students will have a clearer understanding of how to use recombinant DNA techniques to introduce new genes into an organism and have that organism produce new proteins. Using a gene from a sea anemone to express the red fluorescent protein models how the same process can be used with a human gene to produce proteins for a human therapeutic, such as insulin or human growth hormone.

Timings for the sequences of laboratories

We are aware that a major challenge for schools using the kits is working out how to fit the additional practical activities into a timetable already crowded by the curriculum content. Schools participating in the ABE programme have found time:

- in lessons
- in lunchtime clubs
- in after schools clubs
- in off-timetable days
- by swapping lessons with other (Science) subjects to give a more intense period of study

To support you in tailoring the ABE programme to fit your requirements, we have provided a rough guide below for how long each practical activity takes in the classroom. We have not estimated how long you wish to spend on explanations and follow up work. These are guide timings, as obviously each classroom is different, but we hope that they will help with your planning. Black font denotes hands-on activities, blue font is for periods whilst experiments run when other teaching / activities can occur.

Sequence A	Practical activities	Timings (minutes)
Chapter 1	Using a micropipette (1.1) Gel electrophoresis (1.2)	≈ 15 practice ≈ 15 load ≈ 10 gel
Chapter 2	Restriction digestion of plasmids (2)	≈ 20 set up 60 incubation
Chapter 3	Building the pARA-R plasmid (3)	30 denaturation ≈ 10 set up
Chapter 5	Visualisation of products from restriction digestion, ligation and the PCR (5)	≈ 30 set up ≈ 50 gel ≈ 5 transfer 30 stain ≈ 20 photographing

Overview of content

The central tenet of molecular biology, and one that presents considerable challenges for student comprehension is that proteins encoded by DNA are responsible for traits.

Goals for understanding

- Students understand that all the information required by organisms to maintain life is encoded in the arrangement of nucleotides in their DNA.
- Students understand that the coding and decoding of DNA is the same among all organisms, which makes possible the expression of a human gene by bacteria.
- Students understand how the processes of transcription and translation facilitate the transfer of information from DNA to proteins.
- Students recognize that the traits of organisms are determined by the expression of specific genes in their DNA.
- Students understand how gene expression is regulated.
- Students understand how protein functions are responsible for the traits of an organism.
- Students recognize that a change in the DNA sequence can alter the function of a protein and can change the traits of an organism.
- Students know how bacteria can be genetically modified to make new products.
- Students understand the reciprocal relationship between basic science research and technology development: They understand that the discoveries of plasmids, restriction enzymes, and ligases during basic research have generated the tools and techniques of biotechnology; in turn, the tools and techniques of biotechnology are enabling scientists to reach deeper understandings about genes and the function of their products in a cell.
- Students understand the purpose of using controls in scientific investigations.

Learning outcomes

After completing the program, students should be able to do the following:

- Describe the relationship between DNA, genes, proteins, and traits
- Explain how information is transferred from DNA to proteins by transcription and translation
- Explain how the expression of genes in a cell determines the traits of an organism
- Describe how the characteristics of organisms (traits) are the result of protein activity
- Describe how a loss or gain of protein function can alter the traits of an organism
- Discuss how organisms can be engineered to have new characteristics
- Describe the role of biological tools, such as plasmids, restriction enzymes, and DNA ligase, in the genetic engineering process
- Provide examples of how genetic engineering can be used to solve medical problems
- Model the process of producing a recombinant plasmid

Connections to theoretical understanding

A brief overview of the molecular biology theory underpinning the practical activities across the full series of ABE laboratories is given in the table below. Only those labs highlighted feature in this (short) guide

Chapters	Curriculum Connections	Notes
1	DNA structure	• Basic structure and size of DNA • Separation of charged molecules by agarose gel electrophoresis
2, 2a	Genes and DNA Function of enzymes Enzyme reactions	• Relationship between genes and DNA • How enzymes work • Restriction enzymes • Role of restriction enzymes in genetic engineering
3	Enzyme reactions	• Role of ligase in genetic engineering
4, 4a	Genes and DNA Function of enzymes Enzyme reactions	• Relationship between genes and DNA • Specificity of complementary base pairing • How enzymes work • Role of DNA polymerase in the polymerase chain reaction
5, 5a	Universality of DNA	• Visualising DNA
6	Universality of gene expression Transcription Translation Protein activity Bacteria cell structure Variables and controls	• Biology big idea: DNA → Protein → Trait • Process of gene expression • All organisms express genes the same way • Colonies vs. individual cells
7, 7a	Genes and DNA Function of enzymes Enzyme reactions	• Relationship between genes and DNA • Specificity of complementary base pairing • How enzymes work • Role of DNA polymerase in the polymerase chain reaction
9	Inheritance Genes and DNA Function of enzymes Enzyme reactions	• Relationship between genes and DNA • How enzymes work • Restriction enzymes • Role of restriction enzymes in DNA profiling

Overview of preparation

Most of the solutions in your kit are at working concentrations so that you will not have to dilute them before aliquotting for students. However, some preparation is common to multiple laboratories and this section contains guidance about these procedures. Guidelines for aliquotting are included for each lab in the Preparation section

TAE buffer dilution

Tris-Acetate-EDTA (TAE) buffer is sent to you at a 50x stock concentration and needs to be made up at a 1x working concentration. To calculate the dilution from a stock concentration to a working concentration you can use the following method.

- Determine how much of the working solution you will need per group (400 ml).
- Multiply the volume per group times the number of groups (1).
- Then use the formula $C_1V_1 = C_2V_2$ (where C_1 is the concentration supplied, C_2 is the concentration required, V_1 is the volume to use, and V_2 is the volume to prepare) to determine the volume of stock solution (V_1) you will need to use to prepare the final volume of working solution (V_2).

Example: You need to prepare 400 ml of 1x TAE buffer, the stock is a 50x concentration.

- Apply the formula $C_1 \quad V_1 = C_2 \quad V_2$

$$(50x)\ (V_1) = (1x)\ (400\ mL)$$

$$(V_1) = \frac{(1x)\ (400\ mL)}{(50x)}$$

$$(V_1) = 8\ mL\ of\ 50x\ TAE$$

- To make 400 ml of 1x TAE electrophoresis buffer add 8 ml of 50x TAE to 392 ml of dH_2O.
- **Note:** Once used, 1x TAE can remain in the electrophoresis box or be discarded. If you have back-to-back classes, keep the buffer in the electrophoresis boxes for classes doing the same lab. Do not re-use more than twice.

GelRed DNA stain dilution

GelRed is provided as a 10,000 x concentrate, and needs to be used at a 1.5 x concentration (ie; a 6,667 fold dilution).

- To make up 100 ml, which will cover 2 gels in one of the staining trays, add 15 µl of GelRed 10,000 x concentrate to 100 ml of distilled water. Don't forget to wear eye protection and chemical-resistant gloves.

Do not use more than 50 ml per gel as GelRed is expensive and you will run out!

Do label the tray using marker pen on tape, so you know whose gel is which end of the tray.

GelRed stain can be used for more than one gel. We have noticed no reduction in DNA staining for at least 3 gels. You do not have to destain these gels.

If your students are not in the laboratory during photodocumentation, do save at least one of the better gels (in plastic wrap or ziplock bag in the fridge) so that they can see the fluorescence of the GelRed-DNA complex on the UV transilluminator. This generally gets the students excited. The DNA may diffuse a bit in the gel and the bands may be a bit fuzzy, but they will still be visible.

Autoclave gels before disposal as they contain biological material.

Agarose gel preparation

Agarose gels are used to separate the DNA products produced in many of the ABE laboratories. Typically the agarose is at a concentration of 0.8%. The greater the percentage the smaller the holes in the gel matrix, and therefore the smaller the DNA molecules that can be separated. When making the gels, 1% is 1 gram in 100 ml (and therefore 0.8% would be 0.8g in 100 ml).

1 Preparation of electrophoresis gel trays

- Tape the ends of the gel trays
- Insert the comb into the slots

2 Preparation of the agarose solution

- Wearing eye protection and chemical-resistant gloves, make up 400 ml of 1x TAE buffer in a measuring cylinder, labelled "TAE", by mixing 8 ml of 50x TAE buffer with 392 ml of distilled or deionized water (dH2O)
- For each 0.8% agarose gel to be poured, measure 0.28 g agarose and add 35 ml 1x TAE buffer into a glass flask with 3 x the capacity of the volume used (as it will bubble lots when heated).
- Do not swirl as the agarose will be deposited on the walls and not dissolve in the liquid.
- Cover the flask opening with pierced plastic wrap to prevent excessive loss of water vapour.
- Heat the flask in a microwave on high for 1 minute (keep checking it, DO NOT WALK AWAY). Using a heatproof glove, remove the flask and gently swirl.
- Continue microwaving for 5 to 15 second periods until all of the agarose has dissolved. When held to the light and swirled there should be no agarose crystals suspended in the liquid.

Don't forget to cool the agarose after is has melted. If the agarose is poured when it is too hot then it will warp the gel tray. If the solution begins to re-solidify, reheat it in the microwave.

3 Pouring the agarose gels

- When the agarose solution has cooled to the point where you can safely touch the flask containing it, pour about 35 ml of the solution into each gel tray. The solution should cover 2-3 mm of each comb.
- Leave to solidify for at least 15 minutes.
- Remove the comb by pulling straight up, so that the wells are not damaged and remove the masking tape from the ends of the gel.
- Gels can be stored in their electrophoresis trays, in electrophoresis tanks submerged in 1x TAE buffer if they are to be used soon, or the gels can be slid from their gel trays into zip-lock bags with about 10 ml of 1x TAE buffer and stored in the fridge until they are used.

Tip: After the DNA has been drawn into the gel (by at least 5 minutes electrophoresis) it can be removed to a ziplock bag and stored in the fridge until electrophoresis can be completed

Chapter 1: Some tools of the trade

Overview

Chapter 1 introduces students to the use of micropipettes for accurate volumetric measurements. It then proceeds to consider the use of gel electrophoresis for separation of biological materials.

Preparation

This guidance for preparation is divided into three parts:

1. Advanced preparation: activities that can be done several days in advance
2. Prior preparation: preparation required immediately prior to the session
3. Post-lab support: experimental support needed after the practical work

The instructions in Table 1.1 give the equipment and reagents required for a pair / group of students. You will need to increase the amounts to suit the number of pairs that you are teaching.

Note: *C denotes an item that is shared amongst the class and *2 denotes an item that is shared between 2 pairs or groups.

Laboratory	Advance preparation	Prior preparation	Post-lab support
1.1: Using a micropipette	Aliquot solution: • Microfuge tube of red dye (RD) solution Prepare laminated practice sheet	Prepare benches with: • P-20 micropipette • Pipette tips • Microfuge tube rack • Microfuge tube of red dye solution • Laminated sheets • Waste container • Microfuge (shared)	
1.2: Gel electro-phoresis	Aliquot solutions: • Dye solution 1 (S1) • Dye solution 2 (S2) • Dye solution 3 (S3) Dilute TAE buffer to 1 x TAE buffer Pour agarose gel Pour agar-agar practise plates (optional)	Prepare benches with: • P-20 micropipette • Pipette tips • Microfuge tube rack • Microfuge tubes of 3 dye solutions (S1, S2 and S3) • Waste container • Microfuge *C • Electrophoresis tank *2 • 1 x TAE buffer • Gel tray containing an 0.8% agarose gel • Powerpack	

Table 1.1 Preparation for laboratory 1.

The processes for diluting TAE buffer and preparing agarose gels are covered in the overview of preparation section on page I.5/6. If you pour an 8-well, 0.8% agarose gel, then two groups will each be able to load one set of dye samples. If you pour an 15-well, 0.8% agarose gel, then two groups will each be able to load two sets of dye samples.

When aliquotting reagents, we aim to provide sufficient for the number of pairs that you are doing the practical activities with and a bit extra for pipetting errors. Table 1.2 gives a brief summary of the quantity of reagents required by each pair of students for this laboratory.

Tube contents	Tube label	Aliquot (µl)	Actually used (µl)
Red dye solution	RD	150	104
Dye solution 1	S1	24	10 / 20
Dye solution 2	S2	24	10 / 20
Dye solution 3	S3	24	10 / 20

Table 1.2 Aliquotting reagents for laboratory 1.

All of these reagents should be kept at room temperature.

Health and safety

It is assumed that all students follow general laboratory safety precautions including: tying back long hair; not eating or drinking in the lab; keeping lab benches clear of clutter; clearing up spills immediately; handling materials and equipment with care, and; washing hands with soap after completing lab work. In addition, specific Health and Safety information for this lab is outlined in Table 1.3.

Substance / procedure	Hazard	Precautions
Dye solution 1	Low hazard, but stains skin.	Use good laboratory practice to avoid contact with skin.
Dye solution 2	Low hazard, but stains skin.	Use good laboratory practice to avoid contact with skin.
Dye solution 3	Low hazard, but stains skin.	Use good laboratory practice to avoid contact with skin.
Tris-Acetate-EDTA (TAE) buffer	The mixture is an irritant, particularly to skin and eyes. Diluting to 1 x TAE buffer reduces the hazard. At 50x concentration, the mixture is very toxic to aquatic life with long lasting effects and should not be released into water courses until diluted.	When handling, wear eye protection (safety glasses are sufficient) and chemical- resistant gloves. Dilute before student use. A 1 x solution is low hazard, but as a precaution students should wear eye protection and gloves when handling the solution. Skin or eyes should be rinsed with plenty of water.
Agarose gel	Agarose is safe, but will be melted in 1 x TAE buffer, so an agarose gel is low hazard.	An agarose gel made with 1 x TAE is low hazard, but as a precaution students should wear eye protection and gloves when handling.

Table 1.3 Health and Safety information for laboratory 1 (continued on next page).

Substance / procedure	Hazard	Precautions
Gel electrophoresis	Access to liquids carrying a 100 V current. Access to a metal contact which is live at 100 V.	The electrophoresis chamber cuts off the electric supply whenever the lid is removed. All connections are plastic coated.

Table 1.3 Health and Safety information for laboratory 1 (continued from previous page).

Learning objectives

By the end of this laboratory students should be able to:

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

These learning objectives help to address the basic structure and size of DNA and how charged molecules can be separated by agarose gel electrophoresis.

Prior knowledge and skills

It is helpful if students already know:

- The relationship between DNA, genes, proteins, and traits—specifically, that genes contain the code for making a protein and that proteins are molecules that are used in making and running the cell, so they are responsible for traits
- That charged objects, including molecules, move through an electric field

Expected question responses

The question sections include: (i) questions that could be used to prompt students to think about what they know before they start the practical activities; (ii) questions appropriate for after the reading and practical work are complete, and; (iii) questions that are intended to help with results analysis. They can be used to illicit prior knowledge and misunderstandings, as a basis for peer-discussion and/or as a type of formative assessment.

Questions posed in the student guide and some possible answers are given below:

Laboratory 1.1: Using a micropipette

1. When loading or dispensing a solution, why is it important to actually see the solution enter or leave the pipette tip?

   The amounts are so small that it is easy to become confused and do the steps in the wrong order or miss a step, so it is important to check that the solution enters and leaves the tip as you need it to. If a reagent is missing, an entire experiment can be invalid.

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?

   For two reasons. Firstly, the tips and all surfaces need to remain clean so that the plasmids and bacterial cells do not get contaminated with other substances. Secondly, you also do not want contaminate yourself or surfaces with bacterial cells.

3. Why do you think it is necessary to use very small and exact volumes of reagents in biotechnology?
   In this field you would use very small amounts of the reagents because they are so difficult and expensive to purify. Using the correct volumes is required to ensure that the reagents are at the correct concentrations and that the procedures are successful.

Laboratory 1.2: Gel electrophoresis

1. When might it be important to use gel electrophoresis to separate and identify plasmids and short linear pieces of DNA?
   This would be important if you are making a recombinant plasmid and have to verify that you have been successful.
2. What electrical charge do the dyes have? Explain how you know.
   They must be negatively charged, because when they are put into the wells of an agarose gel in an electric field they are repelled by the negative electrode and attracted to the positive electrode. You can see them migrating towards the positive electrode.
3. Study your gel electrophoresis results. Which solution sample contained a single dye: S1, S2, or S3? How do you know?
   S3 contained a single dye because it only had one band.
4. From your gel electrophoresis results, put the dyes into size order from largest to smallest. Explain your reasoning.
   From heaviest to lightest: Xylene cyanole, bromophenol blue, orange G. This order is based on the appearance of the color bands in the agarose gel after running the electrophoresis box—the light blue xylene cyanol is closest to the wells, the purple bromophenol blue is next closest, and the yellow/orange coloured orange G dye moved the farthest from the wells.
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?
   The order students are likely to give will probably not be correct, because of the ratios of charge to mass of the dyes used in this lab. Although heavier molecules will move more slowly than smaller molecules if both have the same electric charge, molecules with more negative electric charge will move faster than molecules with less negative electric charge if both have the same weight. Despite, xylene cyanole (538.62 au) having a smaller size than bromophenol blue (669.98 au) it migrates more slowly than bromophenol blue because xylene cyanol has a smaller charge to mass ratio.
6. Can you suggest any other factors that may have played a role in the separation of these dyes?
   In addition to the ratio of charge to mass, the other factor that affects speed through the gel is shape. Longer or branched molecules will be tangled up in the gel and will move more slowly than shorter or more compact molecules if they have the same weight and electric charge.

Additional resources

There are additional resources to expand upon the concepts and practice of genetic engineering, therapeutic proteins, diabetes, why sea anemones fluoresce and how gel electrophoresis can be used in DNA fingerprinting. These can be used to expand the teaching experience in class, as homework, extension or group work. They are in the 'Additional resources' section at the back of the teacher and technician guide.

Chapter 2: Beginning to clone a gene

Overview

Chapter 2 allows students to use restriction digestion with two enzymes (*Bam*HI and *Hin*dIII) to create linear DNA fragments from 2 known plasmids. The expression plasmid (pARA) and plasmid containing the red fluorescent protein gene (pKAN-R) will each be cut twice, producing 2 linear fragments. This is the first step towards creating a recombinant plasmid for production of protein therapeutics, or in our practical work, red fluorescent protein.

Preparation

This guidance for preparation is divided into three parts:

1. Advanced preparation: activities that can be done several days in advance
2. Prior preparation: preparation required immediately prior to the session
3. Post-lab support: experimental support needed after the practical work

The instructions in table 2.1 give the equipment and reagents required for a pair / group of students. You will need to increase the amounts to suit the number of pairs that you are teaching.

Note: *C denotes an item that is shared amongst the class.

Laboratory	Advance preparation	Prior preparation	Post-lab support
2: Restriction digestion of plasmids	Aliquot solutions: • 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)	Prepare benches with: • P-20 micropipette • Pipette tips • Microfuge tube rack • Microfuge tubes of solutions • 1.5 mL microfuge tubes • Permanent marker • Waste container • Floating tube rack • Microfuge *C • 37°C water bath *C	Remove the restriction digestion reactions to a tube rack labelled with the class details and store in the freezer at –20°C.

Table 2.1 Preparation for laboratory 2.

When aliquotting reagents, we aim to provide sufficient for the number of pairs that you are doing the practical activities with and a bit extra for pipetting errors. Table 2.2 gives a brief summary of the quantity of reagents required by each pair of students for this laboratory.

Tube contents	Tube label	Aliquot (µl)	Actually used (µl)
2.5 x restriction buffer	RB	24	20
pKAN-R plasmid	K	12	10
pARA plasmid	A	12	10
Restriction enzymes *Bam*HI and *Hind*III	RE	5	4
Distilled water	dH_2O	20	4

Table 2.2 Aliquotting reagents for laboratory 2.

The aliquots of distilled water can be kept at room temperature and reused. Restriction buffer and plasmids should be stored in the freezer at –20°C, defrosted to room temperature for aliquotting and then returned to the freezer. The restriction enzymes should be bought out of the freezer in an isofreeze box for aliquotting and returned to the freezer as soon as possible to avoid loss of enzyme activity.

Health and safety

It is assumed that all students follow general laboratory safety precautions including: tying back long hair; not eating or drinking in the lab; keeping lab benches clear of clutter; clearing up spills immediately; handling materials and equipment with care, and; washing hands with soap after completing lab work. In addition, specific Health and Safety information for this lab is outlined in table 2.3.

Substance / procedure	Hazard	Precautions
Restriction buffer	Low hazard	
Plasmids	Biological materials: very low risk of contaminating living cells.	Use in a controlled way, applying techniques that mirror the aseptic techniques used in microbiology. Autoclave materials that come into contact with plasmids before disposal.
Restriction enzymes	Biological materials: very low risk of contaminating living cells.	Use in a controlled way, applying techniques that mirror the aseptic techniques used in microbiology. Autoclave materials that come into contact with plasmids before disposal.

Table 2.3 Health and Safety information for laboratory 2.

Learning objectives

By the end of this laboratory students should 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

These learning objectives help students to explore the relationship between genes and DNA, to consider enzyme function and the role of restriction enzymes both in nature and in genetic engineering.

Prior knowledge and skills

It is helpful if students already know:

- DNA is a double-stranded molecule, and each strand of DNA is made up of covalently linked subunits called nucleotides.
- A nucleotide is made up of a sugar, a phosphate group, and a nitrogenous base. There are four different nitrogenous bases—cytosine, guanine, adenine, and thymine.
- Nucleotides are attached to each other by a sugar-phosphate backbone, while the nitrogenous bases jut out from the backbone.
- The two strands of DNA are connected by hydrogen bonds between adjacent nitrogenous bases, which are called base pairs; cytosine is always paired with guanine, and adenine is always paired with thymine.
- The process of decoding DNA has two steps: a transcription step that transfers the information encoded in DNA into messenger RNA, and a translation step in which the information carried by messenger RNA is used to make proteins.

Expected question responses

The question sections include: (i) questions that could be used to prompt students to think about what they know before they start the practical activities; (ii) questions appropriate for after the reading and practical work are complete, and; (iii) questions that are intended to help with results analysis. They can be used to illicit prior knowledge and misunderstandings, as a basis for peer-discussion and/or as a type of formative assessment.

Questions posed in the student guide and some possible answers are given below:

1. Using Figure 2.1, 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?

   Restriction digestion of pKAN-R with BamHI and HindIII restriction enzymes will produce 2 DNA fragments of 807 bp and 4705 bp (5512 bp – 807 bp = 4705 bp).

   Restriction digestion of pARA with BamHI and HindIII restriction enzymes will produce 2 DNA fragments of 377 bp and 4495 bp (4872 bp – 377 bp = 4495 bp).

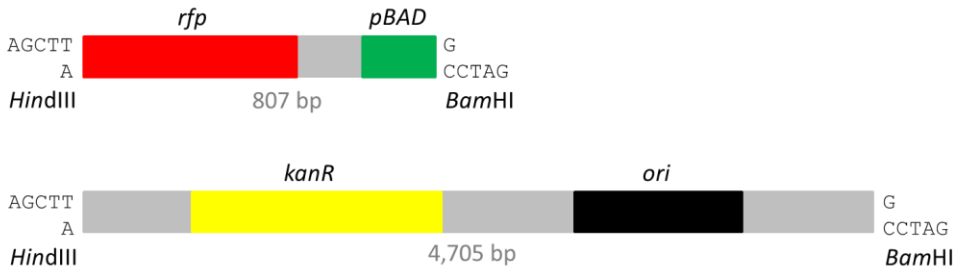
2. Draw the linear fragments, marking on the genes and important sequences. Using table 2.2 below, include the nucleotide sequence of the sticky ends.

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.

Figure 2.1 shows the 4 restriction fragments, the DNA sequences they carry and the ‘sticky ends’ left after restriction digestion with BamHI and HindIII.

pKAN-R digestion fragments:



pARA digestion fragments:

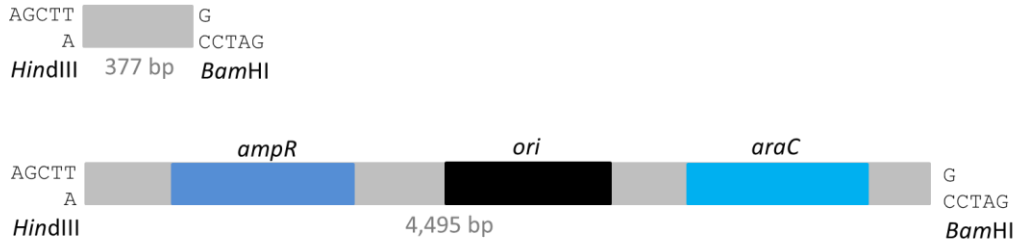


Figure 2.1 The linear restriction fragments.

3. In order to create a plasmid that can produce the red fluorescent protein in bacteria, what components are needed in the plasmid?

   Important features of the plasmid are (1) a sequence for the initiation of DNA replication, which allows the plasmid to replicate in the bacteria (ori); (2) a promoter sequence for initiating transcription of the inserted gene (pBAD); (3) the gene of interest (rfp), and; (4) a gene encoding a protein for antibiotic resistance, which allows for identification of bacteria that have taken in the plasmid (kanR / ampR).

4. Bacteria can be killed by an antibiotic unless they carry a plasmid that has the gene for restance 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?

   You want to know which bacterial cells contain the recombinant plasmid and can make the protein you wish to obtain. The selectable marker will allow you to kill off the bacteria that do not have the plasmid with the gene of interest by growing all bacteria on nutrient agar containing an antibiotic. Only those with the plasmid will be resistant and survive.

5. What role do restriction enzymes have in nature?

   They protect the bacteria from infection by bacteriophage (viruses that infect bacteria).

6. Why might the enzymes work best at 37°C? Why should the unused enzymes then be placed in the freezer?

   The enzymes originally came from bacteria that are at human body temperature and are designed to work at this temperature. Placing the enzymes in the freezer will stop the enzymes from losing activity.

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?

   The tubes without the enzymes are controls. We can compare a control with the tube that has the enzymes in order to compare the uncut plasmid with the cut plasmid. The control will indicate if for some reason the enzymes did not work as expected, as both the control tube and the tube with the enzymes will give the same result when examined by gel electrophoresis.

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?

   The bacteria with the antibiotic resistance gene will reproduce more because they have a selective advantage over other bacteria that do not carry that gene. This selective advantage is a huge concern in medicine because now there are strains of bacteria that cause disease but cannot be killed by antibiotics.

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.

   The DNA code and the processes of transcription and translation are the same in all living organisms. Enzymes such as RNA polymerase are specific for promoters; bacterial RNA polymerase will only recognize bacterial promoters. Once a human or sea anemone gene is placed under the control of a bacterial promoter on a plasmid and the recombinant plasmid is taken up by the bacteria, the bacterial RNA polymerase will transcribe the inserted gene and the protein synthesis machinery will translate the universal triplet codon into protein.

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!)

    The bacteria need to be divided into two batches; one batch should be treated with kanamycin, and the other batch should be treated with ampicillin. Only the bacteria with the kanamycin-resistant gene will survive in the presence of kanamycin, and only bacteria with the ampicillin-resistant gene will survive in the presence of ampicillin.

Additional resources

There are additional resources to expand upon the concepts and practice of genetic engineering, plasmids, bacterial promoters in plasmids, the pARA-R plasmid and restriction enzymes. There is also a paper-based plasmid cloning exercise 'clone that gene', to help students to understand what is going on at the molecular level during the practical laboratory work. These can be used to expand the teaching experience in class, as homework, extension or group work. They are in the Additional resources section at the back of the teacher and technician guide.

Resource	Page
Clone that gene	R.3
What is genetic engineering? Treating disease with gene cloning	R.8
Using plasmids and restriction enzymes in genetic engineering	R.3
Why use bacterial promoters in plasmids?	R.4
Restriction enzymes	R.5

Chapter 3: Building a recombinant plasmid

Overview

In this chapter students learn about another essential biological tool used in genetic engineering: DNA ligases, which they use to catalyse covalent bond formation between the linear fragments with sticky ends that they created by restriction digestion in laboratory 2. The goal is to make the recombinant pARA-R plasmid containing both the *rfp* gene and the *ampR* gene.

Preparation

This guidance for preparation is divided into three parts:

1. Advanced preparation: activities that can be done several days in advance
2. Prior preparation: preparation required immediately prior to the session
3. Post-lab support: experimental support needed after the practical work

The instructions in table 3.1 give the equipment and reagents required for a pair / group of students. You will need to increase the amounts to suit the number of pairs that you are teaching.

Note: *C denotes an item that is shared amongst the class.

Laboratory	Advance preparation	Prior preparation	Post-lab support
3: Building the pARA-R plasmid	Aliquot solutions: • Ligation buffer (LB) • DNA ligase (LIG)	Prepare benches with: • P-20 micropipette • Pipette tips • Microfuge tube rack • Microfuge tubes of solutions • Permanent marker • Waste container • Floating tube rack • Microfuge *C • 70°C water bath *C Retrieve and distribute the digested pKAN-R plasmid (K+) and digested pARA plasmid (A+) tubes from lab 2. Reuse the tubes of distilled water.	Return the digested pKAN-R plasmid (K+) and digested pARA plasmid (A+) tubes from lab 2 to the freezer at –20°C. Label a tube rack and leave with the ligation reactions in it, at room temperature overnight.

Table 3.1 Preparation for laboratory 3.

When aliquotting reagents, we aim to provide sufficient for the number of pairs that you are doing the practical activities with and a bit extra for pipetting errors. Table 3.2 gives a brief summary of the quantity of reagents required by each pair of students for this laboratory.

Tube contents	Tube label	Aliquot (µl)	Actually used (µl)
Ligation buffer	LB	5	4
DNA ligase	LIG	2.5	2.5
Distilled water (inc. lab 5)	dH_2O	50	4

Table 3.2 Aliquotting reagents for laboratory 3.

The aliquots of distilled water can be kept at room temperature and reused. Ligation buffer should be stored in the freezer at –20°C, defrosted to room temperature for aliquotting and then returned to the freezer. The DNA ligase should be bought out of the freezer in an isofreeze box for aliquotting and returned to the freezer as soon as possible to avoid loss of enzyme activity.

Health and safety

It is assumed that all students follow general laboratory safety precautions including: tying back long hair; not eating or drinking in the lab; keeping lab benches clear of clutter; clearing up spills immediately; handling materials and equipment with care, and; washing hands with soap after completing lab work. In addition, specific Health and Safety information for this lab is outlined in table 3.3.

Substance / procedure	Hazard	Precautions
Plasmids digested with restriction enzymes	Biological materials: very low risk of contaminating living cells.	Use in a controlled way, applying techniques that mirror the aseptic techniques used in microbiology. Autoclave materials that come into contact with plasmids before disposal.
Ligation buffer	Low hazard	
DNA ligase enzyme	Biological materials: very low risk of contaminating living cells.	Use in a controlled way, applying techniques that mirror the aseptic techniques used in microbiology. Autoclave materials that come into contact with plasmids before disposal.

Table 3.3 Health and Safety information for laboratory 3.

Learning objectives

By the end of this laboratory students should 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

These learning objectives provide opportunities to explore enzyme reactions and the role of DNA ligase in genetic engineering.

Prior knowledge and skills

It is helpful if students already know:

- DNA is a double-stranded molecule, and each strand of DNA is made up of covalently linked subunits called *nucleotides*.

- A nucleotide is made up of a sugar, a phosphate group, and a nitrogenous base. There are four different nitrogenous bases—cytosine, guanine, adenine, and thymine.
- Nucleotides are attached to each other through a covalent phosphodiester bond, which forms the sugar-phosphate backbone of DNA.
- The two strands of DNA are connected by hydrogen bonds between adjacent nitrogenous bases, which are called *base pairs*; cytosine is always paired with guanine, and adenine is always paired with thymine.

Expected question responses

The question sections include: (i) questions that could be used to prompt students to think about what they know before they start the practical activities; (ii) questions appropriate for after the reading and practical work are complete, and; (iii) questions that are intended to help with results analysis. They can be used to illicit prior knowledge and misunderstandings, as a basis for peer-discussion and/or as a type of formative assessment.

Questions posed in the student guide and some possible answers are given below:

1. What is the function of enzymes in reactions?

   Enzymes catalyse reactions. That is, they cause reactions to occur at a greater rate than they would occur if the enzyme were not present. They do not alter their form while catalysing reactions.

2. Based on your understanding of DNA replication, explain the role of DNA ligase in replication.

   During DNA replication the two strands in the double helix are unwound and separated from each other. The nucleotides in each strand are hydrogen-bonded to complementary single nucleotides. The enzyme DNA polymerase then creates a new strand of DNA. Formation of this new strand is continuous on one strand and discontinuous on the other strand. The role of DNA ligase is to catalyse the joining of the DNA fragments through formation of covalent bonds in the sugar-phosphate backbone of the DNA molecule on the discontinuous strand.

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.

   When two complementary sticky ends join, the bases on each overhanging strand form hydrogen bonds to each other, creating base pairs. DNA ligase creates the covalent bonds that join the DNA backbone together.

4. What are the roles of hydrogen bonds and covalent bonds in the structure of DNA?

   Hydrogen bonds are weak bonds formed between the complementary nucleotide bases in the two strands of the double helix; they hold the two strands together. Covalent bonds are formed between the sugar and phosphate groups of the nucleotides; they create a strong backbone for each DNA strand.

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.

   Ten possible two-fragment plasmids can be made from the four fragments present in the restriction digest. The 4 fragments are shown in figure 2.1 of the teacher and technician guide. The possible 10 plasmids appear below in figure 3.1. Review students' work and present all the possible plasmids if students have not described them all.

6. Why is it important to inactivate the *Bam*HI and *Hin*dIII restriction enzymes before ligating the fragments? What might happen if you did not perform this step?

   If the restriction enzymes are still active, they will break down plasmids formed from the action of the DNA ligase.

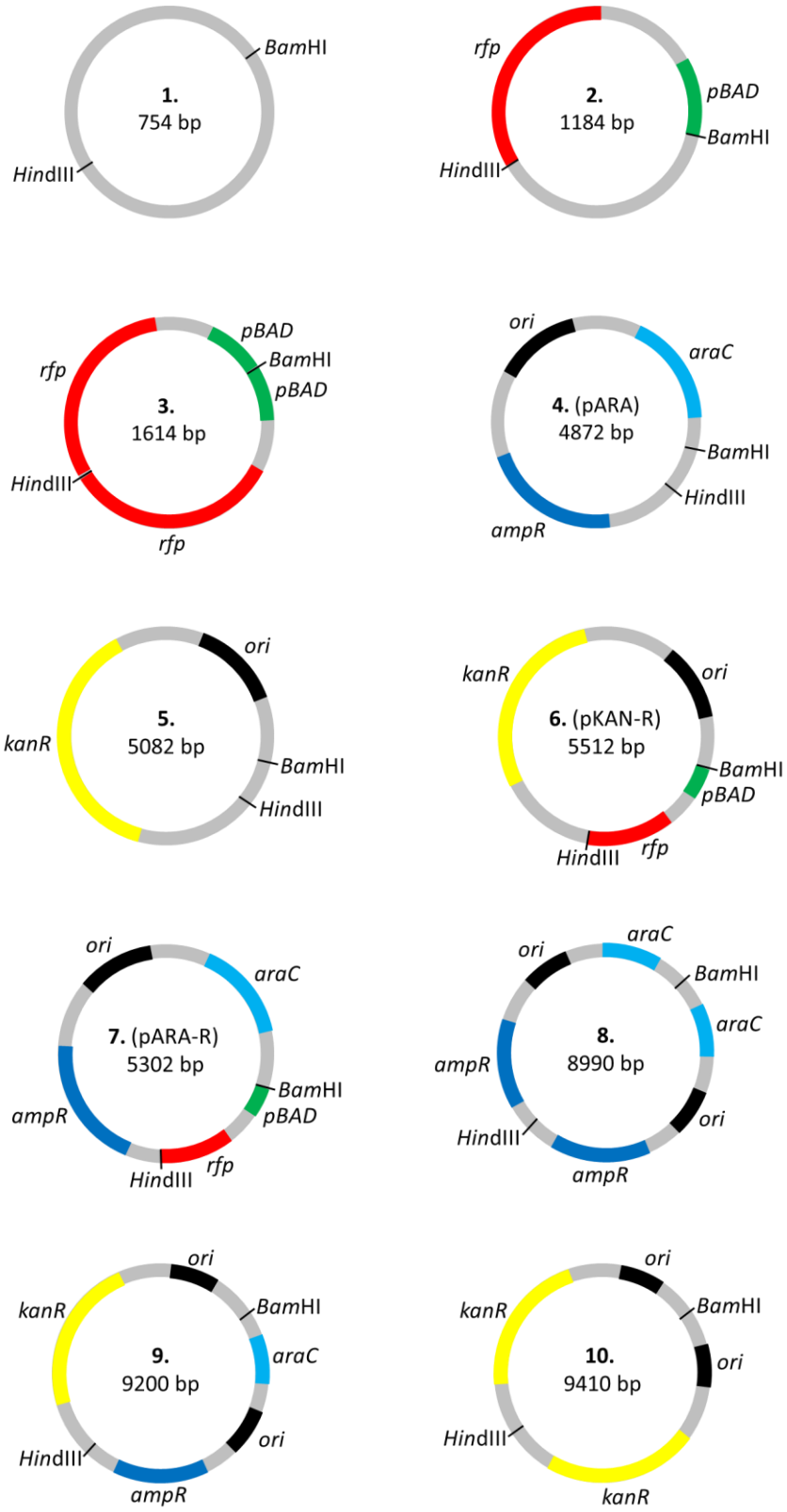


Figure 3.1 The 10 possible plasmids made by recombination.

Additional resources

There are additional resources to expand upon the concepts and practice of genetic engineering, the enzymes of DNA replication and DNA ligases. These can be used to expand the teaching experience in class, as homework, extension or group work. They are in the Additional resources section at the back of the teacher and technician guide.

Resource	Page
The enzymes of DNA replication	R.5
DNA ligases in nature and genetic engineering	R.6

Chapter 5: Checking you've created a recombinant plasmid

Overview

Chapter 5 separates the DNA fragments created in labs 2-4 using agarose gel electrophoresis and then stains the DNA bands so that the success of the previous labs in creating recombinant plasmid pARA-R can be assessed.

Preparation

This guidance for preparation is divided into three parts:

1. Advanced preparation: activities that can be done several days in advance
2. Prior preparation: preparation required immediately prior to the session
3. Post-lab support: experimental support needed after the practical work

The instructions in table 5.1 gives the equipment and reagents required for a pair / group of students. You will need to increase the amounts to suit the number of pairs that you are teaching.

Note: *C denotes an item that is shared amongst the class and *2 denotes an item that is shared between 2 pairs or groups.

Laboratory	Advance preparation	Prior preparation	Post-lab support
5: Visualisation of products from restriction digestion, ligation and the PCR Electrophoresis	Aliquot solutions: • Loading dye (LD) • DNA ladder (1 kb) • Dilute TAE buffer (1 x TAE). • Pour agarose gel	Prepare benches with: • P-20 micropipette • Pipette tips • Microfuge tube rack • Microfuge tubes of solutions • Beakers containing empty microfuge tubes • Permanent marker • Waste container Within the classroom place: • Chemical resistant gloves • Microfuge *C • Electrophoresis tank *2 • 1 x TAE buffer • Gel tray containing a 0.8% agarose gel *2 • Powerpack*2	Ligation reactions should be returned to the fridge (4 °C) ready for lab 6. Electrophoresis may not be complete. You can: • allow samples to electrophorese for 5 -10 minutes and then store the gels to complete electrophoresis later • monitor until finished, then store the gels

Table 5.1 Preparation for laboratory 5 (continued on next page).

Laboratory	Advance preparation	Prior preparation	Post-lab support
5: Visualisation of products from restriction digestion, ligation and the PCR Electrophoresis		Retrieve & distribute: • 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) • The PCR with pARA as template from lab 4 (MMpARA) • The PCR with ligation reaction as template from lab 4 (MMLIG) Reuse the tubes of distilled water.	Once electrophoresis is complete: • gels can be stored in ziplock bags with a little buffer in the fridge (4°C) until needed • gels can be stained, then stored in ziplock bags with a little buffer in the fridge (4°C) until needed • gels can be stained and photographed, so results are ready for analysis in the next teaching session
5: Visualisation of products from restriction digestion, ligation and the PCR Staining and photodocumentation	Dilute GelRed solution	Prepare benches with: • GelRed solution • Plastic gel staining tray *2 • Permanent marker • Tape • Aluminium foil square Within the classroom place: • Chemical resistant gloves • Foil-covered bottle and funnel (if reusing GelRed solution) *C • UV transilluminator*C • Hood *C • Camera *C	Possibly printing gel pictures or transferring to computer(s).

Table 5.1 Preparation for laboratory 5 (continued from previous page).

The processes for diluting TAE buffer, preparing agarose gels and diluting GelRed solution are covered in the overview of preparation section on page I5/6. If you pour an 15-well, 0.8% agarose gel, then two groups will each be able to load one set of 7 samples and there will be one well for the 1 kb DNA ladder. Depending on whether you have completed lab 4, you may wish to provide alternative suggestions for the sample loading order given in figure 5.3 of the student guide. Without the wells

containing the PCRs, there will be enough space for each group to load 1 kb DNA ladder into a well.

When aliquotting reagents, we aim to provide sufficient for the number of pairs that you are doing the practical activities with and a bit extra for pipetting errors. Table 5.2 gives a brief summary of the quantity of reagents required by each pair of students for this laboratory.

Tube contents	Tube label	Aliquot (µl)	Actually used (µl)
Loading dye	LD	18	14
DNA ladder	1 kb	12	10
Distilled Water (from lab 3)	dH20	50	27 (+4)

Table 5.2 Aliquotting reagents for laboratory 5.

The loading dye should be kept at room temperature. DNA ladder should be stored in the freezer at –20°C, defrosted to room temperature for aliquotting and then returned to the freezer until needed.

Health and safety

It is assumed that all students follow general laboratory safety precautions including: tying back long hair; not eating or drinking in the lab; keeping lab benches clear of clutter; clearing up spills immediately; handling materials and equipment with care, and; washing hands with soap after completing lab work. In addition, specific Health and Safety information for this lab is outlined in table 5.3.

Substance / procedure	Hazard	Precautions
Previously prepared: Restriction digest reactions Ligation reaction PCRs	Biological materials: very low risk of contaminating living cells.	Use in a controlled way, applying techniques that mirror the aseptic techniques used in microbiology. Autoclave materials that come into contact with plasmids before disposal.
Loading dye	Low hazard, but stains skin.	Use good laboratory practice to avoid contact with skin.
DNA ladder	Low hazard, but contains biological materials and loading dye included can stain skin	Use in a controlled way, applying techniques that mirror the aseptic techniques used in microbiology. Use good laboratory practice to avoid contact with skin. Autoclave materials that come into contact with plasmids before disposal.
Tris-acetate-EDTA buffer (TAE) buffer, supplied at 50x concentration	The mixture is an irritant, particularly to skin and eyes. Diluting to 1 x TAE buffer reduces the hazard. At 50x concentration, the mixture is very toxic to aquatic life with long lasting effects and should not be released into water courses until diluted	When handling, wear eye protection (safety glasses are sufficient) and chemical- resistant gloves. Dilute before student use. A 1 x solution is low hazard, but as a precaution students should wear eye protection and gloves when handling the solution. Skin or eyes should be rinsed with plenty of water.

Table 5.3 Health and Safety information for laboratory 5 (continued on next page).

Substance / procedure	Hazard	Precautions
Agarose gel	Agarose is safe, but will be melted in 1 x TAE buffer, so an agarose gel is low hazard.	An agarose gel made with 1 x TAE is low hazard, but as a precaution students should wear eye protection and gloves when handling.
Gel electrophoresis	Access to liquids carrying a 100 V current. Access to a metal contact which is live at 100 V.	The electrophoresis chamber cuts off the electric supply whenever the lid is removed. All connections are plastic coated.
GelRed	Whilst **not** mutagenic like some DNA stains, this is a substance that can bind to DNA.	Avoid eye and skin contact by wearing eye protection and chemical-resistant gloves. Dispose of any unwanted dye down the drain with significant additional water to dilute and flush away.
UV transilluminator	UV light can damage the cornea if observed directly.	The UV light boxes used are fitted with a protective shield, which has to make a magnetic connection before the UV light will come on.

Table 5.3 Health and Safety information for laboratory 5 (continued from previous page).

Learning objectives

By the end of this laboratory students should 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

These learning objectives help to address the basic structure and size of DNA and how charged molecules can be separated by agarose gel electrophoresis. Interpretation of the results observed can help students to revise the enzymes involved and procedures used in; restriction digestion, ligation and the polymerase chain reaction.

Prior knowledge and skills

It is helpful if students already know:

- DNA is a double-stranded molecule, the length of which is frequently measured in base pairs (the number of linked nucleotides that make up the DNA molecule).
- That charged objects, including molecules, move through an electric field

Expected question responses

The question sections include: (i) questions that could be used to prompt students to think about what they know before they start the practical activities; (ii) questions appropriate for after the reading and practical work are complete, and; (iii) questions that are intended to help with results analysis. They can be used to illicit prior knowledge and misunderstandings, as a basis for peer-discussion and/or as a type of formative assessment.

Questions posed in the student guide and some possible answers are given below:

1. Why do DNA restriction fragments and plasmids separate when analyzed by gel electrophoresis?
   The DNA molecules, including fragments and plasmids, move through the gel during the procedure of gel electrophoresis. Separation occurs during the procedure because lighter and more compact molecules move faster than heavier and less compact molecules.
2. Why is it important to check each stage in the preparation of a recombinant plasmid?
   You might make errors during a procedure or there might be other problems, such as reagents that are not correct. The products of genetic engineering procedures are not visible, so mistakes may go unnoticed. You should ensure that you have a recombinant plasmid that has the gene you need as well as any other important genes or sequences.
3. When DNA fragments are joined with a DNA ligase an array of products are created. How does this happen?
   Any two fragments that have complementary sticky ends can be ligated, including two fragments that are the same.
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?
   The order of fragments from smallest to largest is B, G, D, F, A, C, and E.
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?
   There are 2 reasons for using a loading dye. Firstly, loading dye contains glycerol, which helps samples to sink to the bottom of the wells. Secondly, the dyes can be seen so they tell you whether your samples are running in the right direction. They do also co-migrate with DNA of a known size through the agarose gel, so they allow you to see how far differently-sized DNA molecules have progressed. In a 1% agarose gel; Orange G dye co-migrates with linear DNA of about 50 bp, bromophenol blue co-migrates with linear DNA of about 500 bp, and xylene cyanol co-migrates with linear DNA of about 4,000 bp.
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?
   The supercoiled and nicked circle plasmids have the same molecular weight, but the supercoiled plasmid moves more quickly through the gel because it takes up less space for its size and therefore can move through the spaces in the gel more quickly. The multimer is very slow because it has multiple copies of the plasmid and therefore has a much greater molecular weight.
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?
   The two plasmids can have all three configurations.
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?
   The ligated plasmids can only have the nicked circle configuration, as the other two configurations can occur only if a plasmid has been replicated in a bacterial cell.
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.

Table 5.4 shows the expected fragments and their sizes in each lane of the agarose gel.

Tube	Fragments and plasmids listed in order of increasing bp size in each tube
K-	***pKAN-R, 5,512 bp*** *The plasmid can have all three configurations. The supercoiled configuration should move the fastest, the multimer configuration will move the slowest and the nicked circle configuration will move at an intermediate speed.*
K+	*(1)* ***pBAD-rfp fragment, 807 bp*** *(2)* ***kanR fragment, 4,705 bp***
A-	***pARA, 4,872 bp*** *The plasmid can have all three configurations. The supercoiled configuration should move the fastest, the multimer configuration will move the slowest and the nicked circle configuration will move at an intermediate speed.*
A+	*(1)* ***BamHI – HindIII restriction fragment, 377 bp*** *(2)* ***ampR-ori-araC fragment, 4,495 bp***
LIG	*(1)* ***double BamHI – HindIII restriction fragment plasmid, 754 bp*** *(2)* ***pBAD-rfp & BamHI – HindIII restriction fragment plasmid, 1,184 bp*** *(3)* ***double pBAD-rfp plasmid, 1,614 bp*** *(4)* ***pARA, 4,872 bp*** *(5)* ***kanR & BamHI – HindIII restriction fragment plasmid, 5,082 bp*** *(6)* ***pARA-R, 5,302 bp*** *(7)* ***pKAN-R, 5,512 bp*** *(8)* ***double ampR-ori-araC plasmid, 8,990 bp*** *(9)* ***ampR-ori-araC & kanR plasmid, 9,200 bp*** *(10)* ***double kanR plasmid, 9,410 bp*** *(For help visualising plasmids see figure 3.1 of the teacher guide.)*
MMpARA	*(1)* ***primers, 22 bp*** *(probably too small to be visible)* *(2)* ***expected PCR product, 662 bp*** *(3)* ***pARA template, 4,872 bp*** *(in all 3 plasmid configurations)*
MMLIG	*(1)* ***primers, 22 bp*** *(probably too small to be visible)* *(2)* ***PCR product of BamHI – HindIII restriction fragment, 662 bp*** *(ie; pARA made)* *(3)* ***PCR product of pBAD-rfp fragment, 1092 bp*** *(ie; pARA-R made)* *(4)* ***PCR product of double ampR-ori-araC plasmid, 4,780 bp*** *(unlikely because primer binding to both parts of plasmid will obstruct PCR)* *(5)* ***PCR product of ampR-ori-araC & kanR plasmid, 4,990 bp*** *(less likely because of length of extension step in PCR thermal cycling)* *Some or all of these PCR products could be present.*

Table 5.4 the DNA fragments and expected sizes from labs 2 to 5.

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).

    The DNA ladder diagram is reproduced in Figure 5.1 with the expected sizes of the DNA bands.

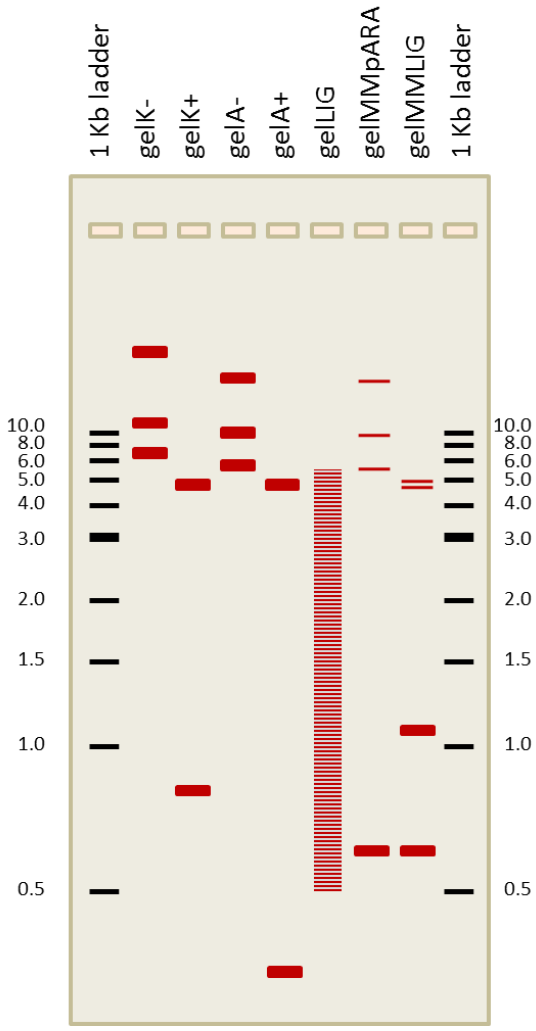


Figure 5.1 The DNA ladder diagram completed with the expected sizes of DNA bands.

The K- lane shows undigested pKAN-R plasmid in the 3 configurations (multimer, nicked circle and supercoiled), whilst the K+ lane shows the 2 products following restriction digestion. The A- lane shows undigested pARA plasmid in the 3 configurations, whilst the A+ lane shows the 2 products following restriction digestion. The many possible ligated products in the LIG lane are frequently only visible as a faint smear, as the amount of any particular DNA product made is often quite small and therefore hard to see. The pARA PCR lane shows the expected 662 bp PCR product and a small amount of pARA plasmid template in the 3 configurations. This template is not always visible and a small band of primer DNA is sometimes visible. The ligation PCR lane shows re-ligated pARA (662 bp), anticipated pARA-R (1092 bp) and small amounts of the other 2 possible ligation products that the PCR would detect (4780 and 4990 bp).

11. When you have had a chance to examine your gel photograph, analyse how your actual gel results compare to your gel predictions.

Answers will vary, but if the procedure was done correctly, students should see the same number of bands as predicted in each lane. It is not uncommon for students to load the gels in a different order than suggested in the protocol. This is okay, as long as they can figure out what is in each lane by reviewing the undigested plasmids and the lengths of the fragments.

More problematically, sometimes a reaction does not work and trying to unpick what the problem was, is very challenging for many students.

12. Do you see any bands that are not expected? What could explain the origin of these unexpected bands?

    Answers will vary depending on how accurately the students had predicted what they would see and how successfully they have performed the practical work.

13. Does the gel show that your restriction digest and ligation procedures were successful? Describe the evidence you used to make this assessment.

    If the restriction digest procedures were successful then there will be a difference between the A- and A+ lanes, and between the K- and K+ lanes. The A- and K- lanes will contain 1-3 bands of undigested plasmid DNA (caused by the 3 plasmid configurations). The A+ and K+ lanes will contain 2 clear bands of digested plasmid DNA, that have migrated further through the gel, due to their linear nature and smaller size. If the ligation procedure was successful then there won't be the same results as for the 2 digested plasmid lanes mixed together. Instead a rather unsatisfying smear, with possibly some faint bands will be apparent.

14. In the gelK– and gelA– lanes, do you see evidence of multiple configurations of plasmids? Explain your answer.

    Students should see two or three different bands in the gelK– and gelA– lanes, which is evidence of multiple plasmid configurations.

15. In the gelK+ and gelA+ lanes, do you see evidence of complete digestion? Explain your answer.

    There are only two bands in each lane, showing that the plasmid was completely digested into its two fragments. No undigested plasmid DNA remains.

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.

    In the gelK+ lane, you would expect to see a band between the 1,000 bp and 500 bp DNA ladder fragments, which is the 807 bp fragment that carries the rfp gene. In the gelA+ lane, we would expect to see a band between the 4,000 bp and 5,000 bp DNA ladder fragments, which is the 4,495 bp fragment that carries the ampR gene. Hopefully students will see both of these bands in these locations.

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

    This answer will obviously depend on which bands are seen. It is likely that either or both of the 662 bp band (representing the re-ligated pARA plasmid) and 1,092 bp band (representing the desired pARA-R plasmid) will be present. It is less likely that the PCR product of a double ampR-ori-araC plasmid, (4,780 bp) or PCR product of ampR-ori-araC & kanR plasmid (4,990 bp) will be present and unlikely that the 22 bp primers will be seen.

Additional resources

There are currently no additional resources to expand upon the concepts and practice of agarose gel electrophoresis.

Additional resources

Overview

Additional resources have been developed and are made available in this section. Some link directly to the laboratories, including: the sheets to be laminated for micropipetting practice on page R.2 and the paper-based 'clone that gene' activity on page R.3. Others provide additional background to the scientific understanding and use of molecular biology, possible questions, internet links, vocabulary exercises and material to help explore the social and ethical dimensions of these techniques.

This section is presented separately so that you can choose parts of it for inclusion as you feel appropriate during your teaching. We hope that you find the resources helpful.

Micropipette Practice

Name	20µL	15µL	10µL	5µL	2µL
1. _____	o	o	o	o	o
2. _____	o	o	o	o	o
3. _____	o	o	o	o	o
4. _____	o	o	o	o	o

Micropipette Practice

Name	20µL	15µL	10µL	5µL	2µL
1. _____	o	o	o	o	o
2. _____	o	o	o	o	o
3. _____	o	o	o	o	o
4. _____	o	o	o	o	o

Micropipette Practice

Name	20µL	15µL	10µL	5µL	2µL
1. _____	o	o	o	o	o
2. _____	o	o	o	o	o
3. _____	o	o	o	o	o
4. _____	o	o	o	o	o

Clone that gene

There are 2 important biological tools for cloning a gene:

1 – plasmids

2 – restriction enzymes

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, as, for example, insulin does.

What are plasmids?

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, ranging in size from 1,000 to 200,000 *base pairs* that are present in multiple copies, separate from the chromosomal DNA. Plasmids are vectors: that is vehicles for carrying DNA sequences from one organism to another. In nature this happens during bacterial conjugation.

For a plasmid to be useful in genetic engineering it needs to have several features (figure C.1):

- A sequence for the initiation of *DNA replication*, called the *origin of replication* or *ori* site, that allows the plasmid to replicate in the bacteria using the host DNA synthesis enzymes
- A gene encoding a protein for *antibiotic resistance*, which allows the bacteria that have taken in the plasmid to be identified by their survival when exposed to the *antibiotic*
- A *promoter* sequence for initiating *transcription* of an inserted gene
- One or more *recognition sites* for *restriction enzymes* that opens the plasmid circle and enables insertion of the gene of interest into the plasmid DNA

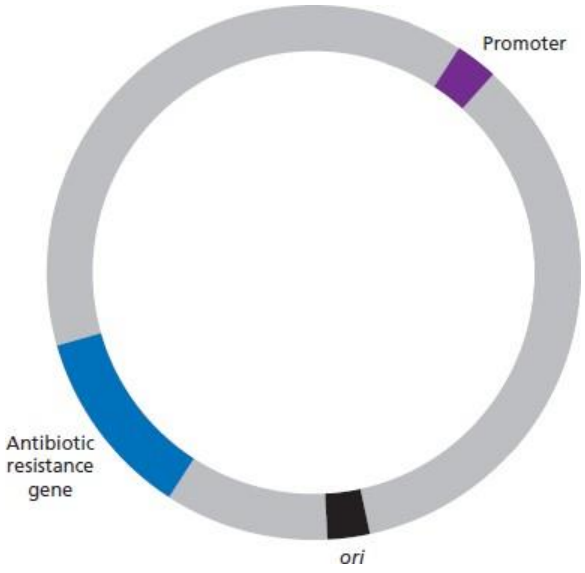


Figure C.1 A plasmid vector with 3 of the desirable characterictics shown.

What are restriction enzymes?

Restriction enzymes are proteins produced by bacteria that restrict the growth of bacteriophage by recognizing and destroying the phage DNA without damaging the host (bacterial) DNA. Figure C.2 provides examples of restriction enzymes isolated from different strains of bacteria and the DNA sequences 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. For example, a cut (or digestion) with *Eco*RI will leave an AATT overhang (or "sticky end") on one strand and a TTAA sticky end on the other strand.

Source	Restriction enzyme	Recognition site
Bacillus amyloliquefaciens	*Bam*HI	↓ 5′ GGATCC 3′ 3′ CCTAGG 5′ ↑
Escherichia coli	*Eco*RI	↓ 5′ GAATTC 3′ 3′ CTTAAG 5′ ↑
Haemophilus influenzae	*Hin*dIII	↓ 5′ AAGCTT 3′ 3′ TTCGAA 5′ ↑

Figure C.2 Restriction enzymes, their sources and recognition sites

For a restriction enzyme to be useful in genetic engineering they need to:

- Cut the plasmid at a site or sites that allow for the insertion of the new gene
- Cut the plasmid near the promoter so that the inserted gene can be expressed
- Cut the plasmid at an appropriate site to ensure that no important genes or sequences are disrupted, including the *ori* site, the promoter, and at least one of the genes encoding antibiotic resistance
- Cut the human DNA as close as possible to both ends of the gene of interest so that it can be inserted into the appropriate site in the plasmid DNA, without cutting within the gene

How are recombinant plasmids created?

In order to create a *recombinant DNA* plasmid, which contains the gene of interest (such as insulin) you need to:

1. Cut the plasmid and the human DNA with the appropriate restriction enzyme
2. Insert the human insulin gene into the plasmid DNA
3. Determine which antibiotic you would use to identify bacteria that have taken in the plasmid

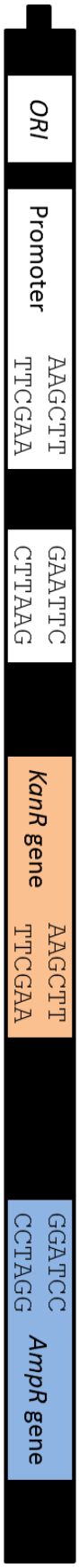
This ‘clone that gene’ activity gives you the opportunity to make a paper model of a recombinant plasmid that contains an insulin gene by completing these 3 stages of the procedure.

Procedure

1. On the Plasmid Diagram (C.3):
    - Use scissors to cut out the plasmid sequence and tape the ends together to make a paper model of the plasmid
    - Locate the positions of the *ori* site, the promoter site, and the genes for antibiotic resistance
    - Locate the positions of each restriction enzyme recognition site
2. Choose the restriction enzyme that should be used to cut the plasmid. Verify that the restriction enzyme meets all the following criteria:
    - The *ori* site on the plasmid is intact
    - The promoter site is intact
    - At least one of the antibiotic resistance genes is intact
    - The enzyme cuts the plasmid only once
    - The cut is close to the promoter sequence
3. Review Table C.2 and use scissors to cut the plasmid at the recognition site exactly as the restriction enzyme would cut it. Write the sequences of the nucleotides that are left on each end of the plasmid.
4. On the Human DNA Sequence (C.4), scan the human DNA sequence and determine where the three restriction enzymes, *Bam*HI, *Eco*RI, and *Hin*dIII, would cut the DNA.
5. Determine whether the restriction enzyme you chose in step 2 is a good choice for cutting out the insulin gene from the human DNA by verifying that it meets all the following criteria:
    - It does not cut within the insulin gene
    - It cuts very close to the beginning and end of the gene
    - It will allow the insulin gene to be inserted into the cut plasmid
6. Review Table C.2 and use scissors to cut the human DNA at the recognition site exactly as the restriction enzyme would cut it. Write the sequences of the nucleotides that are left on each end of the insulin gene after it is cut from the human DNA.
7. Use tape to insert the insulin gene into the cut plasmid. Verify that the *sticky ends* will connect in the correct orientation. (In the lab, a third biological tool, *DNA ligase*, is used to permanently connect the sticky ends together.) This is a paper model of a recombinant plasmid that contains an insulin gene. Once the plasmid replicates (copies) itself, the insulin gene is also copied, or cloned!

Activity questions

1. Which restriction enzyme did you choose? Why did you choose that one? Why is it important that the same enzyme or enzymes be used to cut both the plasmid and the insulin gene from the human DNA?
2. Where would you insert the insulin gene and why?
3. Which antibiotic would you use to determine if the recombinant DNA was taken in?



C.3 Plasmid diagram

C.4 Human DNA sequence

Teacher notes

Students will need scissors and cellotape, as well as copies of the plasmid diagram (C.3) and human DNA sequence (C.4) to create their model of a recombinant plasmid.

Answering the questions

1. Which restriction enzyme did you choose? Why did you choose that one? Why is it important that the same enzyme or enzymes be used to cut both the plasmid and the insulin gene from the human DNA?

   The EcoRI restriction enzyme is the only enzyme that cuts the plasmid once without disrupting a gene. The same enzymes must be used for the plasmid and the insulin gene, so that they have complementary sequences of bases that can match up and the two pieces of DNA can be joined by ligation.

2. Where would you insert the insulin gene and why?

   The gene should be inserted near the promoter sequence, as this sequence will enable the gene to be transcribed in the bacterial cell.

3. Which antibiotic would you use to determine if the recombinant DNA was taken in?

   Either ampicillin or kanamycin can be used, as both genes are part of the final recombinant plasmid.

What is genetic engineering?

by the Education Development Center

Treating disease with gene cloning

Until relatively recently, people with certain diseases had to rely on remedies that were expensive and sometimes difficult to obtain. Amazing as it might seem, many of these diseases are the result of the loss of a single protein function, either because the protein produced is defective or because it is not produced in normal amounts. (A *protein* is a large *biomolecule* that carries out essential functions in cells.) For example, individuals with *haemophilia*, a bleeding disorder in which blood fails to clot normally, make little or no clotting factor protein; a deficiency of human growth hormone can cause poor growth, delayed puberty, and muscle weakness in children, and fatigue, reduced muscle and bone mass, baldness, increased body fat, and memory loss in adults.

By providing the patient with a functional protein, the symptoms of these diseases can be alleviated. Before genetic engineering technology, these therapeutic proteins had to be extracted from natural sources, such as human blood or animal tissue, a process that was generally difficult, inefficient, and expensive. Pharmaceutical companies can use genetic engineering—or *gene cloning*, as it is often called—to make these proteins cost-effectively, in far greater quantities, without the impurities and viruses that can be transmitted from blood and tissue samples. Gene cloning involves inserting the human gene that encodes the protein into bacteria where the protein is made along with all the other bacterial proteins.

The ability to make enough of the proteins to treat diseases is the result of two key discoveries about bacteria made by scientists in the 1970s and ’80s. The first discovery was that bacteria contain tiny circles of DNA, called *plasmids* that sometimes contain genes that can make them resistant to antibiotics. The second discovery was that bacteria also contain proteins called *restriction enzymes* that can cut DNA at very specific places.

The findings made by basic research often lead to fundamental understandings about the nature of life. In some instances these findings can also lead to new technologies that can improve life. With the discovery of plasmids and restriction enzymes, a whole new era of genetic engineering was launched. Scientists now have the ability to generate products that can improve health in ways never before imagined.

One of the first pharmaceutical products produced using these tools was insulin, which is used to treat *diabetes*, a debilitating and sometimes fatal disease. To generate large quantities of human insulin, the sequences of DNA that contain the codes of human insulin are inserted into a plasmid that is introduced into the common intestinal bacterium *Escherichia coli* (*E. coli*), where the new protein is synthesized along with all the other bacterial proteins. The genetically modified bacteria are then grown in large batches, and the insulin is purified for use in the treatment of diabetes.

Why is the ability to produce large quantities of insulin so important, and how exactly is this done? In the following readings you will learn about diabetes: what it is and why insulin is in such demand. Then you will carry out some of the very same procedures that scientists use to produce human insulin in bacteria. But instead of producing insulin, you will engineer *E. coli* to produce a red fluorescent protein. This protein is made by a sea anemone gene that has undergone a mutation that makes the protein brighter in colour. You will give *E. coli* a new protein and a trait it did not have before: *the ability to glow*.

DATELINE: AMERICA **MAY 2012**

Teenage Diabetes on the Rise

The occurrence of type 2 diabetes in teenagers, once a disease found primarily in adults, has increased dramatically over the past 10 years. Nearly one in four teens between the ages of 12 and 19 is pre-diabetic (i.e., shows early signs of diabetes) or already has the disease. To make matters even worse, research suggests that the disease progresses more rapidly in children than in adults. In diabetes, the levels of *glucose* (a type of sugar) in the blood can become dangerously high, causing complications such as loss of vision, kidney failure, and nerve and blood vessel damage. The onset of diabetes early in life could mean serious health issues, such as heart disease, blindness, and amputation, for individuals in their 30s and 40s, far younger than such complications have been seen in the past.

Why this sudden rise in teenage diabetes? Although being overweight or obese can contribute significantly to developing diabetes, weight is not the only factor; 35 percent of teens of normal weight have glucose levels that are higher than normal, which is one indicator of being pre-diabetic. Factors such as lack of exercise in this age of increased computer and mobile device use may be part of the problem. While many pre-diabetics go on to develop full-blown type 2 diabetes, studies indicate that eating less fat and fewer calories and exercising a mere 20 minutes a day can reduce the risk of developing type 2 diabetes by 60 percent.

Diabetes can result from the body's inability to make sufficient insulin (type 1) or to effectively use the insulin that it does produce (type 2). Many patients with diabetes must take in insulin as an injection. More diabetes in the population will mean a greater demand for insulin.

What is it like for a teen to learn she has diabetes? Read the following story about one teenager's struggle with the disease.

Jennifer's story

Jennifer felt hungry all the time, but despite eating whatever she felt like whenever she wanted to, she was losing weight. She was also very thirsty and was constantly drinking water and then needing to pee. Initially, she just thought it was typical for a 15 year old; she was in a growth spurt and very active with soccer and track at school. Of course she was hungry and thirsty! She was also pleased by her weight loss, since she'd been a bit overweight for a while. Jennifer also felt unusually tired and draggy, especially in the afternoon. But again, who wouldn't be, since school started at the ungodly hour of 7:30? When she began to have trouble seeing the board in class, she thought, *Drat! Do I really need glasses?* But it was the cut on her leg that refused to heal and became infected that finally got her to talk to her parents and ultimately go to the doctor's office. There Jennifer was diagnosed with diabetes.

Jennifer had heard about a disease called diabetes but never gave it much thought. Now she really needed to pay attention. Diabetes is the result of too much of a sugar called glucose in the blood and not enough of it getting into *cells*, where it provides the energy to construct the biological molecules the body needs to survive. Jennifer learned that in order for glucose to get into cells, the body makes a hormone called insulin, which binds to the cells and enables glucose to enter them. For some reason, Jennifer's body no longer produced normal amounts of insulin, resulting in Jennifer having very high levels of glucose in her blood and not enough glucose getting into her cells. Jennifer hoped she could control her diabetes by eating more fruits **and** vegetables and getting more exercise. But although this change in habits helped some, it was not sufficient, and Jennifer had to begin injecting herself daily with insulin.

Jennifer now is very aware of what she eats, monitoring exactly how much sugar and other carbohydrates she ingests. She checks the level of her blood glucose several times a day by pricking her finger and testing her blood. She also injects her insulin faithfully. She knows that she can't cure her diabetes and that if the disease progresses further she could suffer very serious complications.

Diabetes: types 1 and 2

What is diabetes?

Diabetes is the result of elevated levels of glucose in the blood. Glucose is a major source of energy and is used to construct biological molecules in the body. What you ate for breakfast or lunch today is rapidly being converted to glucose, which in turn will be used to generate energy, to synthesize new cells and tissues, and to carry out processes required to sustain life. The starch in your bread or potato is made up of long chains of glucose molecules (figure R.1a). As food passes through your mouth, oesophagus, and stomach (figure R.1b), these chains are broken down to release glucose. The glucose is then absorbed through the intestinal wall and enters the bloodstream, where it is carried to all the cells in the body (Figure R.1c).

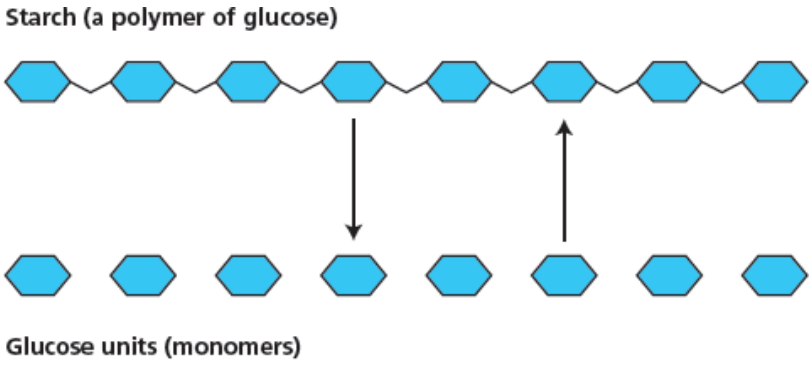


Figure R.1a: Starch is made up of subunits of glucose bonded together.

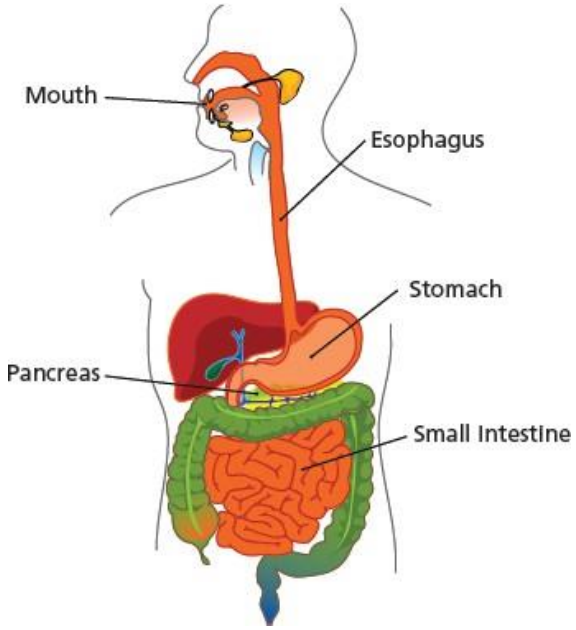


Figure R.1b: Nutrients such as starch are broken down into smaller molecules during digestion in the mouth, oesophagus, and stomach.

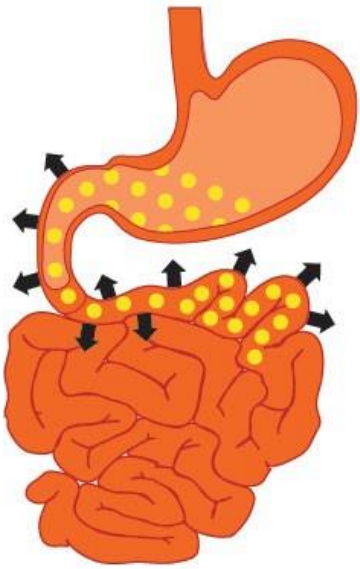


Figure R.1c: Glucose molecules pass through the small intestinal wall into the bloodstream, which delivers the glucose to cells in the body.

Crossing the cellular divide

In order to get inside a cell, glucose must cross the cell membrane that separates the inside of the cell from its environment. Insulin, which is made by beta cells found in the pancreas (see figure R.1b), binds to a special site on the cell called a *receptor*, which causes an opening in the cell membrane and allows glucose to enter the cell (see figure R.2). Without insulin, glucose cannot penetrate this cellular barrier.

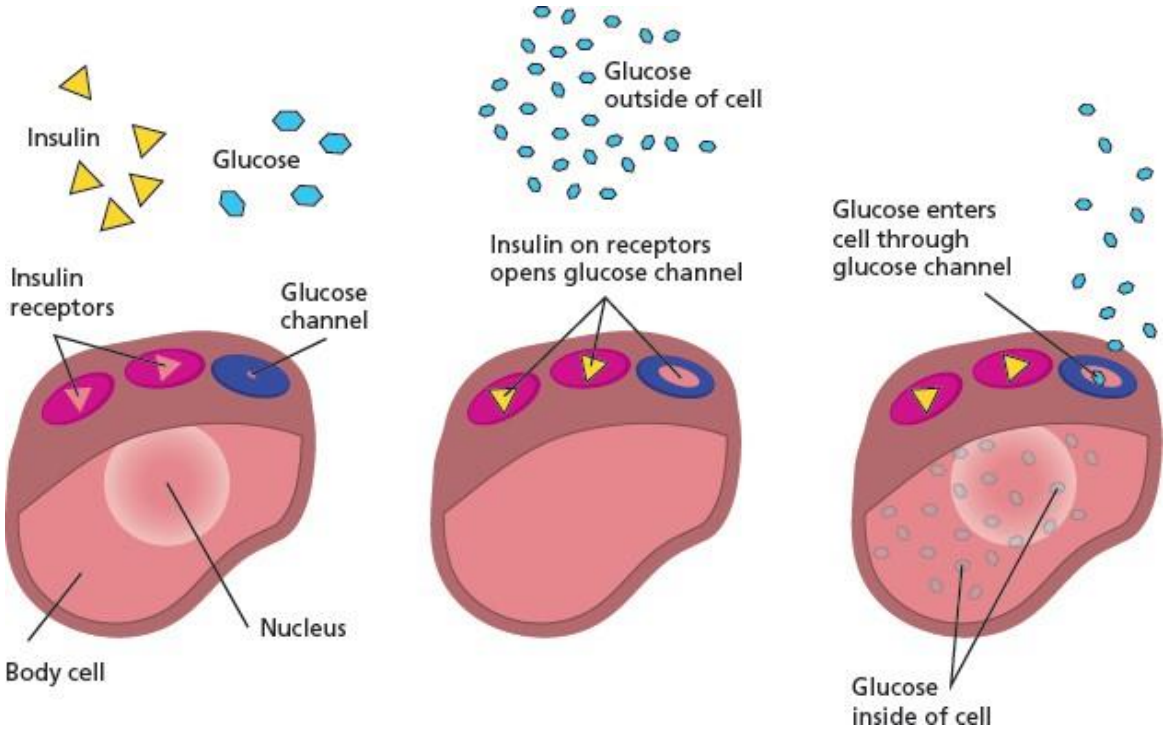


Figure R.2: How glucose crosses the cell membrane. Insulin in the blood binds to specific receptors on the cell. This binding alters the conformation of the cell membrane, resulting in the formation of a glucose channel. Glucose in the blood can now enter the cell through these channels.

In both type 1 and type 2 diabetes, glucose is unable to enter the cells, resulting in elevated levels of

glucose in the blood. In type 1 diabetes, the beta cells in the pancreas are unable to produce insulin. Without insulin to create glucose channels, the glucose remains in the blood (figureR.3a). Type 2 diabetes is the result of a combination of two factors: (1) Cells become resistant to insulin, and the receptors can no longer bind the hormone (figure R.3b). As the blood sugar levels rise, the beta cells pump out more and more insulin to no avail, since the cells cannot use it. (2) Eventually the beta cells are exhausted and can no longer produce insulin, and insulin levels in the blood drop while the sugar levels continue to increase.

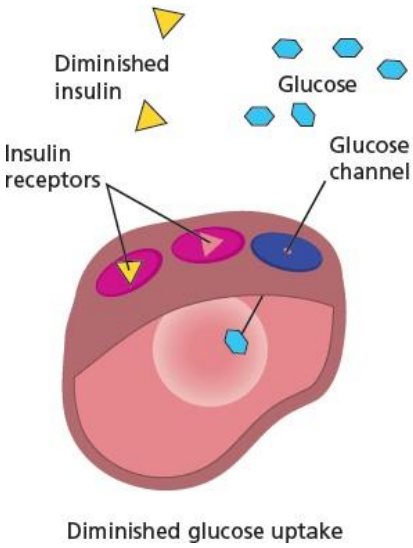


Figure R.3a: In type 1 diabetes, there is a lack of insulin in the body.

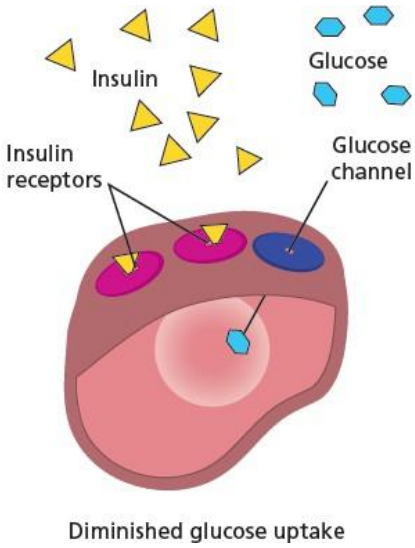


Figure R.3b: In type 2 diabetes, cells cannot bind the insulin.

The problems of too little intracellular glucose

When cells cannot get glucose, they cannot get the energy and biological molecules they need. The body responds by breaking down fats and proteins to obtain its needed energy. Loss of proteins and fats can cause serious damage to tissues and organs, leading to the symptoms of diabetes that patients like Jennifer experience, such as blindness and nerve damage (which can result in amputation).

Treating diabetes

Individuals with type 1 diabetes can regulate their sugar levels by monitoring their blood and injecting insulin as needed. Those with type 2 diabetes can sometimes regulate their blood sugar levels by changing their diet and increasing the amount that they exercise. However, in many cases, medications that reduce insulin-resistance in cells and increase the levels of insulin in the blood are required to maintain normal blood sugar levels.

Currently, there is no cure for either type of diabetes.

E. coli with a human gene

With the rise in diabetes in the population, the need for insulin for treatment is also on the rise. Originally isolated from the pancreases of pigs and cows, most of the insulin used today is genetically engineered human insulin, manufactured by bacteria. DNA sequences encoding human insulin in plasmids are taken up by bacteria, which make the hormone along with all of its bacterial proteins. Insulin is then isolated from the bacteria. In 1982, human insulin was the first commercially successful product made by recombinant DNA technology. (*Recombinant DNA* refers to DNA that contains sequences or genes from two or more sources.)

Using plasmids and restriction enzymes in genetic engineering

Some possible questions and answers to explore the use of plasmids and restriction enzymes in genetic engineering are suggested for use in questioning, discussion or written work.

1. What is the structure and function of DNA? Describe in words or a drawing the structure of a DNA molecule. Be as detailed as possible.

   DNA is a double-stranded molecule, and each strand is made up of nucleotides. There are four different nucleotides, which are distinguished by a subpart called a base*. The bases are cytosine, guanine, adenine, and thymine. The two strands of DNA are connected by hydrogen bonds between adjacent bases, which are called* base pairs*. In the base pairs, cytosine is always paired with guanine, and adenine is always paired with thymine.*

2. All living organisms contain DNA. In what ways is DNA from different organisms the same, and in what ways does it vary?

   All DNA has the same structure and uses the same code and transcription and translation processes. Among different organisms, the DNA sequences will vary because the organisms make different proteins.

3. Using your understanding of genes and how they are expressed, explain why it is possible for a bacterial cell to make a human protein from the instructions encoded in a human gene.

   Because the DNA in all organisms uses the same code and transcription and translation processes, the bacterial cell can create a human protein from a human gene.

4. As described in the Program Introduction, scientists use two biological tools to engineer organisms to make new proteins: plasmids and restriction enzymes. What do you remember about how these tools are used?

   Restriction enzymes can cut out a human gene and can cut a plasmid, and these two pieces can be joined together to make a recombinant plasmid that is inserted into bacteria.

5. Use what you know about natural selection and evolution to describe how plasmids might confer a selective advantage to their host bacteria.

   If some bacteria carry a plasmid with an antibiotic resistance gene, they will survive when exposed to the antibiotic. This gives them a selective advantage over bacteria that do not carry that plasmid; those bacteria will be killed when exposed to the antibiotic.

6. How do bacteria that carry a restriction enzyme avoid cutting up their own DNA?

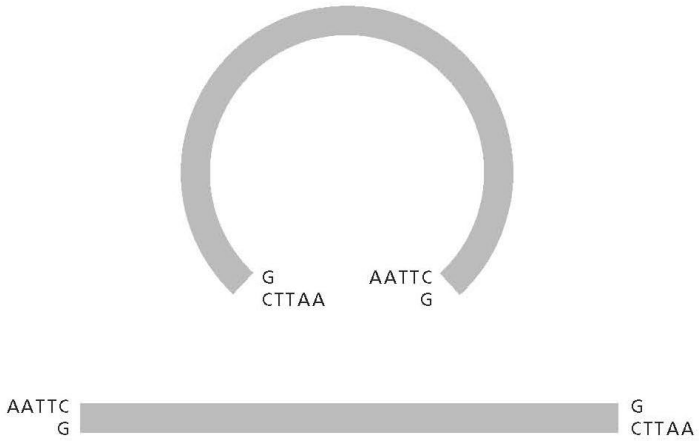
   The bacteria might have a way to protect their own DNA in sequences where it could be cut by the restriction enzyme, or their DNA might not have the sequence that is cut by the restriction enzyme.

7. What is the sequence of the sticky end that results when DNA is cut with BamHI? With HindIII?

   The overhanging end for BamHI is GATC. The overhanging end for HindIII is AGCT.

8. Scientists can modify plasmids to have a single restriction enzyme site. Imagine that you have a plasmid with a single EcoRI site. Draw the structure of the plasmid after it has been cut with the enzyme, and show the nucleotide sequences left at the site of the cut. If you wanted to insert a gene from a plant at this site, what enzyme would you use to cut the plant DNA with? Explain your response.

There are two possible ways to represent a plasmid that has been cut with EcoRI:



You would use the EcoRI restriction enzyme to cut the plant gene. The ends of the gene can then line up with the ends of the plasmid.

Why use bacterial promoters in plasmids?

All genes have their own promoters, so why include a promoter sequence when constructing a plasmid vector? The mechanism of gene transcription is the same in all organisms—RNA polymerase binds to a specific promoter sequence and copies the DNA sequence of the gene into messenger RNA. However, the DNA sequence of promoters and the structure of RNA polymerases may vary; thus, bacterial RNA polymerase will not recognize and bind to a human promoter sequence.

Perhaps more importantly, because many eukaryotic genes contain introns and exons, many human genes, such as insulin, that have been cloned are not excised directly from the genomic DNA, but rather DNA is synthesized from the mRNA of the gene of interest by **reverse transcriptase**. It is this copy DNA (cDNA) that is then cloned. These cDNAs lack a promoter and therefore must be inserted into the plasmid vector near a promoter sequence. For ease of understanding the cloning of the insulin gene, the use of cDNA has been omitted from the reading. You may wish to elaborate on this process with your students

Restriction Enzymes

In the early 1970s, Hamilton Smith and Daniel Nathans were able to purify an unknown immune "agent" found in bacteria. This molecular agent protected bacteria by restricting the growth of bacteriophage viruses. The agent was found to be an enzyme that could cut up the viral DNA into fragments as it was injected into its cell. Smith, Nathans, and Werner Arbor received the Nobel Prize for their discovery and characterization of these important molecules.

There are several classes of restriction enzymes, but the ones that have been most useful are those that consistently recognize and cut a specific nucleotide base sequence. Some restriction enzymes recognize a four-base sequence; others recognize a five- or six-base sequence. The recognition sites are palindromes. This is an important concept that you may want to emphasize to your students, perhaps using the examples "radar" and "Madam, I'm Adam".

Some restriction enzymes will make a "blunt cut," leaving no overhanging bases. Other enzymes, including BamHI and HindIII, will leave overhanging bases, thus creating sticky ends. These enzymes are particularly useful since sticky ends make recombining DNA fragments a fairly simple procedure. The "stickiness" is the result of the extraordinary affinity of complementary nucleotides to form hydrogen bonds between them.

The nomenclature for restriction enzymes is fairly straightforward. The first letter of the enzyme's name is derived from the genus of bacterium from which the enzyme was isolated. The next two letters come from the first two letters of the bacterium's specific epithet. Often there is a fourth letter following the first three, which represents the strain or type of bacterium. Because some strains of bacteria produce several restriction enzymes, a Roman numeral identifies the order in which the enzymes were isolated. Page B.4 of the Student Guide has this table, showing some examples.

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′

The enzymes of DNA replication

DNA ligase is one of several enzymes involved in DNA replication, which occurs at multiple origination sites along the molecule. In order to replicate, the DNA molecule must first unwind to reveal the bases. *Topoisomerase* initiates unwinding by nicking the DNA at the *ori* sites. Unwinding is continued by *helicase*, which breaks the hydrogen bonds between the base pairs. The enzyme *DNA polymerase* copies the sequences into new strands by binding to the DNA strand to be copied and guiding the nucleotides to hydrogen-bond with the complementary bases.

Replication by DNA polymerase can occur only in the 5' to 3' direction. Replication can take place continuously on the *leading* or 5' to 3' strand of the double helix but must occur non-continuously on the 3' to 5' or *lagging* strand. Replication on the lagging strand requires RNA primers that prime replication in the 5' to 3' direction. These primers are attached to the DNA by *primase.* Replication on the lagging strand produces small fragments, which are joined by *DNA ligase*. The RNA primers are removed prior to ligation of the fragments by DNA ligase.

DNA polymerase also has a proofreading function, recognizing mismatched base pairs and excising the incorrect base. DNA ligase catalyses the final step of replication, the formation of the covalent phosphodiester bond to complete the sugar/phosphate backbone of the newly synthesized DNA strand.

DNA ligases in nature and genetic engineering

Some possible questions and answers to explore the role of DNA ligases in nature and uses in genetic engineering are suggested for use in questioning, discussion or written work.

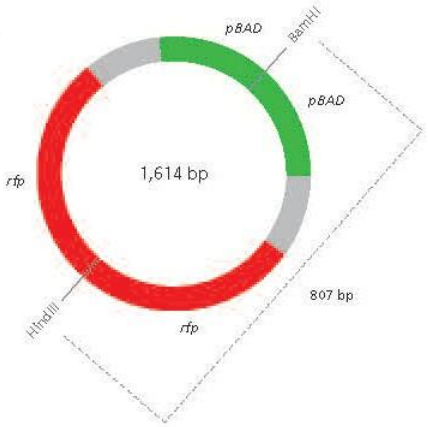
1. What role do DNA ligases have in nature?

   The ligase is needed in one of the steps of DNA replication. During the replication process, one of the daughter strands is made up of DNA fragments. The ligase joins the fragments by catalyzing the formation of a covalent bond between adjacent nucleotides.
2. What role do DNA ligases have in gene cloning?

   The ligase catalyzes bonding along DNA's sugar-phosphate backbone after the bases in complementary sticky ends have formed hydrogen bonds to each other.
3. What properties of the DNA restriction fragments produced in *Laboratory 2* enable ligation of these fragments?

   The fragments have complementary sticky ends. The ligase catalyzes bonding of the backbone only after the base pairs have been created.
4. Could two *rfp* fragments join to form a plasmid during the ligation? If not, what would prevent that? If so, what would be the result?

   Yes, the two fragments can join because they have complementary sticky ends. The resulting plasmid is shown here:



5. During ligation, both hydrogen and covalent bonds form. Which bonds form first? Why do both types of bonds need to form?

   The hydrogen bonds form first. Both types of bonds need to form because the hydrogen bonds ensure that the bases are placed in the right order, while the covalent bonds ensure that the nucleotides are permanently joined along the sugar-phosphate backbone.

The Central Dogma and Reverse Transcriptase

In 1957, Francis Crick laid the intellectual groundwork for understanding how information in DNA results in traits of organisms. He proposed what came to be known as the "central dogma," which states that genetic information stored in DNA is transferred to RNA in the process of transcription. The "messenger" RNA (mRNA) then carries this information to ribosomes, where it is translated into proteins (figure R.5).

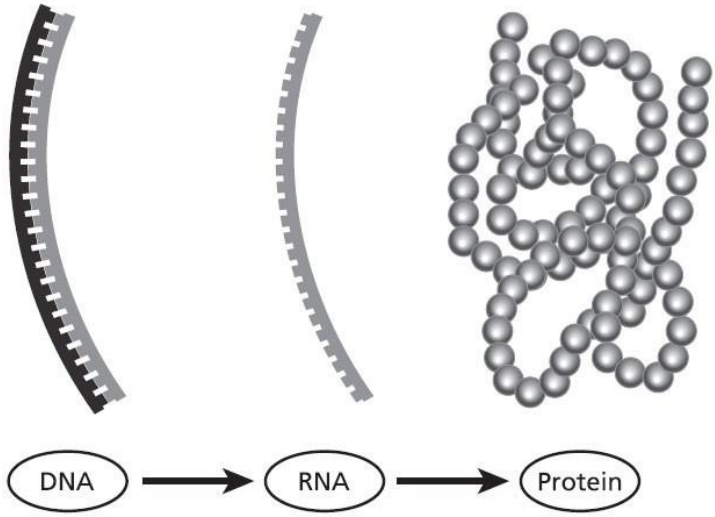


Figure R.5 The central dogma

The discovery that certain viruses do not use DNA but rather RNA as the genetic material led to a significant modification of the central dogma. One group of RNA viruses, called *retroviruses*, store their genetic information as double-stranded RNA, and the information in the RNA is converted to DNA by the enzyme reverse transcriptase. This DNA is integrated into the host cell genome and then transcribed by the host cell RNA polymerase into mRNA, which is translated by the host cell protein synthesis machinery to generate viral proteins. Another group of viruses, called *positive strand viruses*, bypass DNA completely. A single-stranded RNA molecule serves as both the genetic material and as the mRNA. Polio virus is an example of a virus whose genomic RNA has three functions: (1) storing genetic information, (2) serving as a template for replication by an RNA-dependent RNA polymerase, and (3) acting as an mRNA that directs translation of the encoded information into viral proteins.

These discoveries demonstrated that the flow of information as stated in the central dogma has multiple pathways. In addition to the pathway shown above, there are two additional pathways (figure R.6).

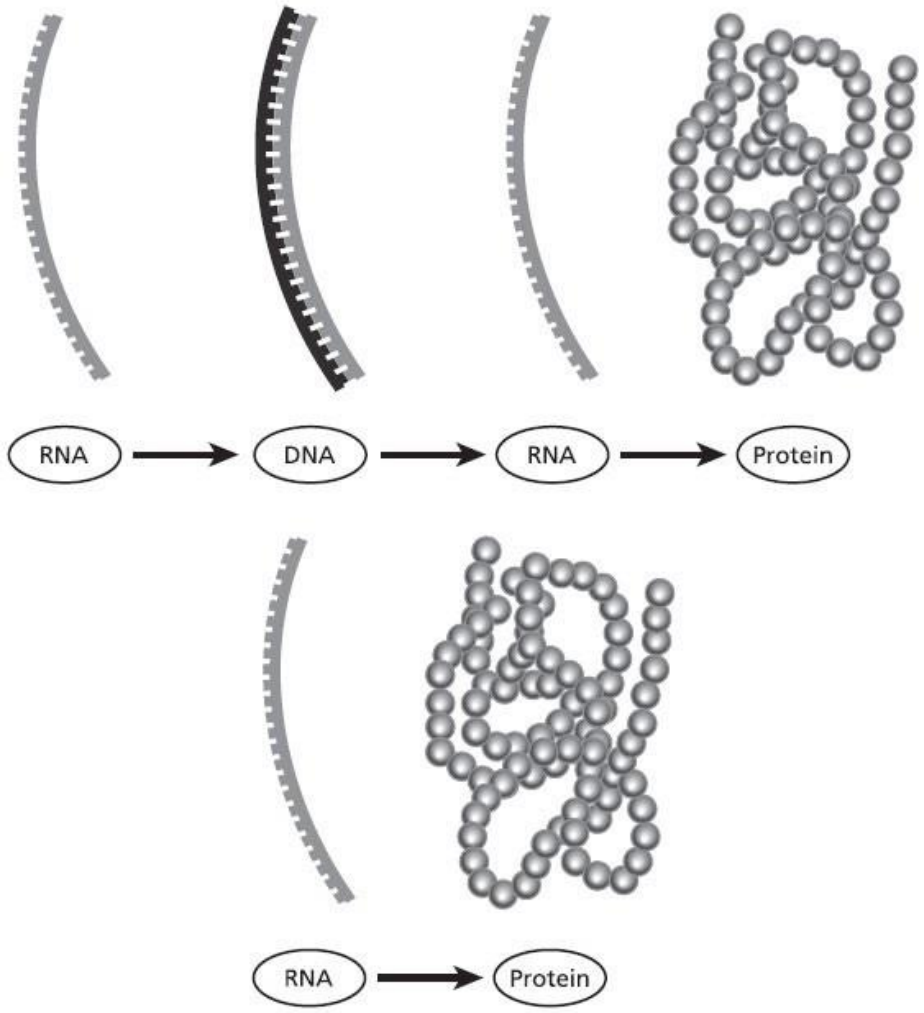


Figure R.6 Alternative pathways for the central dogma

The discovery of reverse transcriptase, which won a Nobel Prize in 1970 for its discoverers, Howard Temin and David Baltimore, provided an invaluable tool for biotechnology. With reverse transcriptase, copies of DNA could be generated directly from mRNA. This copy DNA (cDNA) alleviated the problems presented by the fact that genomic DNA could not be used in bacteria, which do not have the ability to splice (remove) introns from mRNA. The cDNA contains only exons and therefore can be used in bacteria to produce the encoded protein

In addition to carrying genetic information, some RNA molecules can act like enzymes and are involved in the regulation of gene expression. The ability of RNA to play a number of different roles in the cell has led some scientists to propose that the ancestral form of life was RNA-based and subsequently evolved into the forms we know now, with DNA as the major biomolecule of inheritance