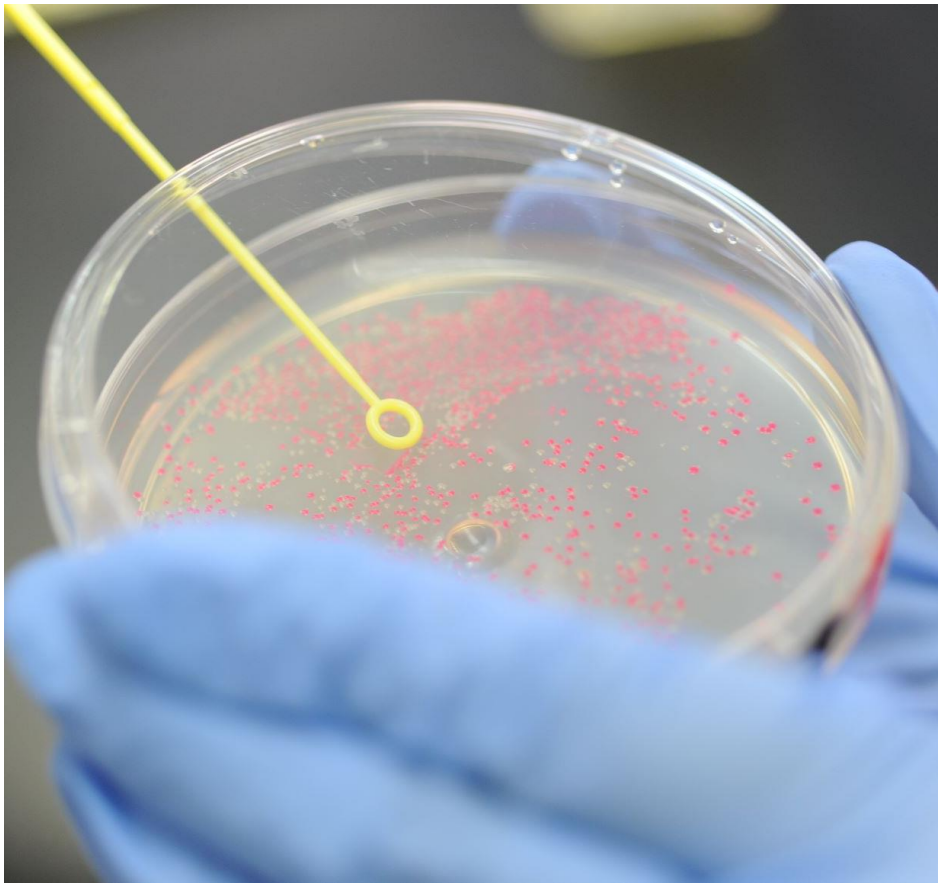
AMGEN® Biotech Experience

Scientific Discovery for the Classroom

Teacher and Technician 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	
Introduction from the Education Development Center	I.1
Sequence of laboratories	I.2
Timings for the sequences of laboratories	I.6
Overview of content	I.12
Connections to theoretical understanding	I.13
Overview of preparation	
TAE buffer dilution	I.14
GelRed DNA stain dilution	I.14
Agarose gel preparation	I.15
Agar plate preparation	I.15
Photodocumentation	I.17
Using the PCR machine	I.17
1 – Some tools of the trade	
Overview	1.1
Preparation	1.1
Health and safety	1.2
Learning objectives	1.3
Prior knowledge and skills	1.3
Expected question responses	1.3
Additional resources	1.5
2 - Beginning to clone a gene	
Overview	2.1
Preparation	2.1
Health and safety	2.2
Learning objectives	2.2
Prior knowledge and skills	2.3
Expected question responses	2.3
Additional resources	2.5

Chapter, section	Page

3 - Building a recombinant plasmid

- Overview 3.1
- Preparation 3.1
- Health and safety 3.2
- Learning objectives 3.2
- Prior knowledge and skills 3.2
- Expected question responses 3.3
- Additional resources 3.4

4 - Verifying pARA-R is present

- Overview 4.1
- Preparation 4.1
- Health and safety 4.2
- Learning objectives 4.2
- Prior knowledge and skills 4.3
- Expected question responses 4.3
- Additional resources 4.4

5 - Checking you've created a recombinant plasmid

- Overview 5.1
- Preparation 5.1
- Health and safety 5.3
- Learning objectives 5.4
- Prior knowledge and skills 5.4
- Expected question responses 5.4
- Additional resources 5.8

6 - Inserting recombinant plasmids into bacteria

- Overview 6.1
- Preparation 6.1
- Health and safety 6.3
- Learning objectives 6.3
- Prior knowledge and skills 6.3
- Expected question responses 6.4
- Additional resources 6.7

Chapter, section	Page

7 - Correlating DNA fragment size (genotype) with phenotype

- Overview 7.1
- Preparation 7.1
- Health and safety 7.4
- Learning objectives 7.6
- Prior knowledge and skills 7.7
- Expected question responses 7.7
- Additional resources 7.11

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

- Overview 2a.1
- Preparation 2a.1
- Health and safety 2a.2
- Learning objectives 2a.2
- Prior knowledge and skills 2a.2
- Expected question responses 2a.3
- Additional resources 2a.5

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

- Overview 4a.1
- Preparation 4a.1
- Health and safety 4a.2
- Learning objectives 4a.2
- Prior knowledge and skills 4a.3
- Expected question responses 4a.3
- Additional resources 4a.4

5a - Verifying the engineered plasmid pARA-R

- Overview 5a.1
- Preparation 5a.1
- Health and safety 5a.3
- Learning objectives 5a.4
- Prior knowledge and skills 5a.4
- Expected question responses 5a.4
- Additional resources 5a.8

Chapter, section	Page
6a - Inserting recombinant plasmids into bacteria	
Overview	6a.1
Preparation	6a.1
Health and safety	6a.2
Learning objectives	6a.3
Prior knowledge and skills	6a.3
Expected question responses	6a.4
Additional resources	6a.7
7a - Correlating DNA fragment size with red fluorescent protein expression	
Overview	7a.1
Preparation	7a.1
Health and safety	7a.4
Learning objectives	7a.6
Prior knowledge and skills	7a.7
Expected question responses	7a.7
Additional resources	7a.10
9 - DNA profiling	
Overview	9.1
Preparation	9.1
Health and safety	9.4
Learning objectives	9.5
Prior knowledge and skills	9.5
Expected question responses	9.6
Additional resources	9.7
10 – Serial dilutions	
Overview	10.1
Preparation	10.1
Health and safety	10.2
Learning objectives	10.2
Prior knowledge and skills	10.2
Expected question responses	10.2

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 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.

Sequence of laboratories

If students complete the full sequence of ABE labs, they will produce a recombinant DNA molecule and then use it to transform *E. coli*. However, when you first begin the program or if your time is limited, this can be challenging. Some alternative routes through the laboratories are suggested below. Sequences A-G are different variations of the series of experiments that mirror the process used in creating protein therapeutics. Generally these sequences are aimed at Year 13 students. Sequence H is a stand-alone lab.

Sequences A-D involve creating a recombinant plasmid (A), then transforming it into bacteria (B), with the possibility of confirming your results using PCR either after the ligation is complete (C), or after the transformation (D) when phenotype can be correlated with genotype. These possibilities are illustrated in figure I.1 and summarised in table form below.

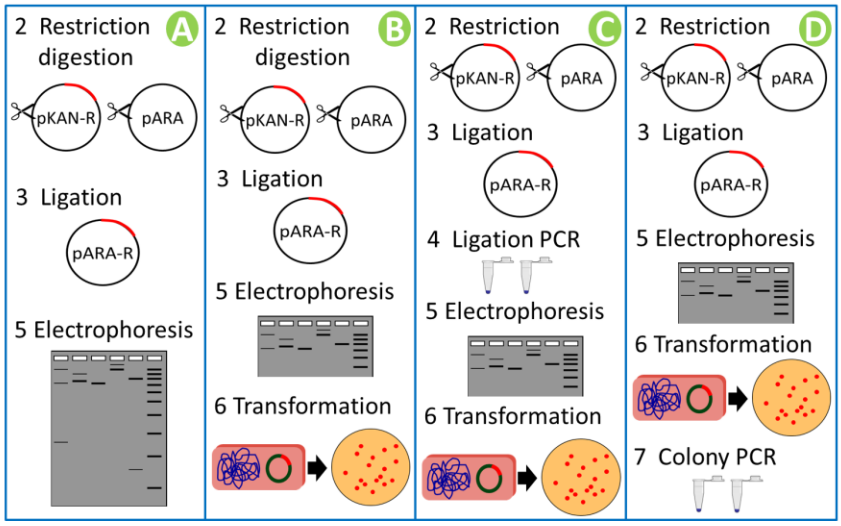


Figure I.1. A summary of the practical work undertaken in sequences A, B, C and D after the initial activities to familiarise students with the equipment.

Sequence A	Practical activities	Summary
Chapter 1	Using a micropipette (1.1) Gel electrophoresis (1.2)	Students can build their own recombinant plasmid and check their progress using agarose gel electrophoresis.
Chapter 2	Restriction digestion of plasmids (2)	
Chapter 3	Building the pARA-R plasmid (3)	
Chapter 5	Visualisation of products from restriction digestion, ligation and the PCR (5)	

Sequence B	Practical activities	Summary
Chapter 1	Using a micropipette (1.1) Gel electrophoresis (1.2)	Students can build their own recombinant plasmid, checking their progress using agarose gel electrophoresis, then transform *E. coli* with their recombinant plasmid.
Chapter 2	Restriction digestion of plasmids (2)	
Chapter 3	Building the pARA-R plasmid (3)	
Chapter 5	Visualisation of products from restriction digestion, ligation and the PCR (5)	
Chapter 6	Transforming bacteria with the ligation products (6)	

Sequence C	Practical activities	Summary
Chapter 1	Using a micropipette (1.1) Gel electrophoresis (1.2)	Students can build their own recombinant plasmid, checking their progress using PCR and agarose gel electrophoresis, then transform *E. coli* with their recombinant plasmid.
Chapter 2	Restriction digestion of plasmids (2)	
Chapter 3	Building the pARA-R plasmid (3)	
Chapter 4	PCR of the recombinant plasmids formed by ligation (4)	
Chapter 5	Visualisation of products from restriction digestion, ligation and the PCR (5)	
Chapter 6	Transforming bacteria with the ligation products (6)	

Sequence D	Practical activities	Summary
Chapter 1	Using a micropipette (1.1) Gel electrophoresis (1.2)	Students can build their own recombinant plasmid, checking their progress using agarose gel electrophoresis, then transform *E. coli* with their recombinant plasmid. The phenotype of colonies can then be correlated to genotype using PCR.
Chapter 2	Restriction digestion of plasmids (2)	
Chapter 3	Building the pARA-R plasmid (3)	
Chapter 5	Visualisation of products from restriction digestion, ligation and the PCR (5)	
Chapter 6	Transforming bacteria with the ligation products (6)	
Chapter 7	Colony PCR from the transformed bacteria (7.1) Visualisation of products from the colony PCR (7.2)	

Sequences E-G use an engineered (pre-made) recombinant plasmid. The identity of the recombinant plasmid can be confirmed using restriction digestion (E), or PCR (F), before *E. coli* cells are transformed and express the Red Fluorescent Protein, or after transformation using PCR (G). These possibilities are illustrated in figure I.2 and summarised in table form below.

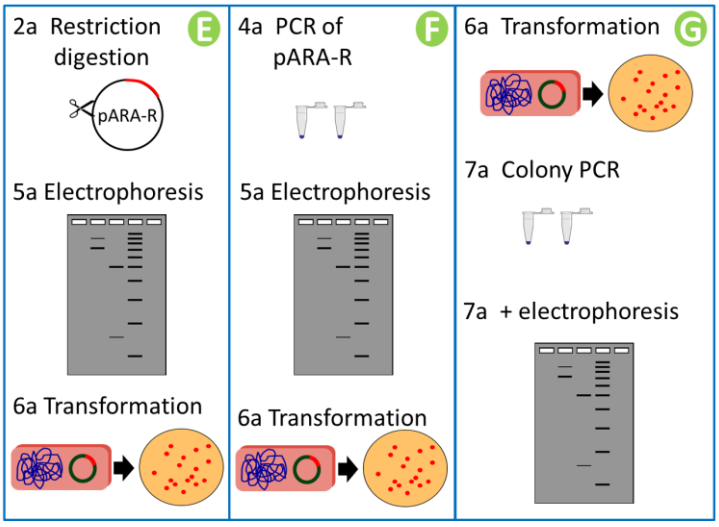


Figure I.2. A summary of the practical work undertaken in sequences E, F and G after the initial activities to familiarise students with the equipment.

Sequence E	Practical activities	Summary
Chapter 1	Using a micropipette (1.1) Gel electrophoresis (1.2)	Students can check the identity of a pre-made recombinant plasmid using restriction digestion and agarose gel electrophoresis, then transform *E. coli* with the recombinant plasmid.
Chapter 2a	Restriction digestion of pARA-R (2a)	
Chapter 5a	Visualisation of products from restriction digestion and the PCR (5a)	
Chapter 6a	Transforming bacteria with the recombinant plasmid pARA-R (6a)	

Sequence F	Practical activities	Summary
Chapter 1	Using a micropipette (1.1) Gel electrophoresis (1.2)	Students can check the identity of a pre-made recombinant plasmid using PCR and agarose gel electrophoresis, then transform *E. coli* with the recombinant plasmid.
Chapter 4a	The PCR with pARA-R (4a)	
Chapter 5a	Visualisation of products from restriction digestion and the PCR (5a)	
Chapter 6a	Transforming bacteria with the recombinant plasmid pARA-R (6a)	

Sequence G	Practical activities	Summary
Chapter 1	Using a micropipette (1.1) Gel electrophoresis (1.2)	Students can transform *E. coli* with a pre-made recombinant plasmid, then check its identity using PCR and agarose gel electrophoresis.
Chapter 6a	Transforming bacteria with the recombinant plasmid pARA-R (6a)	
Chapter 7a	Colony PCR from the transformed bacteria (7a.1) Visualisation of products from the colony PCR (7a.2)	

Sequence H is a shorter, stand-alone laboratory allowing students to undertake a DNA profiling activity. This sequence would typically be aimed at Y10 or Y11 students.

Sequence H	Practical activities	Summary
Chapter 1	Using a micropipette (1.1) Gel electrophoresis (1.2)	Students can use restriction digestion and agarose gel electrophoresis to create DNA profiles.
Chapter 9	Cutting DNA by restriction digestion (9.1) DNA separation by agarose gel electrophoresis (9.2)	

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.

Laboratory	Practical activity	Timing (minutes)
1.1 1.2	Using a micropipette Gel electrophoresis	≈ 15 practice ≈ 15 load gel ≈ 10 gel
2	Restriction digestion of plasmids	≈ 20 set up 60 incubation[*1]
3	Building the pARA-R plasmid	30 denaturation ≈ 10 set up
4	PCR of the recombinant plasmids formed by ligation	≈ 15 set up 90 PCR [*1]
5	Visualisation of products from restriction digestion, ligation and the PCR	≈ 30 set up ≈ 50 gel [*2] ≈ 5 transfer[*3] 30 stain [*3] ≈ 20 photographing [*3]
6	Transforming bacteria with the ligation products	≈ 50-60 36 hour incubation ≈ 20 photographing
7.1 7.2	Colony PCR from the transformed bacteria Visualisation of products from the colony PCR	≈ 20 set up 90 PCR ≈ 20 set up ≈ 45 gel ≈ 5 transfer [*3] 30 stain [*3] ≈ 20 photographing [*3]

[*1] during the incubation step of the restriction digestion, or the PCR thermal cycling, students can pour the agarose gels

[*2] while electrophoresis takes place students can progress to the bacterial transformation

[*3] to save time teachers or technicians may wish to perform these steps, although we'd recommend allowing the students to see their gels on the UV transilluminator as this has a 'wow' factor

Laboratory	Practical activity	Timing (minutes)
2a	Restriction digestion of pARA-R	≈ 15 set up 60 incubation [*1]
4a	The PCR with pARA-R	≈ 15 set up 90 PCR [*1]
5a	Visualisation of products from restriction digestion and the PCR	≈ 25 set up ≈ 50 gel [*2] ≈ 5 transfer [*3] 30 stain [*3] ≈ 20 photographing [*3]
6a	Transforming bacteria with the recombinant plasmid pARA-R	≈ 50-60 set up 36 hour incubation ≈ 20 photographing
7a.1	Colony PCR from the transformed bacteria	≈ 15 set up 90 PCR
7a.2	Visualisation of products from the colony PCR	≈ 20 set up ≈ 45 gel ≈ 5 transfer [*3] 30 stain [*3] ≈ 20 photographing [*3]
9.1	Cutting DNA by restriction digestion	≈ 25 set up 60 incubation [*2]
9.2	DNA separation by agarose gel electrophoresis	≈ 25 set up ≈ 60 gel ≈ 5 transfer [*3] 30 stain [*3] ≈ 20 photographing [*3]

[*1] during the incubation step of the restriction digestion, or the PCR thermal cycling, students can pour the agarose gels

[*2] while electrophoresis takes place students can progress to the bacterial transformation

[*3] to save time teachers or technicians may wish to perform these steps, although we'd recommend allowing the students to see their gels on the UV transilluminator as this has a 'wow' factor

These guide timings have also been split to provide ideas for the timings of the sequences of labs described in the previous section, which are given over the next pages.

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

Sequence B	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
Chapter 6	Transforming bacteria with the ligation products (6)	≈ 50-60 set up 36 hour incubation ≈ 20 photographing

Sequence C	Practical activities	Timings (minutes)
Chapter 1	Using a micropipette (1.1) Gel electrophoresis (1.2)	≈ 15 set up ≈ 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 4	PCR of the recombinant plasmids formed by ligation (4)	≈ 15 set up 90 PCR
Chapter 5	Visualisation of products from restriction digestion, ligation and the PCR (5)	≈ 30 set up ≈ 50 gel ≈ 5 transfer 30 stain ≈ 20 photographing
Chapter 6	Transforming bacteria with the ligation products (6)	≈ 50-60 set up 36 hour incubation ≈ 20 photographing

Sequence D	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
Chapter 6	Transforming bacteria with the ligation products (6)	≈ 50-60 set up 36 hour incubation ≈ 20 photographing
Chapter 7	Colony PCR from the transformed bacteria (7.1) Visualisation of products from the colony PCR (7.2)	≈ 20 set up 90 PCR ≈ 20 set up ≈ 45 gel ≈ 5 transfer 30 stain ≈ 20 photographing

Sequence E	Practical activities	Timings (minutes)
Chapter 1	Using a micropipette (1.1) Gel electrophoresis (1.2)	≈ 15 practice ≈ 15 load ≈ 10 gel
Chapter 2a	Restriction digestion of pARA-R (2a)	≈ 15 set up 60 incubation
Chapter 5a	Visualisation of products from restriction digestion and the PCR (5a)	≈ 25 set up ≈ 50 gel ≈ 5 transfer 30 stain ≈ 20 photographing
Chapter 6a	Transforming bacteria with the recombinant plasmid pARA-R (6a)	≈ 50-60 set up 36 hour incubation ≈ 20 photographing

Sequence F	Practical activities	Timings (minutes)
Chapter 1	Using a micropipette (1.1) Gel electrophoresis (1.2)	≈ 15 practice ≈ 15 load ≈ 10 gel
Chapter 4a	The PCR with pARA-R (4a)	≈ 15 set up 90 PCR
Chapter 5a	Visualisation of products from restriction digestion and the PCR (5a)	≈ 25 set up ≈ 50 gel ≈ 5 transfer 30 stain ≈ 20 photographing
Chapter 6a	Transforming bacteria with the recombinant plasmid pARA-R (6a)	≈ 50-60 set up 36 hour incubation ≈ 20 photographing

Sequence G	Practical activities	Timings (minutes)
Chapter 1	Using a micropipette (1.1) Gel electrophoresis (1.2)	≈ 15 practice ≈ 15 load ≈ 10 gel
Chapter 6a	Transforming bacteria with the recombinant plasmid pARA-R (6a)	≈ 50-60 set up 36 hour incubation ≈ 20 photographing
Chapter 7a	Colony PCR from the transformed bacteria (7a.1) Visualisation of products from the colony PCR (7a.2)	≈ 15 set up 90 PCR ≈ 20 set up ≈ 45 gel ≈ 5 transfer 30 stain ≈ 20 photographing

Sequence H	Practical activities	Timings (minutes)
Chapter 1	Using a micropipette (1.1) Gel electrophoresis (1.2)	≈ 15 practice ≈ 15 load ≈ 10 gel
Chapter 9	Cutting DNA by restriction digestion (9.1) DNA separation by agarose gel electrophoresis (9.2)	≈ 25 set up 60 incubation ≈ 25 set up ≈ 60 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 in the different laboratories is given in the table below.

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 $C_1 \quad V_1 = C_2 \quad V_2$

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

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

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

- 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

Agar plate preparation

Preparing agar plates for laboratory 6, requires about 20 ml of agar per petri dish (so 1 litre of agar will be enough for about 50 plates). For the transformation protocol 3 types of agar plate will be needed;

- LB (Luria Bertani) agar
- LB amp (Luria Bertani agar with ampicillin)
- LB amp ara (Luria Bertani agar with ampicillin and arabinose)

Preparing 12 LB plates

(1) Suspend 8.75 g of LB agar powder in 250 ml of distilled water inside a vessel suitable for autoclaving.

(2) Heat to boiling while stirring to dissolve all ingredients completely (a microwave is helpful for doing this, make sure there is a plug in the neck of the vessel – cotton wool or sponge – to prevent loss of liquid).

(3) Autoclave for 15 minutes at 121°C, with lids on, but loosened.

(4) Allow the agar to cool to about 55°C (the point when you can pick it up without a glove).

(5) Prepare an area for plate pouring (ideally it wants to be away from draughts, surfaces should be disinfected, and it is good practice to pour plates around the base of a lit Bunsen burner).

(6) Remove sterile petri dishes from plastic wrap (this bag should be saved for plate storage) and label the petri dish lids with single stripe by running the permanent marker pen down a pile.

(7) Pour a thin layer of LB agar into each plate, being careful not to lift the lid excessively (you should open up just enough to pour the agar in).

(8) Swirl the plate in a circular motion to distribute the agar evenly in the petri dish. If the agar was about 55°C when poured the lid should be able to go on again now, without excessive condensation forming. If condensation becomes an issue, rest the lid on the side of the petri dish, so that about 2/3 of the plate is covered, but so there is a gap for condensation to escape.

(9) Allow the plates to cool (becoming opaque and solid). This takes 20-30 minutes.

(10) With the lids back on invert the plates, so any condensation doesn't drip onto the agar.

(11) Store plates in the fridge (4°C) until needed.

Preparing 12 LB amp plates

Follow the above procedure, with these additions;

(4a) Add 250 μl of ampicillin stock (50 mg/ml) to the agar once it is cooled to below 55°C. Swirl the vessel to mix in the ampicillin, but do not shake violently as this will introduce air bubbles, which do persist in the petri dishes and make it hard for students to spread their bacterial cells.

(6a) Label the petri dishes with two stripes (by running 2 pens down the side of a stack of plates).

(11a) LB agar plates with ampicillin can be stored in the fridge at 4°C for up to 2 weeks before use without the ampicillin losing activity.

Preparing 12 LB amp ara plates

Follow the above procedure, with these additions;

(4a) Add 250 μl of ampicillin stock solution (50 mg/ml) **and** 2.5 ml of arabinose stock solution (500 mg/ml) to the agar once it is cooled to below 55°C. Swirl the vessel to mix in the ampicillin and arabinose, but do not shake violently as this will introduce air bubbles, which do persist in the petri dishes and make it hard for students to spread their bacterial cells.

(6a) Label the petri dishes with three stripes (by running 3 pens down the side of a stack of plates).

(11a) LB agar plates with ampiciliin and arabinose can be stored in the fridge at 4°C for up to 2 weeks before use without the ampicillin losing activity.

Storage

Once poured, plates can be stored at 4°C (in the fridge) until an hour or so before they are needed to prevent growth of any microorganisms, when they should be removed and allowed to return towards room temperature before use. Alternatively they can be stored at room temperature.

Photodocumentation

The GelRed-DNA complex will fluoresce on the UV transilluminator so you can see the results before photographing. When you wish to take a photo, place the camera hood over the gel on the UV transilluminator. The easiest way to photograph the gel is:

- Turn on the transilluminator.
- Turn the camera on using the button on top of the camera.
- Select the 'C' setting on the top dial of the camera (this is the **c**ustom set up for use with gels at this short distance from the lens). Once set to 'C' it requires no further adjustment.
- Focus the image by depressing the shutter button on top of the camera halfway. Then depress the shutter button all the way to expose the image.
- To view your images on the camera screen, press the button on the back, which has a blue rectangle around a blue triangle (top left button on the back of the camera).

To transfer and save your images to a computer, remove the card from the camera and place it in the card reader or connect the camera directly to your computer using the USB lead. PLEASE DO NOT ERASE THE IMAGES FROM THE SD CARD FOR THE CAMERA! We check the images to make sure that your labs are working correctly.

Using the PCR machine

Before initiating the thermal cycler:

- Mark the PCR tubes on the 'shoulder', as marker can transfer from the PCR tube lid to the heated lid of the thermal cycler.
- Keep the PCR tubes on ice until the PCR machine is ready to start to maintain the activity of DNA polymerase.

Starting the thermal cycler:

- Make sure the power cord is attached and the machine is switched on.
- Press the 'Standby' button on the front panel to start the thermal cycler.
  The thermal cycler will complete a self-test.
- Press the F1 key to access the 'protocol library'. You need to access the programme called '**pARA**' if you are using the PCR to amplify a region inserted in the pARA plasmid (labs 4, 4a, 7 or 7a) – ie. The rfp gene. If you need to enter a username it is '**Amgen**', and the password is the numbers corresponding to spelling AMGEN on the keypad.
- Select 'run protocol' and press 'enter'.
  The screen will change to 'run setup '.
- Place the PCR tubes in the heating block. Close and lock the lid.
- Press 'F5' to 'begin run'.

The thermal cycling programme

- On the Biorad MJ mini thermal cycler it takes about 2 hours to run this programme.
- The PCR programme for amplifying the region between the *Bam*HI and *Hin*dIII restriction sites in the pARA expression plasmid (the rfp gene) is shown below – Labs 4(4a) and 7 (7a)

Reagent		**Temperature (°C)**	**Time (sec)**
Initial denaturation		95	200
30 cycles of	Denaturation	95	30
	Annealing	53	30
	Extension	68	60
Final extension		68	300
Hold		4	Indefinite

- On the Biorad MJ mini thermal cycler it takes about 1 ½ hours to run this programme.
- When they are complete both programmes hold the tubes at 4°C indefinitely, so samples can be left in the PCR machine overnight, without ill effects, if needed.

Stopping the thermal cycling programme:

- When you remove the PCR tubes, press 'F4' to return to the 'Home screen'.
- Then turn off the thermal cycler by holding down the 'standby' key for 2 or 3 seconds.

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) 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 I10. 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

Copies of a Micropipetting practice sheet are provided in the back of this Teacher & Technician Guide . Simply photocopy (and if desired laminate). If you wish to prepare students for micropipetting you can ask them to view an excellent video prepared by the University of Leicester or use resources available on LabXchange. Any of these can also be used directly in your lab.

Resource	Page
Micropipetting practice sheet	R.2
University of Leicester – video - https://youtu.be/uEy_NGDfo_8?si=DpLVkVDhumg8O2eH	

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).

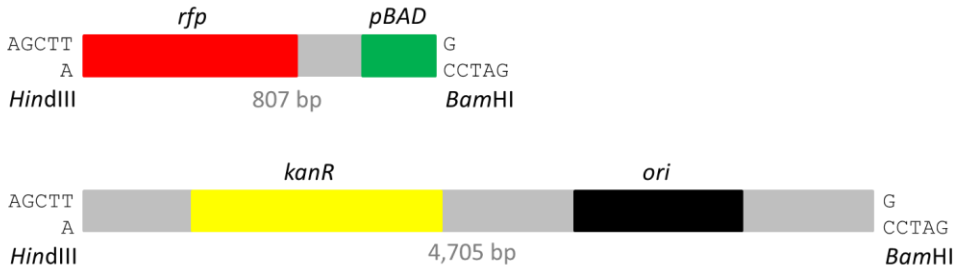
2. Draw the linear fragments, marking on the genes and important sequences. Using table 2.2 below, include the nucleotide sequence of the sticky ends.

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:

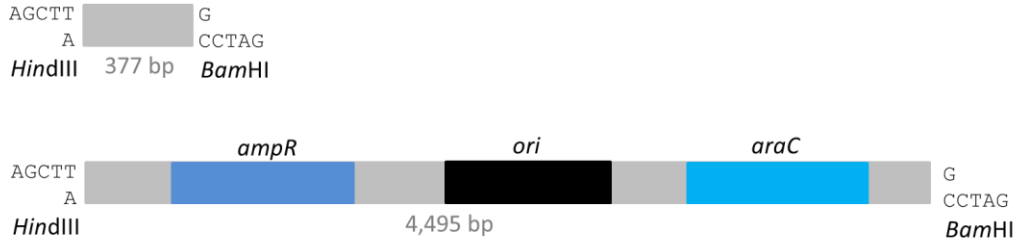


Figure 2.1 The linear restriction fragments.

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.4
What is genetic engineering? Treating disease with gene cloning	R.16
Red fluorescent protein in sea anemones: why glow?	R.21
Using plasmids and restriction enzymes in genetic engineering	R.22
Why use bacterial promoters in plasmids?	R.23
The components of the pARA-R plasmid	R.24
Restriction enzymes	R.25

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	dH_2O	20	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.

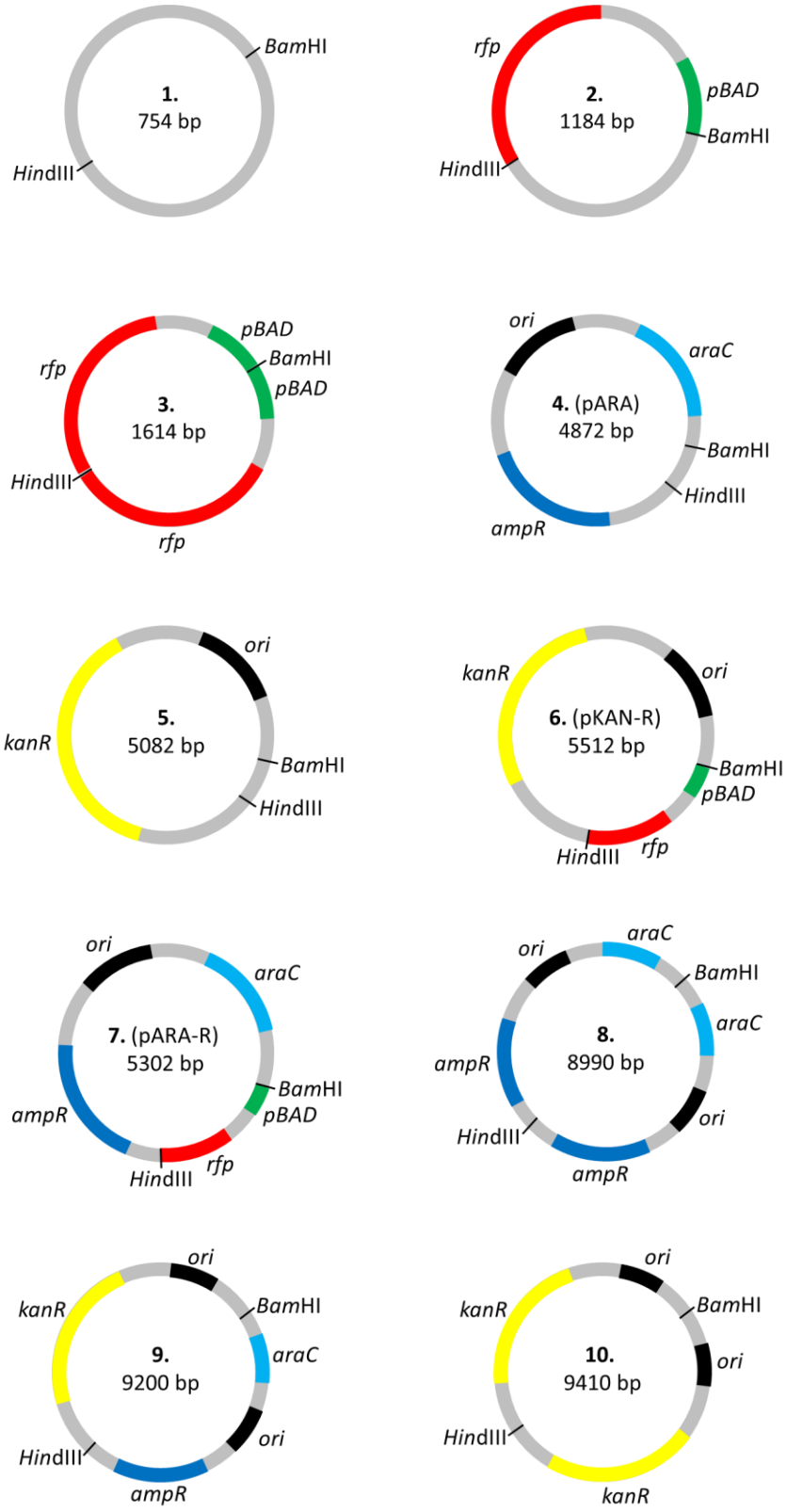


Figure 3.1 The 10 possible plasmids made by recombination.

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.25
DNA ligases in nature and genetic engineering	R.26

Chapter 4: Verifying pARA-R is present

Overview

In this chapter students explore one of the many uses of the polymerase chain reaction (PCR), learning about the enzyme DNA polymerase, sequence specific primers and thermal cycling. PCR is used to amplify the DNA fragments ligated to the larger restriction digest fragment from the expression plasmid pARA, to find out whether pARA-R has been created.

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 4.1 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
4: PCR of the recombinant plasmids formed by ligation	Aliquot solutions: • Combined forward and reverse primers (PRI) • pARA plasmid (A) • nuclease-free water (H_2O) • 2 PCR tubes of 2 x PCR master mix (MM) Make sure that there will be plenty of ice available.	Prepare benches with: • P-20 micropipette • Pipette tips • Microfuge tube rack • Microfuge tubes of solutions • Polystyrene cup of ice containing PCR tubes of master mix • Permanent marker • Waste container • Microfuge *C • PCR machine *C Retrieve and distribute the ligation reactions (LIG) from lab 3.	Remove students' PCR tubes when thermal cycling is complete and store in fridge (4°C) until required for electrophoresis

Table 4.1 Preparation for laboratory 4.

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 4.2 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 x PCR master mix	MM	12.5 + 12.5	12.5 + 12.5
Nuclease-free water	H_2O	17	14
Combined primers	PRI	5	4
pARA plasmid	A	3	2

Table 4.2 Aliquotting reagents for laboratory 4.

The nuclease-free water can be kept at room temperature. Combined primers and pARA plasmid should be stored in the freezer at –20°C, defrosted to room temperature for aliquotting and then returned to the freezer. The 2 x PCR master mix should be bought out of the freezer in an isofreeze box for aliquotting **into the PCR tubes** and returned to the freezer as soon as possible to avoid loss of DNA polymerase 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 4.3.

Substance / procedure	Hazard	Precautions
pARA plasmid and ligation reaction	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.
Primers	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.
PCR master mix	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 4.3 Health and Safety information for laboratory 4.

Learning objectives

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

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

During practical activities students can consider the relationship between genes and DNA, specificity of

complementary base pairing, function of enzymes and role of DNA polymerase in the polymerase chain reaction.

Prior knowledge and skills

It is helpful if students already know:

- DNA is a double-stranded molecule, with a sugar-phosphate backbone and 4 nitrogenous bases (cytosine, guanine, adenine, and thymine) that form complementary base pairs.
- That cytosine only base pairs with guanine, and that adenine only base pairs with thymine.
- The order of the nitrogenous bases is known as the DNA sequence.
- That enzymes catalyse reactions in a cell and have roles in DNA replication.

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. Based on your understanding of DNA replication, explain the role of DNA polymerase in replication.
   During DNA replication the two, parent DNA strands separate at origin of replication, or ori sites. The enzyme DNA polymerase adds new nucleotides complementary to the single-stranded parent DNA to form base pairs held together by hydrogen bonds. On one parent DNA strand the new, complementary daughter strand forms continuously in a 5' to 3' direction. On the other parent strand of DNA the new, complementary nucleotides are added discontinuously in short fragments extended in a 5' to 3' direction.
   During the PCR, DNA polymerase catalyses the extension of the short double-stranded DNA molecules (created by primer binding to the genomic DNA) continuously, in a 5' to 3' direction.
2. Why do you keep the PCR master mix, which contains DNA polymerase, on ice until the PCR is begun?
   The PCR master mix contains the enzyme DNA polymerase. At room temperature the DNA polymerase starts to lose activity, so keeping the PCR master mix on ice helps to ensure that the enzyme retains activity until the PCR is started.
3. Why does the *Taq* DNA polymerase not denature during the denaturation step at 95°C in your PCR programme?
   DNA polymerase from Thermus aquaticus (Taq), a thermophilic bacteria adapted to survive the high temperatures found in deep sea hydrothermal vents is used, as it is evolved to operate in high temperatures without denaturing.
4. Each DNA molecule is replicated during each cycle of the PCR programme. There are 30 cycles on the PCR programme that you use. If you started with 6 DNA molecules, how many molecules would there be at the end of the PCR?
   $6 \times 2^{30} = 6{,}442{,}450{,}944$
   That is roughly 6 ½ billion DNA molecules.

5. What is the purpose of the pARA PCR, given that the expected size of the PCR product is known to be 662 bp long?

   The pARA PCR provides a control to check that the reagents are active, the correct primers have been used and the PCR thermal cycling is working correctly.

6. What type of chemical bonds will form when the single stranded primer DNA base pairs with the complementary sequence on the recombinant plasmid?

   Hydrogen bonds form between the complementary nucleotides of the single stranded primer DNA and the recombinant plasmid. (Covalent bonds form between the sugar-phosphate backbone of the DNA molecule.)

7. The primers bind to specific DNA sequences. On the larger pARA restriction fragment one primer binding site is 136 bp from the *Bam*HI restriction site and the other is 149 bp from the *Hin*dIII restriction site. Can you suggest 3 sizes of PCR product that you may see?

   There are 4 possible recombinant plasmids created when ligating the DNA molecules from restriction digestion to the large pARA restriction fragment, to form a 2 DNA molecule recombinant plasmid. These possibilities and the size of PCR product that would result are given in table 4.5 below.

Possible fragment inserted	Size of insert (bp)	Size of PCR product (bp)
Small pARA restriction fragment	*377*	*377 + 136 + 149 = 662*
Small pKAN-R restriction fragment	*807*	*807 + 136 + 149 = 1092*
Large pARA restriction fragment	*4,495*	*4,495 + 136 + 149 = 4,780*
Large pKAN-R restriction fragment	*4,705*	*4,705 + 136 + 149 = 4,990*

Table 4.5 The fragments that could become ligated to the large pARA restriction fragment and the sizes that would result if these constructs were amplified by PCR.

In fact the length of the extension step in the PCR thermal cycling programme means that the two small fragments are most likely to be detected, so you are likely to (1) demonstrate the presence of re-ligated pARA plasmid (662 bp), and / or (2) detect the presence of the desired pARA-R plasmid (1092 bp).

Additional resources

There are additional resources providing internet links to PCR videos and a possible 'articulate' exercise to encourage peer-learning of vocabulary. 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
Video resources for PCR	R.29
Articulate for PCR	R.30

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 to (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 I10. 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

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.

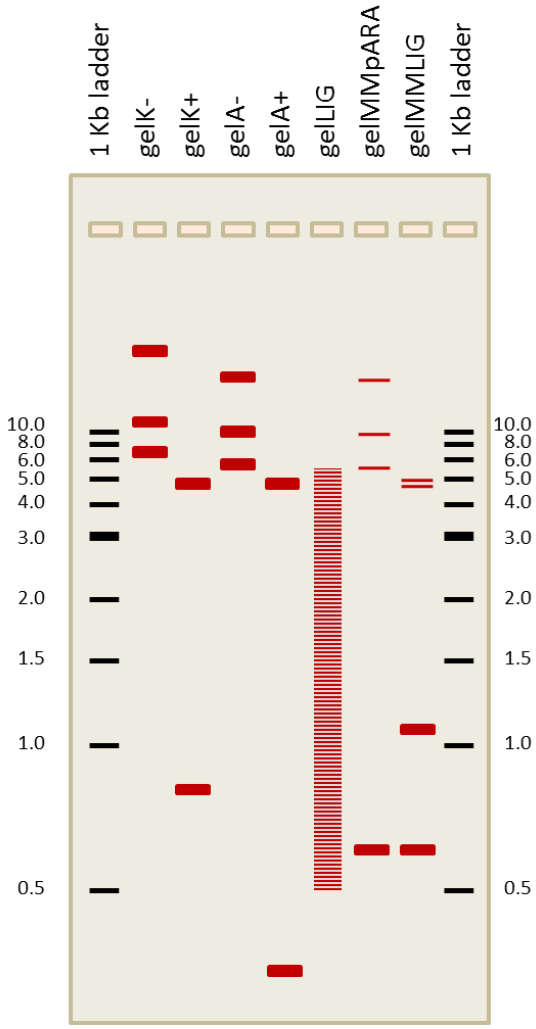


Figure 5.1 The DNA ladder diagram completed with the expected sizes of DNA bands.

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 in the Teacher & Technician Guide. However, an excellent simulation can be found on LabXchange (www.labxchange.org) ; free to register and use.

Chapter 6: Inserting recombinant plasmids into bacteria

Overview

Chapter 6 allows students to introduce their recombinant plasmid, made in the previous labs, into *E. coli*, using the process of transformation. Using antibiotic resistant genes as selectable markers, bacteria that have been successfully transformed are identified. Successfully transformed bacteria that have been supplied with the activator arabinose will turn red and when placed on onto the UV transilluminator will fluoresce red.

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 6.1 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
6: Transforming bacteria with the ligation products	Aliquot solution: • Luria Bertani broth (LB) Prepare petri dishes of LB, LB/amp and LB/amp/ara.	Prepare benches with: • P-20 micropipette • P-200 micropipette • Pipette tips • Microfuge tube rack • Microfuge tube of LB broth • 1.5 mL microfuge tubes • Permanent marker • Waste container • 42°C water bath *C • Stopwatch • Sticking tape • Pack of cell spreaders *2 • 37 °C incubator *C	If the class is not able to view the plates after 24- 36 hours incubation, remove, seal with parafilm and store in the fridge (4 °C) until the class is ready to observe.

Table 6.1 Preparation for laboratory 6 (continued on next page).

Laboratory	Advance preparation	Prior preparation	Post-lab support
6: Transforming bacteria with the ligation products		Retrieve and distribute the students' ligation reactions from lab 3. Provide autoclave bag for disposal of contaminated waste *C Defrost competent cells in wet ice (for about 10 – 15 minutes), resuspend and aliquot just prior to use. Supply to students in a polystyrene cup of wet ice.	
6: Transforming bacteria with the ligation products		Within the classroom place: • UV transilluminator *C Retrieve and distribute students' transformation plates.	Autoclave bacterial plates prior to disposal. Possibly printing gel pictures or transferring to computer(s).

Table 6.1 Preparation for laboratory 6 (continued from previous page).

The process for preparing the agar plates (LB, LB/amp and LB/amp/ara) is covered in the overview of preparation section on page I14.

During transformation the temperature of the water bath must be correct at 42 °C: small fluctuations will affect the transformation efficiency, so set the waterbath temperature well in advance and check carefully before use.

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 6.2 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)
LB broth	LB	320	300
Competent cells	CC	100	100

Table 6.2 Aliquotting reagents for laboratory 6.

LB broth should be prepared in advance and stored in the fridge (4 °C).

It is ***very important to keep the competent cells cold*** before the heat shock procedure. They should be defrosted in wet ice, resuspended and aliquotted just before use. Tubes should be held by the rim to avoid warming the cells with fingers, kept and supplied to students in wet ice.

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 6.3.

Substance / procedure	Hazard	Precautions
Recombinant 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.
E. coli competent cells	Bacteria: low risk of infection.	Use in a controlled way, applying aseptic techniques. CLEAPSS does **not** recommend gloves or eye protection as a matter of course in any Microbiology. Autoclave materials that come into contact with bacteria before disposal.
Growth of microorganisms on agar plates	Opening agar plates with unknown microorganisms	Tape lids onto agar plates in 3, evenly spaced places around the circumference of the petri dish. Keep taped during incubation and viewing.

Table 6.3 Health and Safety information for laboratory 6.

Learning objectives

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

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

The central dogma of Biology about how DNA encodes protein, which produce traits is illustrated in this laboratory. Learning objectives associated with the practical work can also relate to the process of gene expression, techniques required in microbiology, bacterial cell structure and genetic engineering.

Prior knowledge and skills

It is helpful if students already know:

- The process of aseptic technique, used working with microorganisms.
- Prokaryotic cell structure.
- 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.
- DNA is a double-stranded molecule, and each strand of DNA is made up of covalently linked subunits called *nucleotides*, which are abbreviated according the nitrogenous base they contain (C, G, A, T).

- *Transcription* is the process by which information encoded in DNA is transferred to messenger RNA, a single-stranded ribonucleic acid.
- *Translation* is the process by which information encoded in messenger RNA is decoded and transformed into protein.
- Some information about gene expression, particularly regarding promoters and operators.

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 are the steps involved in transcription and translation of a gene?

   *During **transcription**, DNA is copied into messenger RNA. During **translation**, the messenger RNA is decoded at a cell organelle called the ribosome. Each codon (a group of three bases) in the sequence corresponds to one amino acid, the building blocks of proteins. Transfer RNA molecules match up the codons and the amino acids, and the enzyme peptidyl transferase catalyses the bonding of the amino acids to form a polypeptide chain, which folds to form the protein.*

2. What is the relationship among genes, proteins, and traits (or observable characteristics)?

   A gene contains the code for making a protein. Protein molecules make and run the cell, so they are responsible for traits. Often a trait is the outcome of multiple protein actions and interactions.

3. What do bacteria and humans have in common that makes it possible for a human gene to be expressed in bacteria?

   The DNA structure and code are the same in bacteria and humans. The cell machinery that carries out transcription and translation is the same in bacteria and humans.

4. Why is it important that the membranes of *E. coli* bacteria carefully regulate which substances can enter and exit the cell?

   The cell regulates the substances that can enter and exit because some substances might affect it adversely.

5. How are the P+ bacteria treated differently from the P– bacteria? What is the purpose of the P– bacteria?

   The P+ bacteria culture is mixed with the recombinant plasmids, while the P– bacteria culture is not mixed with the recombinant plasmids. The purpose of the P– bacteria culture is to make sure that the cells and growing conditions work as expected. The P– bacteria culture should grow well in Luria Broth, showing that the bacteria were viable, and should die when exposed to the antibiotic ampicillin, allowing successfully transformed bacteria to be distinguished by their selectable marker: ampicillin resistance.

6. Why is it important to use aseptic technique in this lab?

   Aseptic technique is important for 2 reasons; (i) stopping cultures of bacteria from becoming contaminated, and (ii) stopping cultures of bacteria from contaminating us.

7. Why do the cells need time to recover after the heat shock?

   The sudden heating associated with being transferred from ice to having a heat shock stresses the bacteria. They need time to recover; repairing their membranes and returning to their usual state before being manipulated further.

8. Why are the cells incubated at 37°C?

   E. coli bacteria are adapted to growing inside the human body, which maintains a temperature of 37°C by homeostasis.

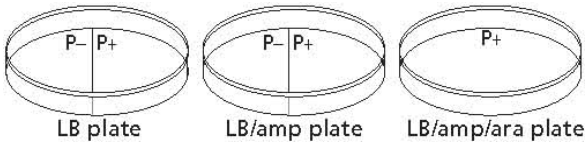
9. Ampicillin is an antibiotic that kills bacterial cells by disrupting the formation of cell walls. However, the pARA-R plasmid has the ampicillin resistance gene, which produces a protein that breaks down ampicillin. What is the purpose of growing bacteria that have been transformed in the presence of ampicillin?

   Only cells that have a plasmid containing the gene for ampicillin resistance will be able to grow in the presence of ampicillin. You can select the cells that have been transformed with the recombinant plasmid including the ampicillin resistance gene, which may number very few.

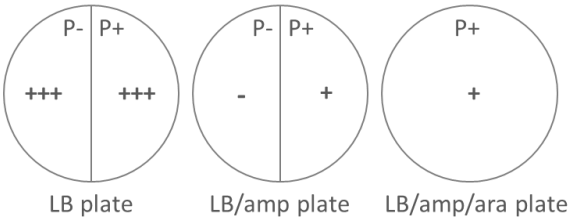
10. What will happen when bacterial cells that contain the pARA-R plasmid are not given arabinose?

    The rfp gene, under the control of the pBAD promoter, cannot be expressed unless the cell is given arabinose. When arabinose is <u>not present</u> the AraC activator protein binds just upstream of the pBAD promoter, preventing the RNA polymerase from transcribing the arabinose operon (which comprises the three genes araB, araA and araD) or other downstream sequences. As a result these genes are not expressed. When arabinose <u>is present</u> it alters the conformation of the AraC protein, so that it guides the RNA polymerase onto the pBAD promoter, to begin transcription of downstream genes. In the recombinant plasmid the rfp gene is downstream of the pBAD promoter, so can only be transcribed and translated to produce an active protein when arabinose is present.

11. You add samples of the control group P– and the treatment group P+ to plates that contain various combinations of Luria Bertani agar (LB), ampicillin, and the sugar arabinose. What are your predictions for the growth you expect on each of the plates?



The predictions for growth expected (using +++ to indicate prolific growth, + to indicate some growth and – to indicate no growth) are:



Some students may struggle to predict these results and breaking down the expectations for the control bacteria (P-) and transformed bacteria (P+) may help.

Expected results for control bacteria (from P- tube):

Agar plate	Predicted growth	Conclusion: if prediction correct	Conclusion: if prediction not correct
LB	*High / +++*	*The bacterial competent cells are viable and able to survive the transformation procedure.*	*No growth signals either (i) the bacterial competent cells are not viable, or (ii) the bacteria are unable to survive the transformation procedure.*
LB/amp	*None / -*	*Without plasmids containing the ampicillin resistance gene, untransformed bacteria die.*	*If there is growth it signals that the ampicillin is not active in the agar plates.*

Expected results for transformed bacteria (from P+ tube):

Agar plate	Predicted growth	Conclusion: if prediction correct	Conclusion: if prediction not correct
LB	*High / +++*	*The bacterial competent cells are viable and able to survive the transformation procedure.*	*No growth signals either (i) the bacterial competent cells are not viable, or (ii) the bacteria are unable to survive the transformation procedure.*
LB/amp	*Low / +* *white*	*Some transformed bacteria have plasmids with the ampicillin resistance gene.*	*No growth signals that the transformation was not successful and there are no bacteria with plasmids containing the ampicillin resistance gene.*
LB/amp/ara	*Low / +* *white and red*	*Some transformed bacteria have plasmids with the ampicillin resistance gene. You may also see expression of the rfp gene in some bacteria.*	*No growth signals that the transformation was not successful and there are no bacteria with plasmids containing the ampicillin resistance gene. No red colonies could results from (i) a problem with the arabinose, or (ii) unsuccessful transformation with the pARA-R recombinant plasmid (see question 13).*

12. Look at the results of your transformation. Do your actual results match your predicted results? If not, what differences do you see, and what are some explanations for these differences?

Answers will vary. If unexpected results occur, help students think through what might have happened. There are many opportunities during the transformation procedure for errors to occur that would affect the results.

13. How many red colonies were present on your LB/amp/ara plate?

Answers will vary. All groups should get a few colonies expressing red fluorescent protein, unless an error was made in carrying out the procedures. If a group fails to get any red colonies, but they do have colonies on the LB/amp/ara plate, discuss with the whole class what might have happened. Students should realise that it is possible that a plasmid was taken up by the bacteria that had the ampR gene but that was not the pARA-R plasmid containing the rfp gene.

14. Why did the red colonies only appear on the LB/amp/ara plate and not the LB/amp plate?

The rfp gene cannot be expressed unless the cell is given arabinose, as the arabinose activator protein will only alter in conformation to allow transcription from the pBAD promoter of the downstream rfp gene in the presence of arabinose.

15. Recombinant plasmids are engineered so that they can replicate in the cell independently of the chromosome replication. Why is it important to have multiple copies of a recombinant plasmid within a cell?

In genetic engineering the goal is to produce a lot of product, such as insulin. More copies of the plasmid and its gene will result in the production of more product within the cell.

16. How is the information encoded in the *rfp* gene expressed as a trait? Be sure to use what you have previously learned about gene expression and the relationship between DNA, RNA, protein, and traits.

    The rfp gene in the plasmid is made up of DNA, and the gene is copied into messenger RNA. This process is called transcription. The messenger RNA moves to the ribosome where transfer RNA molecules match up codons and amino acids, with the enzyme peptidyl transferase catalysing the bonding of the amino acids to form a polypeptide chain. This process is called translation. The polypeptide chain folds to form a protein. The protein can contribute to a trait, and in this case the red fluorescent protein created makes the cells fluoresce red.

17. Why is it possible for bacteria to make a human protein, such as insulin, or a sea anemone protein, such as the red fluorescent protein?

    The DNA structure and code, and the cell machinery that carries out transcription and translation, are the same across these organisms.

18. The only bacteria that could produce the red fluorescent protein in Laboratory 6 were bacteria that were transformed with the pARA-R plasmid. Why?

    Only bacteria that were transformed with a plasmid containing the rfp gene, the ampR gene, and the AraC activator (which activates the promoter for the rfp gene in the presence of arabinose), can grow on the *LB/amp/ara plate and produce the red fluorescent protein. Of the possible plasmids that could be made in the ligation procedure, the pARA-R plasmid is the only plasmid that has all of these sequences. You may want to review the possible plasmids with students (shown on page 3.4).*

Additional resources

There are additional resources to expand upon the concepts and practice of genetic engineering, therapeutic proteins, diabetes and why sea anemones fluoresce. 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 central dogma and reverse transcriptase	R.27
More on the AraC activator protein in '*The components of the pARA-R plasmid*'	R.24
Aseptic technique	Student guide A.1

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

Overview

In this chapter students explore one of the many uses of the polymerase chain reaction (PCR), learning about the enzyme DNA polymerase, sequence specific primers and thermal cycling. A colony PCR, using bacteria grown during lab 6 to provide the DNA template, is employed to discover the size of the restriction fragments ligated to the larger pARA restriction fragment in the plasmids of red colonies and white colonies. This neatly links DNA fragment size (genotype) with colony colour (phenotype).

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 7.1 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
7.1: Colony PCR from the transformed bacteria	Aliquot solutions: • Combined forward and reverse primers (PRI) • nuclease-free water (H_2O) • 2 PCR tubes of 2 x PCR master mix (MM) Make sure that there will be plenty of ice available. Set up 3 control PCRs: • pARA-R template *C • pARA template *C • negative control *C	Prepare benches with: • P-20 micropipette • Pipette tips • Microfuge tube rack • Microfuge tubes of solutions • Polystyrene cup of ice containing PCR tubes of master mix • Permanent marker • Waste container • Microfuge *C • PCR machine *C Retrieve and distribute the agar plates with transformed bacteria from lab 6.	Remove students' PCR tubes when thermal cycling is complete and store in fridge (4°C) until required for electrophoresis

Table 7.1 Preparation for laboratory 7 (continued on next page).

Laboratory	Advance preparation	Prior preparation	Post-lab support
7.2: Visualisation of products from the colony PCR Electrophoresis	Aliquot solutions: • Loading dye (LD) • DNA ladder (1 kb) Prepare control PCRs for loading onto the agarose gel *C. Dilute TAE buffer to (1 x TAE) Pour 0.8% agarose gels	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 an 0.8% agarose gel*2 • Powerpack*2 Retrieve & distribute from lab 7.1: • White colony PCR (MMwhite) • Red colony PCR (MMred) Retrieve the 3 control PCRs from lab 7.1: • pARA-R template *C • pARA template *C • negative control *C Reuse the tubes of distilled water.	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 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

Table 7.1 Preparation for laboratory 7 (continued from previous and on next page).

Laboratory	Advance preparation	Prior preparation	Post-lab support
7.2: Visualisation of products from the colony PCR Staining and photodocumentation	Dilute GelRed solution	Prepare benches with: • GelRed solution • Plastic gel staining tray *2 • Permanent marker • Tape Within the classroom place: • Chemical resistant gloves • Foil-covered bottle and funnel (if reusing GelRed solution) • UV transilluminator*C • Hood *C • Camera *C	Possibly printing gel pictures or transferring to computer(s).

Table 7.1 Preparation for laboratory 7 (continued from previous pages).

Setting up 3 control PCRs

It is good practice to perform control PCRs with: (i) DNA template known to contain the *rfp* gene (pARA-R) – this confirms that the PCR is working and the expected DNA size (1092 bp); (ii) DNA template known not to contain the *rfp* gene (pARA) – this confirms that the PCR is working and the expected DNA size (662 bp), and (iii) no DNA template (neg) – this is a negative control, used to confirm that the reagents are not contaminated with DNA.

Rather than get each pair to set up these additional reactions, you will set up these 3 PCRs, run them in the PCR machine with the students' reactions, then allow students to use your PCR samples for agarose gel electrophoresis. Set up the 3 reactions as shown in table 7.2.

Reagent	MMpARA-R	MMpARA PCR	MMneg PCR
2 X PCR master mix (MM)	12.5 µL	12.5 µL	12.5 µL
Nuclease-free water (H_2O)	8.5 µL	8.5 µL	10.5 µL
Combined primers (PRI)	2.0 µL	2.0 µL	2.0 µL
pARA-R (R)	2.0 µL		
pARA (A)		2.0 µL	

Table 7.2 Reagents to add to the control PCRs.

Preparing the control PCRs for agarose gel electrophoresis

In a fresh 1.5 mL microfuge tube, prepare the control PCRs for agarose gel electrophoresis. The preparation required is shown in table 7.3. You can aliquot this for the students if you wish (they will load 10 µl per gel), or get them to pass around the prepared samples whilst loading their gels.

Reagent	gelMMpARA-R	gelmmpARA	gelMMneg
Distilled water (dH_2O)	60.0 µL	60.0 µL	60.0 µL
Loading dye (LD)	20.0 µL	20.0 µL	20.0 µL
The PCR with pARA-R template (MMpARA-R)	20.0 µL		
The PCR with pARA template (MMpARA)		20.0 µL	
The negative PCR (MMNEG)			20.0 µL

Table 7.3 Preparation of control PCR samples for gel electrophoresis

The processes for diluting TAE buffer and preparing agarose gels are covered in the overview of preparation section on page I14/15. If you pour an 8-well, 0.8% agarose gel, then two groups will each be able to load their pair of samples (gelwhite and gelred), one set of the control PCR samples (from table 7.3) and one lane of 1 kb ladder (shown in the student guide, figure 7.2). If you pour a 15-well, 0.8% agarose gel, then four groups will each be able to load their pair of samples (gelwhite and gelred), alongside one set of the control PCR samples and four lanes of 1 kb ladder.

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 7.4 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 x PCR master mix	MM	12.5 + 12.5	12.5 + 12.5
Nuclease-free water	H_2O	22	19
Combined primers	PRI	5	4
5 x loading dye	LD	6	4
1 kb DNA ladder	1kb	12	10

Table 7.4 Aliquotting reagents for laboratory 7.

The nuclease-free water and loading dye should be kept at room temperature. Combined primers and DNA ladder should be stored in the freezer at –20°C, defrosted to room temperature for aliquotting and then returned to the freezer. The 2 x PCR master mix should be bought out of the freezer in an isofreeze box for aliquotting **into the PCR tubes** and returned to the freezer as soon as possible to avoid loss of DNA polymerase 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 7.5.

Substance / procedure	Hazard	Precautions
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.
Primers	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.
PCR master mix	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.
Micro-organisms on agar plates	Opening agar plates with unknown microorganisms. It is usually recommended by CLEAPSS that agar plates with cultured bacteria are not opened. However, if these agar plates only contain the expected red and white *E. coli* colonies then using these bacteria in a colony PCR is judged an acceptably low risk activity for sixth form students.	Direct students to work where lower school students will not observe and be tempted to copy this procedure. Use in a controlled way, applying aseptic techniques whilst touching the pipette tip to the bacterial colony and transferring it into the PCR tube. CLEAPSS does **not** recommend gloves or eye protection as a matter of course in any Microbiology. Autoclave materials that come into contact with bacteria 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.

Table 7.5 Health and Safety information for laboratory 7 (continued on next page).

Substance / procedure	Hazard	Precautions
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.
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 be closed, making a magnetic connection before the UV light will come on.

Table 7.5 Health and Safety information for laboratory 7 (continued from previous page).

Learning objectives

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

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

The primary objective for this laboratory is facilitating understanding of the link between genotype and phenotype and how genes encode proteins that cause traits or characteristics in organisms; a concept many students struggle with. Additionally, students can consider the relationship between genes and DNA, specificity of complementary base pairing, function of enzymes and role of DNA polymerase in the polymerase chain reaction.

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
- DNA is a double-stranded molecule, with a sugar-phosphate backbone and 4 nitrogenous bases (cytosine, guanine, adenine, and thymine) that form complementary base pairs.
- That cytosine only base pairs with guanine, and that adenine only base pairs with thymine.
- The order of the nitrogenous bases is known as the DNA sequence.
- That enzymes catalyse reactions in a cell and have roles in DNA replication.
- 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 7.1: Colony PCR from the transformed bacteria

1. What is the structure of a prokaryotic cell? Do prokaryotes have cell walls? What about cell membranes and nuclear membranes?

   Prokaryotes are small, unicellular organisms. The majority of prokaryotes do have a (peptidoglycan) cell wall outside of their cell membrane, but have no membrane-bound organelles, such as a nucleus or mitochondria.

2. Why does DNA need to be extracted from animal cells for use in PCR (laboratory 8), but not from bacterial cells?

   There are two reasons why the DNA needs to be extracted from animal cells, but not bacterial cells: cell structure and nucleases. Firstly, the cell structure is different, and whilst the thermal cycling of the PCR to 95 °C is sufficient to lyse the bacteria, releasing the DNA, it is not sufficient to lyse the eukaryotic cells and release the DNA from inside the membrane-bound nucleus. Secondly, the nucleases released from the cytoplasm of bacteria within the PCR are sequence specific and will not cut the bacterial DNA, whilst the nucleases released from the cells in a mouth swab that probably contains bacteria, food matter and cheek cells will probably cut the desired human DNA and may interfere with the PCR.

3. What is aseptic technique? Why do you need to use it when performing colony PCR? What constitutes good aseptic technique?

   Aseptic technique is the name of procedures that are used to stop pure bacterial cultures from becoming contaminated. Aseptic technique is important when performing colony PCR for 2 reasons; (i) stopping the bacterial colonies from becoming contaminated with other micro-organisms, and (ii) stopping the bacterial colonies from contaminating us. There is a section on aspetic technique in the student guide (page A.1). Top tips for aseptic technique include;

   - *Organise your work area, to avoid spillages and confusion*
   - *Use your Bunsen burner flame to sterilise glass equipment and create an updraft around the base that leaves a 'sterile zone' (as hot air rises taking airborne contaminants with it).*

- *Keep equipment sterile: close tip boxes when not in use and when removing plasticware from packaging try to touch only the items you intend to use to keep the remaining items from contamination.*
- *Disinfect equipment that has come into contact with bacteria and autoclave prior to disposal*

4. Why does the *Taq* DNA polymerase not denature during the denaturation step at 95°C in your PCR programme?

   DNA polymerase from Thermus aquaticus (Taq), a thermophilic bacteria adapted to survive the high temperatures found in deep sea hydrothermal vents is used, as it is evolved to operate in high temperatures without denaturing.

5. What plasmid constructs could be present in the transformed bacteria that produce white colonies on the LB/amp/ara plates? Can you suggest 3 possible constructs?

   The plasmid construct must contain the ampicillin resistance gene, in order to be able to survive on a plate containing ampicillin. The plasmid should not contain the rfp gene, or else it would produce a red colony. If you consider the 10 possible plasmids made by recombination, shown in figure 3.1 of this guide) only 3 possibilities exist. These are shown in figure 7.1. Either the large fragment from pARA (containing the ampR gene): re-ligates to the smaller pARA fragment (4); ligates to itself (8), or; ligates to the large fragment containing the kanR gene from the pKAN-R plasmid (9).

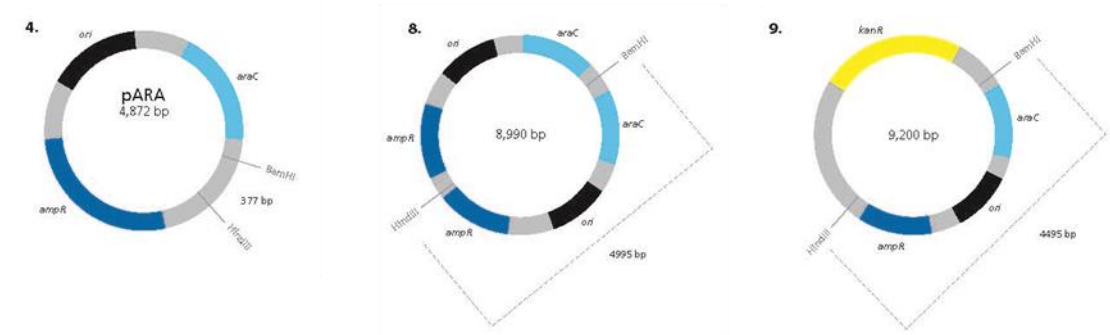


Figure 7.1 Possible plasmid constructs in transformed bacteria producing white colonies on the LB/amp/ara plates

Laboratory 7.2: Visualisation of products from the colony PCR

1. What is the purpose of including a negative control PCR, without a DNA template?

   The negative control PCR demonstrates that the reagents are not contaminated with template DNA and there is not a 'false positive' result.

2. What is the purpose of the PCRs that have pARA and pARA-R as template? What size fragments would you expect to see produced by PCR with these plasmids as template?

   The pARA and pARA-R PCRs provide a control to check that the reagents are active, the correct primers have been used and the PCR thermal cycling is working correctly. The pARA PCR is expected to produce a band of 662 bp and the pARA-R PCR is expected to produce a band of 1,092 bp. These PCR product sizes are shown in figure 7.2.

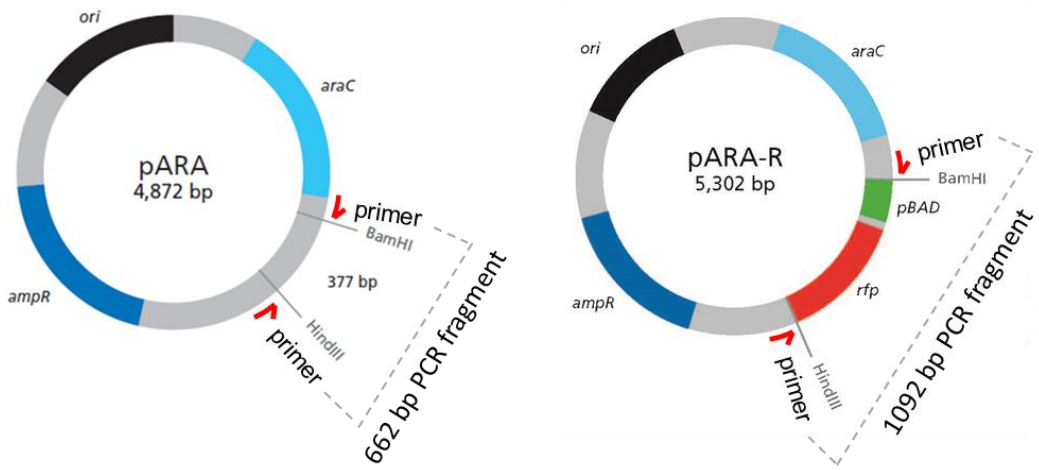


Figure 7.2 Expected PCR product sizes from plasmids pARA and pARA-R.

3. What products might you expect to see from the gelwhite and gelred tubes? Include the length (bp size) of each possible PCR product.

   As described in question 5 of laboratory 7.1, there are 3 possible plasmids that would produce white colonies on the LB/amp/ara plate. Either the large fragment from pARA (containing the ampR gene): re-ligates to the smaller pARA fragment (4); ligates to itself (8), or; ligates to the large fragment containing the kanR gene from the pKAN-R plasmid (9). The only plasmid that contains both the ampicillin resistance gene and the rfp gene is pARA-R. The PCR product sizes for each of these plasmid constructs are given in table 7.6.

Tube	Fragments and plasmids listed in order of increasing bp size in each tube
Gel white	***(1) pARA, 662 bp*** *(2)* ***PCR product of double ampR-ori-araC plasmid, 4,780 bp*** *(unlikely because primer binding to both parts of plasmid will obstruct PCR)* *(3)* ***PCR product of ampR-ori-araC & kanR plasmid, 4,990 bp*** *(less likely because of length of extension step in PCR thermal cycling)*
Gel red	***pARA-R, 1,092 bp***

Table 7.6 The DNA fragments and expected sizes from PCRs in lab 7.1.

4. The DNA ladder serves as a standard because it contains a mixture of DNA molecules of known sizes. By running your samples and the DNA ladder side by side in your gel, you can estimate the actual size in base pairs of unknown DNA molecules. The DNA ladder diagram below shows 10 DNA bands of known sizes. Their sizes are given in kilobase (kb) pairs. 1 kb is 1,000 base pairs (bp). On the diagram predict the positions of DNA bands produced by the possible products found in the gelMMpARA, gelMMpARA-R, gelwhite and gelred tubes by indicating their position on the DNA Ladder Diagram (figure 7.3).

   The DNA ladder diagram is reproduced in Figure 7.3 with the expected sizes of the DNA bands.

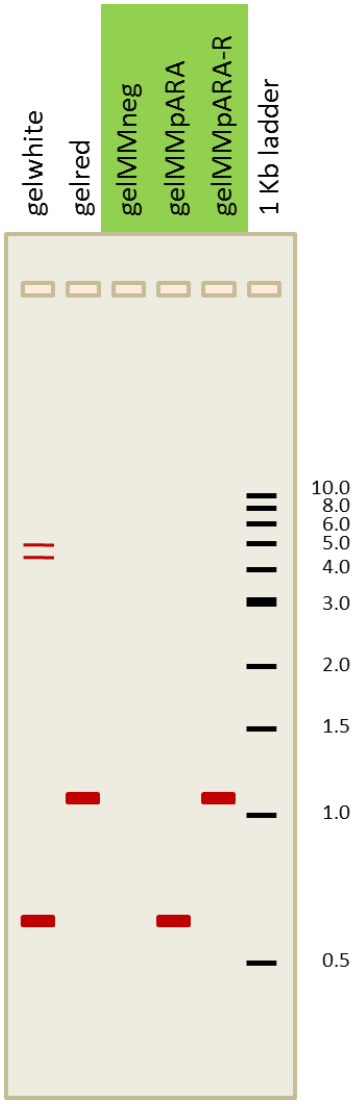


Figure 7.3 The DNA ladder diagram completed with the expected sizes of DNA bands.

The negative control PCR lane should be empty. The pARA PCR and pARA-R PCRs should show PCR products of 662 bp and 1,092 bp respectively. The red colony PCR would be expected to have a PCR product of 1,092 bp. The white colony PCR would probably have a PCR product of 662 bp, but may have a PCR product of 4,780 bp or 4,990 bp. All three of these possibilities are shown in the 'gelwhite' lane to demonstrate their migration through the agarose gel, although only one construct would be expected to be present in any bacterial colony.

5. 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, depending on predictions and results. If PCRs from both the red colony and white colony have failed, it is likely to be a problem with technique or reagents. If the red colony PCR has been successful, but not the white colony PCR it could be that the white bacterial colony had a plasmid construct that was (1) a double ampR-ori-araC plasmid, or (2) a ampR-ori-araC & kanR plasmid and that the PCR amplification was not successful because of the primer binding to both parts of the double ampR-ori-araC plasmid or insufficient time during the extension step of the thermal cycling for the larger products to be made.

6. 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.

7. Can you work out what the plasmid constructs in the colonies you have analysed must be to produce the size of the DNA fragments after PCR that you observe? Does the genotype you have suggested correlate with the phenotype of the bacterial colony?

   The potential PCR product sizes and the plasmid construct corresponding to each, is given in table 7.7

Size of PCR product (bp)	Plasmid construct detected
1,092	*pARA-R*
662	*pARA*
4,780	*Double ampR-ori-araC plasmid*
4,990	*ampR-ori-araC & kanR plasmid*

Table 7.7 PCR product size and corresponding recombinant plasmid construct.

The 1,092 bp band from the pARA-R plasmid should be produced from red colonies. The other 3 bands could be produced from white colonies. Genotypes should correlate with phenotypes of the bacterial colonies.

8. How does the genetic information, in the form of DNA, produce the phenotypes that you observe?

   The rfp gene in the plasmid is made up of DNA, and the gene is copied into messenger RNA. This process is called transcription. The messenger RNA moves to the ribosome where transfer RNA molecules match up codons and amino acids, with the enzyme peptidyl transferase catalysing the bonding of the amino acids to form a polypeptide chain. This process is called translation. The polypeptide chain folds to form a protein. The protein can contribute to a trait, and in this case the red fluorescent protein created makes the cells fluoresce red.

   The genotype : containing rfp gene

   The phenotype : fluorescing red

Additional resources

There are additional resources to expand upon the concepts and practice of PCR. 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
Video resources for PCR	R.29
Articulate for PCR	R.30

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

Overview

Chapter 2a allows students to confirm that two linear DNA fragments of the expected size are present from the engineered plasmid pARA-R, using restriction digestion with two enzymes (*Bam*HI and *Hin*dIII).

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 2a.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
2a: Restriction digestion of pARA-R	Aliquot solutions: • 2.5 x restriction buffer (RB) • pARA-R plasmid (R) • 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 2a.1 Preparation for laboratory 2a.

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 2a.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	12	10
pARA-R plasmid	R	12	10
Restriction enzymes *Bam*HI and *Hin*dIII	RE	3	2
Distilled water	dH_2O	20	2

Table 2a.2 Aliquotting reagents for laboratory 2a.

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 2a.3.

Substance / procedure	Hazard	Precautions
Restriction buffer	Low hazard	
Plasmid	Biological material: 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 2a.3 Health and Safety information for laboratory 2a.

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 2a.2, work out how many linear fragments will be produced from the plasmid pARA-R when it is digested with *Bam*HI and *Hin*dIII. What size will these linear fragments be?

   Restriction digestion of pARA-R with BamHI and HindIII restriction enzymes will produce 2 DNA fragments of 807 bp and 4495 bp (5302 bp – 807 bp = 4495 bp).

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

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

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

Figure 2a.1 shows the 2 restriction fragments, the DNA sequences they carry and the ‘sticky ends’ left after restriction digestion with BamHI and HindIII.

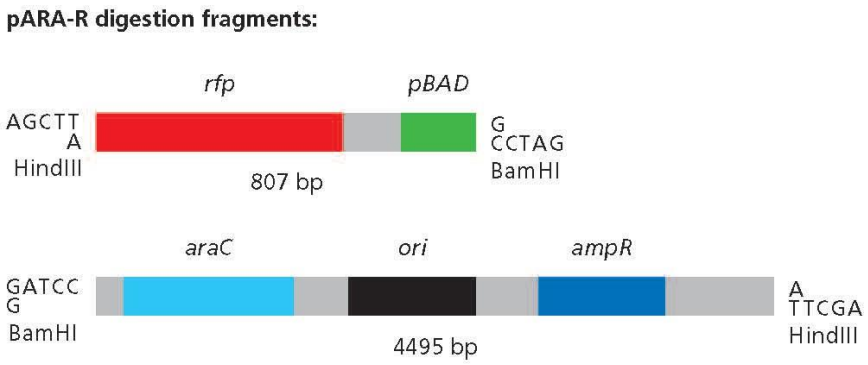


Figure 2a.1 The linear restriction fragments.

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 resistance to that antibiotic. Biotechnologists call these genes *selectable markers* because only bacteria that carry the gene will survive an antibiotic. If the uptake of DNA by bacteria is inefficient (as discussed in the reading), 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.4
What is genetic engineering? Treating disease with gene cloning	R.16
Red fluorescent protein in sea anemones: why glow?	R.21
Using plasmids and restriction enzymes in genetic engineering	R.22
Why use bacterial promoters in plasmids?	R.23
The components of the pARA-R plasmid	R.24
Restriction enzymes	R.25

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

Overview

In this chapter students explore one of the many uses of the polymerase chain reaction (PCR), learning about the enzyme DNA polymerase, sequence specific primers and thermal cycling. PCR is used to amplify part of the engineered plasmid pARA-R, confirming that the expected size DNA fragment is present.

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 4a.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
4a: The PCR with pARA-R	Aliquot solutions: • Combined forward and reverse primers (PRI) • pARA-R plasmid (R) • nuclease-free water (H_2O) • 2 PCR tubes of 2 x PCR master mix (MM) Make sure that there will be plenty of ice available.	Prepare benches with: • P-20 micropipette • Pipette tips • Microfuge tube rack • Microfuge tubes of solutions • Polystyrene cup of ice containing PCR tubes of master mix • Permanent marker • Waste container • Microfuge *C • PCR machine *C	Remove students' PCR tubes when thermal cycling is complete and store in fridge (4°C) until required for electrophoresis

Table 4a.1 Preparation for laboratory 4a.

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 4a.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)	Acually used (µl)
2 x PCR master mix	MM	12.5 + 12.5	12.5 + 12.5
Nuclease-free water	H_2O	22	19
Combined primers	PRI	5	4
pARA-R plasmid	R	3	2

Table 4a.2 Aliquotting reagents for laboratory 4a.

The nuclease-free water can be kept at room temperature. Combined primers and pARA-R plasmid should be stored in the freezer at –20°C, defrosted to room temperature for aliquotting and then returned to the freezer. The 2 x PCR master mix should be bought out of the freezer in an isofreeze box for aliquotting **into the PCR tubes** and returned to the freezer as soon as possible to avoid loss of DNA polymerase 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 4a.3.

Substance / procedure	Hazard	Precautions
Plasmid	Biological material: 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.
Primers	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.
PCR master mix	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 4a.3 Health and Safety information for laboratory 4a.

Learning objectives

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

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

During practical activities students can consider the relationship between genes and DNA, specificity of complementary base pairing, function of enzymes and role of DNA polymerase in the polymerase chain reaction.

Prior knowledge and skills

It is helpful if students already know:

- DNA is a double-stranded molecule, with a sugar-phosphate backbone and 4 nitrogenous bases (cytosine, guanine, adenine, and thymine) that form complementary base pairs.
- That cytosine only base pairs with guanine, and that adenine only base pairs with thymine.
- The order of the nitrogenous bases is known as the DNA sequence.
- That enzymes catalyse reactions in a cell and have roles in DNA replication.

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. Based on your understanding of DNA replication, explain the role of DNA polymerase in replication.
   During DNA replication the two, parent DNA strands separate at origin of replication, or ori sites. The enzyme DNA polymerase adds new nucleotides complementary to the single-stranded parent DNA to form base pairs held together by hydrogen bonds. On one parent DNA strand the new, complementary daughter strand forms continuously in a 5' to 3' direction. On the other parent strand of DNA the new, complementary nucleotides are added discontinuously in short fragments extended in a 5' to 3' direction.

   During the PCR, DNA polymerase catalyses the extension of the short double-stranded DNA molecules (created by primer binding to the genomic DNA) continuously, in a 5' to 3' direction.
2. Why do you keep the PCR master mix, which contains DNA polymerase, on ice until the PCR is begun?

   The PCR master mix contains the enzyme DNA polymerase. At room temperature the DNA polymerase starts to lose activity, so keeping the PCR master mix on ice helps to ensure that the enzyme retains activity until the PCR is started.
3. Why does the *Taq* DNA polymerase not denature during the denaturation step at 95°C in your PCR programme?

   DNA polymerase from Thermus aquaticus (Taq), a thermophilic bacteria adapted to survive the high temperatures found in deep sea hydrothermal vents is used, as it is evolved to operate in high temperatures without denaturing.
4. Each DNA molecule is replicated during each cycle of the PCR programme. There are 30 cycles on the PCR programme that you use. If you started with 6 DNA molecules, how many molecules would there be at the end of the PCR?

   $6 \times 2^{30} = 6{,}442{,}450{,}944$

   That is roughly 6 ½ billion DNA molecules.
5. What is the purpose of the pARA-R PCR, given that the expected size of the PCR product is known to be 1092 bp long?

   If a PCR product of the expected 1092 bp size is obtained by PCR it provides strong evidence that the

pARA-R plasmid has been correct engineered, and that this recombinant plasmid is as you would expect.

6. What is the purpose of the NEG PCR tube?

   The NEG PCR tube is a control to prove that the reagents are not contaminated with DNA template and that the PCR thermal cycling is working so that the results of the pARA-R PCR are more reliable.

7. What type of chemical bonds will form when the single stranded primer DNA base pairs with the complementary sequence on the recombinant plasmid?

   Hydrogen bonds form between the complementary nucleotides of the single stranded primer DNA and the recombinant plasmid. (Covalent bonds form between the sugar-phosphate backbone of the DNA molecule.)

8. How many fragments would you get if you used the restriction enzymes *Bam*HI and *Hind*III to digest the DNA fragment amplified from pARA-R using the PCR? What size would they be?

   Unlike cutting a circular DNA fragment in 2 places, which creates 2 DNA fragments, cutting a linear DNA fragment in 2 places creates 3 DNA fragments. The PCR product from pARA-R, and 3 fragments created by restriction digestion of this PCR product are shown in figure 4a.1.

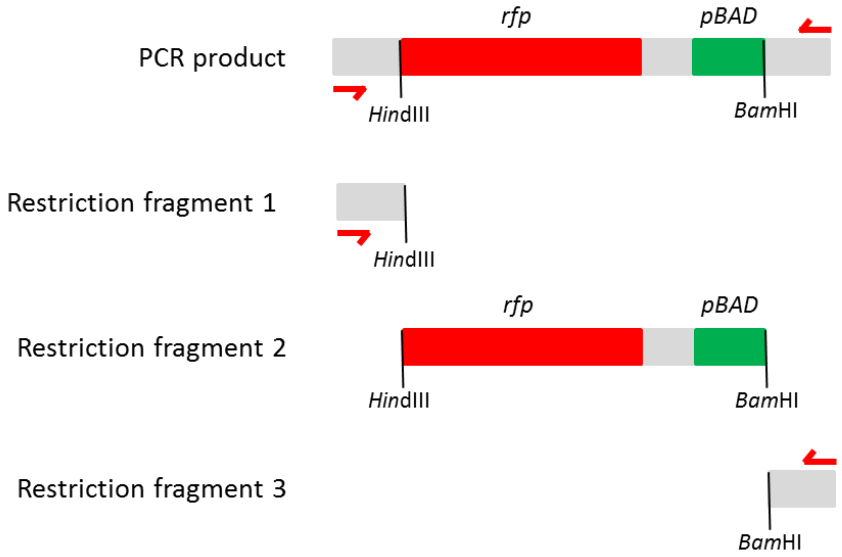


Figure 4a.1. The PCR product from pARA-R and DNA fragments resulting from restriction digestion.

Additional resources

There are additional resources providing internet links to PCR videos and a possible 'articulate' exercise to encourage peer-learning of vocabulary. 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
Video resources for PCR	R.29
Articulate for PCR	R.30

Chapter 5a: Verifying the engineered plasmid pARA-R

Overview

Chapter 5a separates the DNA fragments created in labs 2a and/or 4a using agarose gel electrophoresis and then stains the DNA bands so that the presence of recombinant plasmid pARA-R can be verified.

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 5a.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
5a: Visualisation of products from restriction digestion and the PCR Electrophoresis	Aliquot solutions: • Loading dye (LD) • DNA ladder (1 kb) Dilute TAE buffer to (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 Once electrophoresis is complete: • gels can be stored in ziplock bags with a little buffer in the fridge (4°C) until needed

Table 5a.1 Preparation for laboratory 5a (continued on next page).

Laboratory	Advance preparation	Prior preparation	Post-lab support
5: Visualisation of products from restriction digestion and the PCR Electrophoresis		Retrieve & distribute: • Undigested pARA-R from lab 2a (R-) • Digested pARA-R from lab 2a (R+) • The PCR with pARA-R as template from lab 4a (MMpARA-R) • The negative control PCR from lab 4a (MMNEG) Reuse the tubes of distilled water.	• 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 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 5a.1 Preparation for laboratory 5a (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 I10. If you pour an 15-well, 0.8% agarose gel, then two or three groups will each be able to load one set of 4 samples and a 1 kb DNA ladder. If you have completed one of the labs 2a and 4a, you may wish to provide alternative suggestions for the sample loading order given in figure 5a.3 of the student guide. If students are only loading 2 samples from lab 2a or lab 4a, then 6 groups could use one gel with 3 lanes of 1 kb DNA ladder.

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 5a.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	10	8
DNA ladder	1 kb	12	10

Table 5a.2 Aliquotting reagents for laboratory 5a.

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 5a.3.

Substance / procedure	Hazard	Precautions
Previously prepared: Restriction digest reactions 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.
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 5a.3 Health and Safety information for laboratory 5a (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.
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 5a.3 Health and Safety information for laboratory 5a (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 during 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 student to revise the enzymes involved and procedures used in restriction digestion 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 on the next pages:

1. Why do DNA restriction fragments and plasmids separate when analysed 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 that the restriction fragment and PCR product sizes from a recombinant plasmid match the expected sizes?
   Laboratory reagents can get mixed up. The procedures involved in (i) transforming a bacteria with a recombinant plasmid, (ii) culturing bacteria to express the protein and (iii) purifying the protein from the bacterial culture, are time consuming and expensive, so it's best to check you are working with the correct plasmid before undertaking them!
   As the products of genetic engineering procedures are not visible, mistakes may go unnoticed unless scientists check that they are working with the plasmid that they intended. Observing the sizes of DNA fragments from restriction digestion or PCR is a way to check that you have the correct plasmid. (A third, more definitive method, would be to sequence the plasmid.)
3. After different DNA fragments and plasmids have been separated by gel electrophoresis, the gel is stained to show bands that indicate the location of each kind of fragment and plasmid. The drawing of a stained gel opposite shows a series of bands that have been labelled with letters. The locations of the wells are also shown. What is the order of the fragments, from smallest to largest?
   The order of fragments from smallest to largest is B, G, D, F, A, C, and E.
4. The DNA is not visible as it moves through the gel. The loading dye contains the three dyes that you separated in Laboratory 1.2. Why is it useful to use the loading dye in this lab?
   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.
5. If you used gel electrophoresis to separate the same plasmid that has all three configurations, the supercoiled plasmid would move the fastest, while the multimer would move the slowest. Why do the different plasmid configurations move the way they do through the gel?
   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.
6. The pARA-R plasmid you digested in Laboratory 2a was replicated in a bacterial cell. What configurations—supercoiled, nicked circle, and multimer—might this plasmid have before digestion?
   This plasmid can have all three configurations.
7. What products might you expect to see from the gelR-, gelR+, gelMM-R and gelMMNEG tubes? Create a table that shows the possible fragments and plasmids by tube. Include the length (bp size) of each fragment or plasmid, and arrange the products found in each microfuge tube by size, from smallest to largest. Include any possible plasmid configurations, and arrange them first by size and next by speed through the gel, from fastest to slowest.
   Table 5a.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
R-	***pARA-R, 5,302 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.*
R+	*(1)* ***pBAD-rfp fragment, 807 bp*** *(2)* ***ampR-ori-AraC fragment, 4,495 bp***
MMpARA-R	*(1)* ***primers, 22 bp*** *(probably too small to be visible)* *(2)* ***expected PCR product, 1,092 bp*** *(3)* ***pARA-R template, 5,302 bp*** *(in all 3 plasmid configurations)*
MMNEG	*(1)* ***primers, 22 bp*** *(probably too small to be visible)* *No other product should be visible. If it is it suggests that there has been cross-contamination of the PCR.*

Table 5a.4 the DNA fragments and expected sizes from labs 2a and 4a.

8. The DNA ladder serves as a standard because it contains a mixture of DNA molecules of known sizes. By running your samples and the DNA ladder side by side in your gel, you can estimate the actual size in base pairs of unknown DNA molecules. The DNA Ladder Diagram below shows 10 DNA bands of known sizes. Their sizes are given in kilobase (kb) pairs. 1 kb is 1,000 base pairs (bp). Predict the positions of DNA bands produced by the possible products found in the gelR-, gelR+, gelMM-R and gelMMNEG tubes by drawing them on the DNA Ladder Diagram (figure 5a.5).

   The DNA ladder diagram is reproduced in Figure 5a.1 with the expected sizes of the DNA bands.

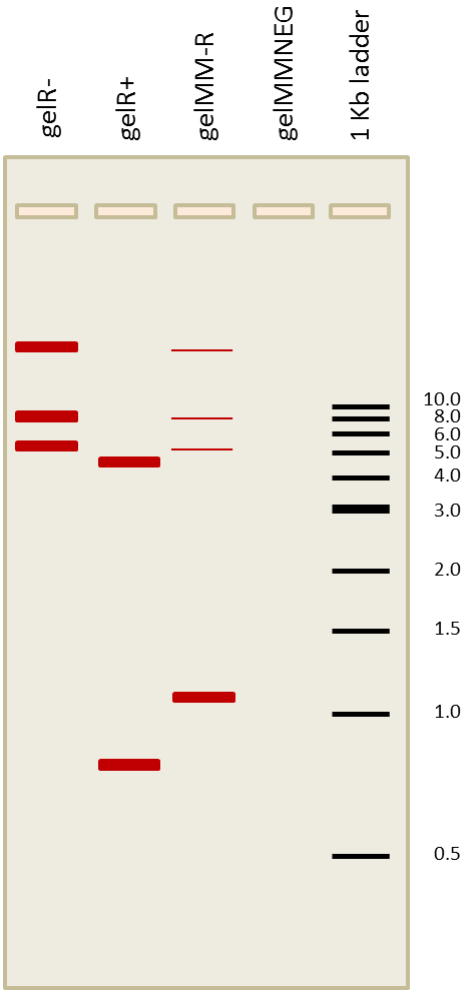


Figure 5a.1 The DNA ladder diagram completed with the expected sizes of DNA bands.

The R- lane shows undigested pARA-R plasmid in the 3 configurations (multimer, nicked circle and supercoiled), whilst the R+ lane shows the 2 products following restriction digestion. The pARA-R PCR lane shows the expected 1,092 bp PCR product and a small amount of pARA-R plasmid template in the 3 configurations. This template is not always visible and a small band of primer DNA is sometimes visible. The negative control PCR lane should show no amplified DNA product.

9. 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.

10. 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.

11. Does the gel show that your restriction digest and PCR 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 R- and R+ lanes. The R- lane will contain 1-3 bands of undigested plasmid DNA (caused by the 3 plasmid configurations). The R+ 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 PCR procedure was successful then you would expect to see one bright band of DNA at 1,092 bp, with other faint bands possible from the undigested DNA template or primers.

12. In the gelR– lane, do you see evidence of multiple configurations of plasmids? Explain your answer.

    Students should see two or three different bands in the gelR– lane, which is evidence of multiple plasmid configurations.

13. In the gelR+ lane, do you see evidence of complete digestion? Explain your answer.

    If there are only two bands in the gelR+ lane, this shows that the plasmid was completely digested into its two fragments. No undigested plasmid DNA remains.

14. Does the gel show that you are using the correct recombinant plasmid, pARA-R? Describe the evidence that you used to make this assessment.

    This answer will obviously depend on which bands are seen. If the restriction digestion produces two bands of 4,495 bp and 807 bp then the restriction mapping shows it is likely to be the pARA-R plasmid. If the PCR product produced is 1,092 bp then this provides additional evidence that the plasmid is pARA-R. To be absolutely certain about the identity of the plasmid being used, the plasmid would need to be sequenced.

Additional resources

There are currently no additional resources to expand upon the concepts and practice of agarose gel electrophoresis in the Teacher & Technician Guide. However, an excellent simulation can be found on LabXchange (www.labxchange.org) ; free to register and use.

Chapter 6a: Inserting recombinant plasmids into bacteria

Overview

Chapter 6a allows students to introduce the recombinant plasmid pARA-R into *E. coli*, using the process of transformation. Using antibiotic resistant genes as selectable markers, bacteria that have been successfully transformed are identified. Successfully transformed bacteria that have been supplied with the activator arabinose will turn red and when placed on the UV transilluminator will fluoresce red.

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 6a.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
6a: Transforming bacteria with the recombinant plasmid pARA-R	Aliquot solutions: • Luria Bertani broth (LB) • pARA-R plasmid (R) Prepare petri dishes of LB, LB/amp and LB/amp/ara.	Prepare benches with: • P-20 micropipette • P-200 micropipette • Pipette tips • Microfuge tube rack • Microfuge tube of LB broth • 1.5 mL microfuge tubes • Permanent marker • Waste container • 42°C water bath *C • Stopwatch • Sticking tape • Pack of cell spreaders *2 • 37 °C incubator *C	If the class is not able to view the plates after 24- 36 hours incubation, remove, seal with parafilm and store in the fridge (4 °C) until the class is ready to observe.

Table 6a.1 Preparation for laboratory 6a (continued on next page).

Laboratory	Advance preparation	Prior preparation	Post-lab support
6a: Transforming bacteria with the recombinant plasmid pARA-R		Provide autoclave bag for disposal of contaminated waste *C Defrost competent cells in wet ice (for about 10 – 15 minutes), resuspend and aliquot just prior to use. Supply to students in a polystyrene cup of wet ice.	
6a: Transforming bacteria with the recombinant plasmid pARA-R		Within the classroom place: • UV transilluminator *C Retrieve and distribute students' transformation plates.	Autoclave bacterial plates prior to disposal. Possibly printing gel pictures or transferring to computer(s).

Table 6a.1 Preparation for laboratory 6a (continued from previous page).

The process for preparing the agar plates (LB, LB/amp and LB/amp/ara) is covered in the overview of preparation section on page I14.

During transformation the temperature of the water bath must be correct at 42 °C: small fluctuations will affect the transformation efficiency, so set the waterbath temperature well in advance and check carefully before use.

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 6a.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)
LB broth	LB	320	300
pARA-R plasmid	R	12	10
Competent cells	CC	100	100

Table 6a.2 Aliquotting reagents for laboratory 6a.

LB broth should be prepared in advance and stored in the fridge (4 °C).

It is ***very important to keep the competent cells cold*** before the heat shock procedure. They should be defrosted in wet ice, resuspended and aliquotted just before use. Tubes should be held by the rim to avoid warming the cells with fingers, kept and supplied to students in wet ice.

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

6a.3.

Substance / procedure	Hazard	Precautions
Recombinant plasmid	Biological material: 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.
E. coli competent cells	Bacteria: low risk of infection.	Use in a controlled way, applying aseptic techniques. CLEAPSS does **not** recommend gloves or eye protection as a matter of course in any Microbiology. Autoclave materials that come into contact with bacteria before disposal.
Growth of microorganisms on agar plates	Opening agar plates with unknown microorganisms	Tape lids onto agar plates in 3, evenly spaced places around the circumference of the petri dish. Keep taped during incubation and viewing.

Table 6a.3 Health and Safety information for laboratory 6a.

Learning objectives

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

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

The central dogma of Biology about how DNA encodes protein, which produce traits is illustrated in this laboratory. Learning objectives associated with the practical work can also relate to the process of gene expression, techniques required in microbiology, bacterial cell structure and genetic engineering.

Prior knowledge and skills

It is helpful if students already know:

- The process of aseptic technique, used working with microorganisms.
- Prokaryotic cell structure.
- 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.
- DNA is a double-stranded molecule, and each strand of DNA is made up of covalently linked subunits called *nucleotides*, which are abbreviated according the nitrogenous base they contain (C, G, A, T).
- *Transcription* is the process by which information encoded in DNA is transferred to messenger RNA, a single-stranded ribonucleic acid.
- *Translation* is the process by which information encoded in messenger RNA is decoded and transformed into protein.
- Some information about gene expression, particularly regarding promoters and operators.

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 are the steps involved in transcription and translation of a gene?

   *During **transcription**, DNA is copied into messenger RNA. During **translation**, the messenger RNA is decoded at a cell organelle called the ribosome. Each codon (a group of three bases) in the sequence corresponds to one amino acid, the building blocks of proteins. Transfer RNA molecules match up the codons and the amino acids, and the enzyme peptidyl transferase catalyses the bonding of the amino acids to form a polypeptide chain, which folds to form the protein.*

2. What is the relationship among genes, proteins, and traits (or observable characteristics)?

   A gene contains the code for making a protein. Protein molecules make and run the cell, so they are responsible for traits. Often a trait is the outcome of multiple protein actions and interactions.

3. What do bacteria and humans have in common that makes it possible for a human gene to be expressed in bacteria?

   The DNA structure and code are the same in bacteria and humans. The cell machinery that carries out transcription and translation is the same in bacteria and humans.

4. Why is it important that the membranes of *E. coli* bacteria carefully regulate which substances can enter and exit the cell?

   The cell regulates the substances that can enter and exit because some substances might affect it adversely.

5. How are the P+ bacteria treated differently from the P– bacteria? What is the purpose of the P– bacteria?

   The P+ bacteria culture is mixed with the recombinant plasmids, while the P– bacteria culture is not mixed with the recombinant plasmids. The purpose of the P– bacteria culture is to make sure that the cells and growing conditions work as expected. The P– bacteria culture should grow well in Luria Broth, showing that the bacteria were viable, and should die when exposed to the antibiotic ampicillin, allowing successfully transformed bacteria to be distinguished by their selectable marker: ampicillin resistance.

6. Why is it important to use aseptic technique in this lab?

   Aseptic technique is important for 2 reasons; (i) stopping cultures of bacteria from becoming contaminated, and (ii) stopping cultures of bacteria from contaminating us.

7. Why do the cells need time to recover after the heat shock?

   The sudden heating associated with being transferred from ice to having a heat shock stresses the bacteria. They need time to recover; repairing their membranes and returning to their usual state before being manipulated further.

8. Why are the cells incubated at 37°C?

   E. coli bacteria are adapted to growing inside the human body, which maintains a temperature of 37°C by homeostasis.

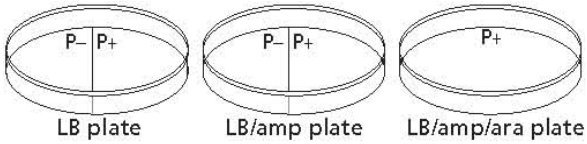
9. Ampicillin is an antibiotic that kills bacterial cells by disrupting the formation of cell walls. However, the pARA-R plasmid has the ampicillin resistance gene, which produces a protein that breaks down ampicillin. What is the purpose of growing bacteria that have been transformed in the presence of ampicillin?

   Only cells that have a plasmid containing the gene for ampicillin resistance will be able to grow in the presence of ampicillin. You can select the cells that have been transformed with the recombinant plasmid including the ampicillin resistance gene, which may number very few.

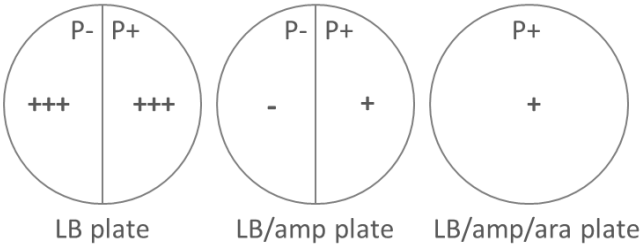
10. What will happen when bacterial cells that contain the pARA-R plasmid are not given arabinose?

    The rfp gene, under the control of the pBAD promoter, cannot be expressed unless the cell is given arabinose. When arabinose is <u>not present</u> the AraC activator protein binds just upstream of the pBAD promoter, obstructing the RNA polymerase from transcribing the arabinose operon (which comprises the three genes araB, araA and araD) or other downstream sequences. As a result these genes are not expressed. When arabinose <u>is present</u> it alters the conformation of the AraC protein, so that it guides the RNA polymerase onto the pBAD promoter, to begin transcription of downstream genes. In the recombinant plasmid the rfp gene is downstream of the pBAD promoter, so can only be transcribed and translated to produce an active protein when arabinose is present.

11. You add samples of the control group P– and the treatment group P+ to plates that contain various combinations of Luria Bertani agar (LB), ampicillin, and the sugar arabinose. What are your predictions for the growth you expect on each of the plates?



The predictions for growth expected (using +++ to indicate prolific growth, + to indicate some growth and – to indicate no growth) are:



Some students may struggle to predict these results and breaking down the expectations for the control bacteria (P-) and transformed bacteria (P+) may help.

Expected results for control bacteria (from P- tube):

Agar plate	Predicted growth	Conclusion: if prediction correct	Conclusion: if prediction not correct
LB	*High / +++*	*The bacterial competent cells are viable and able to survive the transformation procedure.*	*No growth signals either (i) the bacterial competent cells are not viable, or (ii) the bacteria are unable to survive the transformation procedure.*
LB/amp	*None / -*	*Without plasmids containing the ampicillin resistance gene, untransformed bacteria die.*	*If there is growth it signals that the ampicillin is not active in the agar plates.*

Expected results for transformed bacteria (from P+ tube):

Agar plate	Predicted growth	Conclusion: if prediction correct	Conclusion: if prediction not correct
LB	*High / +++*	*The bacterial competent cells are viable and able to survive the transformation procedure.*	*No growth signals either (i) the bacterial competent cells are not viable, or (ii) the bacteria are unable to survive the transformation procedure.*
LB/amp	*Low / +* *white*	*Some transformed bacteria have plasmids with the ampicillin resistance gene.*	*No growth signals that the transformation was not successful and there are no bacteria with plasmids containing the ampicillin resistance gene.*
LB/amp/ara	*Low / +* *red*	*Some transformed bacteria have recombinant plasmid pARA-R, containing the ampicillin resistance gene and the rfp gene.*	*No growth signals that the transformation was not successful and there are no bacteria with the plasmid pARA-R. White colonies could result from (i) a problem with the arabinose, or (ii) mutation of the rfp gene in the recombinant plasmid.*

12. Look at the results of your transformation. Do your actual results match your predicted results? If not, what differences do you see, and what are some explanations for these differences?

    Answers will vary. If unexpected results occur, help students think through what might have happened. There are many opportunities during the transformation procedure for errors to occur that would affect the results.

13. How many red colonies were present on your LB/amp/ara plate?

    Answers will vary. All groups should get a few colonies expressing red fluorescent protein, unless an error was made in carrying out the procedures.

14. Why did the red colonies only appear on the LB/amp/ara plate and not the LB/amp plate?

    The rfp gene cannot be expressed unless the cell is given arabinose, as the arabinose activator protein will only alter in conformation to allow transcription from the pBAD promoter of the downstream rfp gene in the presence of arabinose.

15. Recombinant plasmids are engineered so that they can replicate in the cell independently of the chromosome replication. Why is it important to have multiple copies of a recombinant plasmid within a cell?

    In genetic engineering the goal is to produce a lot of product, such as insulin. More copies of the plasmid and its gene will result in the production of more product within the cell.

16. How is the information encoded in the *rfp* gene expressed as a trait? Be sure to use what you have previously learned about gene expression and the relationship between DNA, RNA, protein, and traits.

    The rfp gene in the plasmid is made up of DNA, and the gene is copied into messenger RNA. This process is called transcription. The messenger RNA moves to the ribosome where transfer RNA molecules match up codons and amino acids, with the enzyme peptidyl transferase catalysing the

bonding of the amino acids to form a polypeptide chain. This process is called translation. The polypeptide chain folds to form a protein. The protein can contribute to a trait, and in this case the red fluorescent protein created makes the cells fluoresce red.

17. Why is it possible for bacteria to make a human protein, such as insulin, or a sea anemone protein, such as the red fluorescent protein?

    The DNA structure and code, and the cell machinery that carries out transcription and translation, are the same across these organisms.

Additional resources

There are additional resources to expand upon the concepts and practice of genetic engineering, therapeutic proteins, diabetes and why sea anemones fluoresce. 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 central dogma and reverse transcriptase	R.27
More on the AraC activator protein in 'The components of the pARA-R plasmid	R.24
Aseptic technique	Student guide A.1

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

Overview

In this chapter students explore one of the many uses of the polymerase chain reaction (PCR), learning about the enzyme DNA polymerase, sequence specific primers and thermal cycling. A colony PCR, using bacteria grown during lab 6a to provide the DNA template, is employed to verify the presence of the *rfp* gene in the plasmid construct transformed into the bacteria. This neatly links DNA fragment size (genotype) with colony colour (phenotype).

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 7a.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
7a.1: Colony PCR from the transformed bacteria	Aliquot solutions: • Combined forward and reverse primers (PRI) • nuclease-free water (H_2O) • PCR tube of 2 x PCR master mix (MM) Make sure that there will be plenty of ice available. Set up 2 control PCRs: • pARA-R template *C • negative control *C	Prepare benches with: • P-20 micropipette • Pipette tips • Microfuge tube rack • Microfuge tubes of solutions • Polystyrene cup of ice containing PCR tubes of master mix • Permanent marker • Waste container • Microfuge *C • PCR machine *C Retrieve and distribute the agar plates with transformed bacteria from lab 6a.	Remove students' PCR tubes when thermal cycling is complete and store in fridge (4°C) until required for electrophoresis

Table 7a.1 Preparation for laboratory 7a (continued on next page).

Laboratory	Advance preparation	Prior preparation	Post-lab support
7a.2: Visualisation of products from the colony PCR Electrophoresis	Aliquot solutions: • Loading dye (LD) • DNA ladder (1 kb) Prepare control PCRs for loading onto the agarose gel *C. Dilute TAE buffer to (1 x TAE) Pour 0.8% agarose gels	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 an 0.8% agarose gel*2 • Powerpack*2 Retrieve & distribute from lab 7a.1: • Red colony PCR (MMred) Retrieve the 2 control PCRs from lab 7a.1: • pARA-R template *C • negative control *C Reuse the tubes of distilled water.	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 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

Table 7a.1 Preparation for laboratory 7a (continued from previous and on next page).

Laboratory	Advance preparation	Prior preparation	Post-lab support
7a.2: Visualisation of products from the colony PCR Staining and photodocumentation	Dilute GelRed solution	Prepare benches with: • GelRed solution • Plastic gel staining tray *2 • Permanent marker • Tape Within the classroom place: • Chemical resistant gloves • Foil-covered bottle and funnel (if reusing GelRed solution) • UV transilluminator*C • Hood *C • Camera *C	Possibly printing gel pictures or transferring to computer(s).

Table 7a.1 Preparation for laboratory 7a (continued from previous pages).

Setting up 2 control PCRs

It is good practice to perform control PCRs with (i) DNA template known to contain the *rfp* gene (pARA-R) – this confirms that the PCR is working and the expected DNA size (1092 bp), and (ii) no DNA template (neg) – this is a negative control, used to confirm that the reagents are not contaminated with DNA.

Rather than get each pair to set up these additional reactions, you will set up these 2 PCRs, run them in the PCR machine with the students' reactions, then allow students to use your PCR samples for agarose gel electrophoresis. Set up the 2 reactions as shown in table 7a.2.

Reagent	MMpARA-R PCR	MMneg PCR
2 X PCR master mix (MM)	12.5 µL	12.5 µL
Nuclease-free water (H_2O)	8.5 µL	10.5 µL
Combined primers (PRI)	2.0 µL	2.0 µL
pARA-R (R)	2.0 µL	

Table 7a.2 Reagents to add to the control PCRs.

Preparing the control PCRs for agarose gel electrophoresis

In a fresh 1.5 mL microfuge tube, prepare the control PCRs for agarose gel electrophoresis. The preparation required is shown in table 7a.3 below. You can aliquot this for the students if you wish (they will load 10 µl per gel), or get them to pass around the prepared samples whilst loading their gels.

You will prepare a bulk mix of loading dye, water and sample (100 µL total volume) and 10 µL should be loaded each time. Thus, 10 replicate samples for each control can be loaded.

Reagent	gelMMpARA-R	gelMMneg
Distilled water (dH_2O)	60.0 µL	60.0 µL
Loading dye (LD)	20.0 µL	20.0 µL
The PCR with pARA-R template (MMpARA-R)	20.0 µL	
The negative PCR (MMNEG)		20.0 µL

Table 7a.3 Preparation of control PCR samples for gel electrophoresis

The processes for diluting TAE buffer and preparing agarose gels are covered in the overview of preparation section on page I14/15. If you pour an 8-well, 0.8% agarose gel, then four groups will each be able to load their sample (gelred), one set of the control PCR samples (from table 7a.3) and two lanes of 1 kb ladder (shown in the student guide, figure 7a.2). If you pour a 15-well, 0.8% agarose gel, then nine groups will each be able to load their sample (gel red), alongside one set of the control PCR samples and four lanes of 1 kb ladder.

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 7a.4 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 x PCR master mix	MM	12.5	12.5
Nuclease-free water	H_2O	12	9.5
Combined primers	PRI	3	2
5 x loading dye	LD	3	2
1 kb DNA ladder	1kb	12	10

Table 7a.4 Aliquotting reagents for laboratory 7a.

The nuclease-free water and loading dye should be kept at room temperature. Combined primers and DNA ladder should be stored in the freezer at –20°C, defrosted to room temperature for aliquotting and then returned to the freezer. The 2 x PCR master mix should be bought out of the freezer in an isofreeze box for aliquotting **into the PCR tubes** and returned to the freezer as soon as possible to avoid loss of DNA polymerase 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 7a.5.

Substance / procedure	Hazard	Precautions
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.
Primers	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.
PCR master mix	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.
Micro-organisms on agar plates	Opening agar plates with unknown microorganisms. It is usually recommended by CLEAPSS that agar plates with cultured bacteria are not opened. However, if these agar plates only contain the expected red and white *E. coli* colonies then using these bacteria in a colony PCR is judged an acceptably low risk activity for sixth form students.	Direct students to work where lower school students will not observe and be tempted to copy this procedure. Use in a controlled way, applying aseptic techniques whilst touching the pipette tip to the bacterial colony and transferring it into the PCR tube. CLEAPSS does **not** recommend gloves or eye protection as a matter of course in any Microbiology. Autoclave materials that come into contact with bacteria 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.

Table 7a.5 Health and Safety information for laboratory 7a (continued on next page).

Substance / procedure	Hazard	Precautions
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.
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 be closed, making a magnetic connection before the UV light will come on.

Table 7a.5 Health and Safety information for laboratory 7a (continued from previous page).

Learning objectives

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

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

The primary objective for this laboratory is facilitating understanding of the link between genotype and phenotype and how genes encode proteins that cause traits or characteristics in organisms; a concept many students struggle with. Additionally, students can consider the relationship between genes and DNA, specificity of complementary base pairing, function of enzymes and role of DNA polymerase in the polymerase chain reaction.

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
- DNA is a double-stranded molecule, with a sugar-phosphate backbone and 4 nitrogenous bases (cytosine, guanine, adenine, and thymine) that form complementary base pairs.
- That cytosine only base pairs with guanine, and that adenine only base pairs with thymine.
- The order of the nitrogenous bases is known as the DNA sequence.
- That enzymes catalyse reactions in a cell and have roles in DNA replication.
- 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 7a.1: Colony PCR from the transformed bacteria

1. What is the structure of a prokaryotic cell? Do prokaryotes have cell walls? What about cell membranes and nuclear membranes?

   Prokaryotes are small, unicellular organisms. The majority of prokaryotes do have a (peptidoglycan) cell wall outside of their cell membrane, but have no membrane-bound organelles, such as a nucleus or mitochondria.

2. Why does DNA need to be extracted from animal cells for use in PCR (laboratory 8), but not from bacterial cells?

   There are two reasons why the DNA needs to be extracted from animal cells, but not bacterial cells: cell structure and nucleases. Firstly, the cell structure is different, and whilst the thermal cycling of the PCR to 95 °C is sufficient to lyse the bacteria, releasing the DNA, it is not sufficient to lyse the eukaryotic cells and release the DNA from inside the membrane-bound nucleus. Secondly, the nucleases released from the cytoplasm of bacteria within the PCR are sequence specific and will not cut the bacterial DNA, whilst the nucleases released from the cells in a mouth swab that probably contains bacteria, food matter and cheek cells will probably cut the desired human DNA and may interfere with the PCR.

3. What is aseptic technique? Why do you need to use it when performing colony PCR? What constitutes good aseptic technique?

   Aseptic technique is the name of procedures that are used to stop pure bacterial cultures from becoming contaminated. Aseptic technique is important when performing colony PCR for 2 reasons; (i) stopping the bacterial colonies from becoming contaminated with other micro-organisms, and (ii) stopping the bacterial colonies from contaminating us. There is a section on aspetic technique in the student guide (page A.1). Top tips for aseptic technique include;

   - *Organise your work area, to avoid spillages and confusion*
   - *Use your Bunsen burner flame to sterilise glass equipment and create an updraft around*

the base that leaves a 'sterile zone' (as hot air rises taking airborne contaminants with it).

- *Keep equipment sterile: close tip boxes when not in use and when removing plasticware from packaging try to touch only the items you intend to use to keep the remaining items from contamination.*
- *Disinfect equipment that has come into contact with bacteria and autoclave prior to disposal*

4. Why does the *Taq* DNA polymerase not denature during the denaturation step at 95°C in your PCR programme?

   DNA polymerase from Thermus aquaticus (Taq), a thermophilic bacteria adapted to survive the high temperatures found in deep sea hydrothermal vents is used, as it is evolved to operate in high temperatures without denaturing.

5. What size of DNA fragment do you expect to be amplified by PCR from the transformed bacteria that produce red colonies on the LB/amp/ara plates?

   The plasmid used during the transformation procedure was pARA-R, a construct that contains the ampicillin resistance gene, to survive on the ampicillin, and the rfp gene for production of red fluorescent protein. Using primers that bind to the DNA sequence flanking the restriction fragment, PCR using the pARA-R plasmid as template is expected to produce a 1,092 bp DNA fragment. This is shown in figure 7a.1.

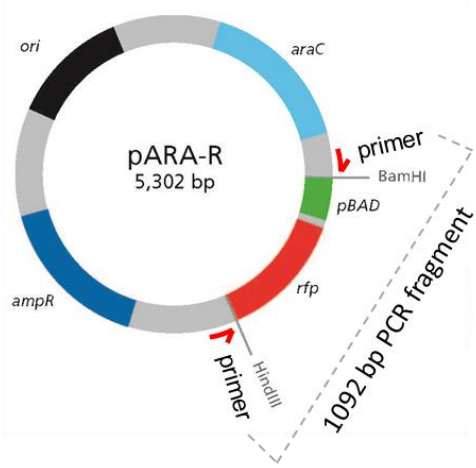


Figure 7a.1 The pARA-R plasmid and expected PCR product size.

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

1. What is the purpose of including a negative control PCR, without a DNA template?

   The negative control PCR demonstrates that the reagents are not contaminated with template DNA and there is not a 'false positive' result.

2. What is the purpose of the PCR that has pARA-R as template? What size fragments would you expect to see produced by PCR with these plasmids as template?

   The pARA-R PCR provides a control to check that the reagents are active, the correct primers have been used and the PCR thermal cycling is working correctly. The pARA-R PCR is expected to produce a band of 1,092 bp (see figure 7a.1).

3. What product would you expect to see from the red colony PCR? Include the length (bp size) of the PCR product.

   As described in question 5 of laboratory 7a.1, the pARA-R plasmid was used during the

transformation procedure and this plasmid construct would be amplified by PCR. The PCR product is expected to be 1,092 bp long.

4. The DNA ladder serves as a standard because it contains a mixture of DNA molecules of known sizes. By running your samples and the DNA ladder side by side in your gel, you can estimate the actual size in base pairs of unknown DNA molecules. The DNA ladder diagram below shows 10 DNA bands of known sizes. Their sizes are given in kilobase (kb) pairs. 1 kb is 1,000 base pairs (bp). On the diagram predict the positions of DNA bands produced from the gelMMpARA-R and gelred tubes by drawing them on the DNA Ladder Diagram (figure7a.3).

   The DNA ladder diagram is reproduced in Figure 7a.2 with the expected sizes of the DNA bands.

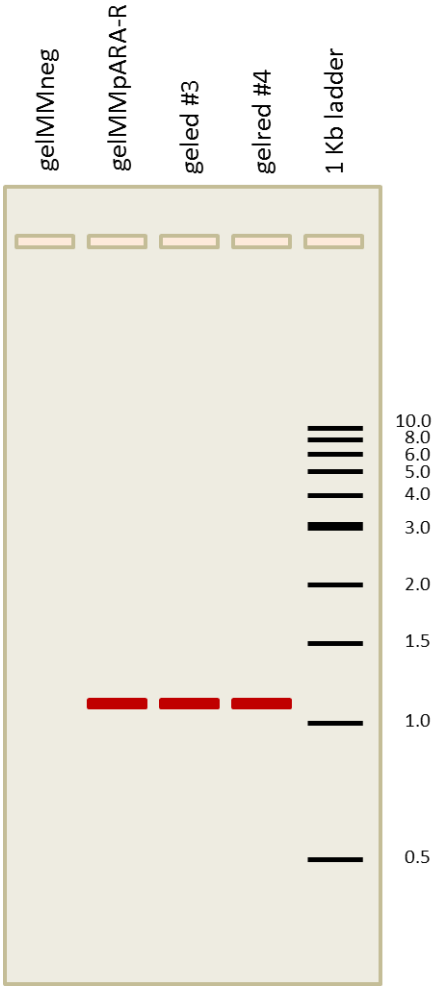


Figure 7a.2 The DNA ladder diagram completed with the expected sizes of DNA bands.

The negative control PCR lane should be empty. The pARA-R PCR should show a PCR product of 1,092 bp, which the red colony PCRs would be expected to match.

5. 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, depending on predictions and results. If the control PCRs show the expected results, but not the colony PCRS, it is likely to be a problem with technique or with absent reagents.

6. 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.

7. Can you work out what the plasmid constructs in the colonies you have analysed must be to produce the size of the DNA fragments after PCR that you observe? Does the genotype you have suggested correlate with the phenotype of the bacterial colony?

   The expected PCR product size and the plasmid construct that it corresponds to, is given in table 7a.6

Size of PCR product (bp)	Plasmid construct detected
1,092	*pARA-R*

Table 7a.6 PCR product size and corresponding recombinant plasmid construct.

The 1,092 bp band from the pARA-R plasmid should be produced from red colonies. Genotype (containing the rfp gene) should correlate with (fluorescing red) phenotype of the bacterial colonies.

8. How does the genetic information, in the form of DNA, produce the phenotype that you observe?

   The rfp gene in the plasmid is made up of DNA, and the gene is copied into messenger RNA. This process is called transcription. The messenger RNA moves to the ribosome where transfer RNA molecules match up codons and amino acids, with the enzyme peptidyl transferase catalysing the bonding of the amino acids to form a polypeptide chain. This process is called translation. The polypeptide chain folds to form a protein. The protein can contribute to a trait, and in this case the red fluorescent protein created makes the cells fluoresce red.

   The genotype : containing rfp gene

   The phenotype : fluorescing red

Additional resources

There are additional resources to expand upon the concepts and practice of PCR. 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
Video resources for PCR	R.29
Articulate for PCR	R.30

Chapter 9: DNA profiling

Overview

Chapter 9 introduces students to the scientific techniques used in DNA profiling. This sequence of practical activities is aimed at capable KS4 students, as the concepts of DNA profiling, some knowledge of restriction enzymes and agarose gel electrophoresis are frequently introduced at this level.

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 9.1 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
9.1: Cutting DNA by restriction digestion	Aliquot solutions: • 2.5 X restriction buffer (BF) • Lambda DNA (ENZ) • *Eco*RI (DNAref) • *Pvu*II (DNA1) • *Bam*HI (DNA2) • *Hind*III (DNA3) • *Eco*RI (DNA4) • *Pst*I (DNA5)	Prepare benches with: • P-20 micropipette • Pipette tips • Microfuge tube rack • Microfuge tubes of solutions • A polystyrene cup of ice containing the DNA samples and 'ENZ' sample • 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 9.1 Preparation for laboratory 9 (continued on next pages).

Laboratory	Advance preparation	Prior preparation	Post-lab support
9.2: DNA separation by agarose gel electrophoresis Electrophoresis	Aliquot solutions: • Loading dye (LD) • DNA ladder (1 kb) Dilute TAE buffer to (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 an 0.8% agarose gel*2 • Powerpack*2 Retrieve & distribute from lab 9.1: • Digested DNAref • Digested DNA1 • Digested DNA2 • Digested DNA3 • Digested DNA4 • Digested DNA5 Reuse the tubes of distilled water.	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 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

Table 9.1 Preparation for laboratory 9 (continued from previous and on next page).

Laboratory	Advance preparation	Prior preparation	Post-lab support
9.2: DNA separation by agarose gel electrophoresis Staining and photodocumentation	Dilute GelRed solution	Prepare benches with: • GelRed solution • Plastic gel staining tray *2 • Permanent marker • Tape Within the classroom place: • Chemical resistant gloves • Foil-covered bottle and funnel (if reusing GelRed solution) • UV transilluminator*C • Hood *C • Camera *C	Possibly printing gel pictures or transferring to computer(s).

Table 9.1 Preparation for laboratory 9 (continued from previous pages).

The processes for diluting TAE buffer and preparing agarose gels are covered in the overview of preparation section on page I10. If you pour an 15-well, 0.8% agarose gel, then two groups will each be able to load their set of samples onto it, with one well remaining empty.

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)
2.5x restriction buffer	BF	30	24
Lambda DNA (80 ng/ µl)	ENZ	15	12
*Eco*RI (2 U/µl)	DNAref	2	2
*Pvu*II (2 U/µl)	DNA1	2	2
*Bam*HI (2 U/µl)	DNA2	2	2
*Hind*III (2 U/µl)	DNA3	2	2
*Eco*RI (2 U/µl)	DNA4	2	2
*Pst*I (2 U/µl)	DNA5	2	2
5x Loading dye	LD	20	15
1 kb DNA ladder	1 kb	12	10

Table 9.2 Aliquotting reagents for laboratory 9.

IMPORTANT NOTE: You will reverse label the DNA and enzyme, because we supply you with 1 type of DNA and 5 different enzymes, rather than 1 type of enzyme and 5 different DNA samples, as you would use for real DNA profiling.

The loading dye should be kept at room temperature. Restriction buffer, BSA, lambda DNA and 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. Keep the enzymes in the isofreeze box whilst aliquotting to retain their 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 9.3.

Substance / procedure	Hazard	Precautions
Restriction buffer	Low hazard	
Lambda DNA	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.
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 9.3 Health and Safety information for laboratory 9 (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 be closed, making a magnetic connection before the UV light will come on.

Table 9.3 Health and Safety information for laboratory 9 (continued from previous page).

Learning objectives

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

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

These learning objectives relate to: inheritance of DNA; the DNA content of cells; the role of restriction enzymes in molecular biology; generation and application of DNA profiles, and; separation of charged molecules.

Prior knowledge and skills

It is helpful if students already know:

- Human somatic cells are diploid. DNA is inherited from parents, who each contribute one of the pair of homologous chromosomes carried in each somatic cell.
- DNA is a double-stranded molecule, with a sugar-phosphate backbone and 4 nitrogenous bases (cytosine, guanine, adenine, and thymine) that form complementary base pairs.
- The order of the nitrogenous bases is known as the DNA sequence.
- 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 was a loading dye mixed with the samples before they were loaded onto the agarose gel?

   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.

2. Why was the DNA loaded by the negative electrode on the agarose gel?

   DNA is negatively-charged (due to the phosphates in the backbone of the structure) and will therefore migrate towards the positive electrode.

3. How does agarose gel electrophoresis separate DNA molecules by size?

   Agarose is a polysaccharide from seaweed. It can be purified and used to make an agarose gel, which is a porous, jelly-like matrix. Differently-sized DNA molecules move / migrate through the pores in the jelly-like matrix at different rates when an electric field is applied (electrophoresis).

   DNA is negatively-charged, due to the phosphate groups that form part of the backbone. It is therefore attracted to the positive electrode. The speed at which the DNA molecule moves / migrates through the gel relates to the size / weight of the molecule: larger molecules are impeded more than smaller molecules and therefore migrate more slowly and less distance, while smaller molecules move faster and further. Molecular shape and electronegativity will also have an effect.

4. How many copies of each VNTR does an individual have in a somatic cell? How many copies of a VNTR would an individual have in a gamete?

   A somatic cell is any cell in the body that is not a sex cell / gamete. Somatic cells in humans are diploid: having two sets of chromosomes. One set is inherited from the mother and one set is inherited from the father. Given that a VNTR is found in the same position on homologous chromosomes, an individual will have 2 copies of each VNTR in a somatic cell (one from each parent). As gametes are haploid (containing only one set of chromosomes), they would only contain one copy of a VNTR.

5. VNTRs are always found in the same location in the DNA sequence, but vary in length. How does this help when we want to generate a DNA profile?

   Since a VNTR is always found in the same location, the DNA either side of it can be sequenced (either experimentally, or now that the Human Genome Project is complete, from online data). If the DNA either side of a VNTR is found to be conserved (it doesn't vary much between individuals) then restriction enzymes that cut within the conserved sequence can be found and used to excise the VNTR from the DNA. The DNA bands can be used to generate a DNA profile.

   To check that the bands observed are from the VNTR and not another part of the genome, a fluorescent probe, complementary to the VNTR sequence is usually used. This part of the procedure is omitted here as it is tricky and time consuming.

6. DNA profiling is frequently used in crime scene investigations. Can you describe 2 or more other applications for DNA profiling?

   As well as being used in criminal investigations DNA profiling can be used: to monitor the spread of

animal or plant populations for conservation purposes; to ensure that zoo animals do not become inbred, and; to test for paternity.

7. When you have had a chance to examine your gel photograph, analyse whether the restriction digests were successful. A successfully digested sample of DNA will have discrete bands visible, an unsuccessful digest will still have most of the DNA in one large band near the wells in the gel. If your digest did not work, can you identify what the problem was?

   Hopefully it will have worked! If not, reasons will vary and may include; not adding a reagent to the original mix, mixing up reagents when setting up the reactions, incubating the restriction digest reactions at the wrong temperature or missing or piercing the well when loading into the agarose gel so that the sample was lost in the buffer and couldn't be analysed.

8. Compare the 5 DNA samples to the reference DNA sample. Can you identify the banding pattern, or DNA profile', that matches? Use this information to write a short conclusion about the outcome of your investigation.

   Sample "DNA4" should create a profile that matches the "crime scene" DNA profile.

9. How could a fluorescently-labelled DNA probe be used to show the presence of a specific DNA sequence?

   Answers could refer to the Southern blotting procedure described in the student guide introduction. They could also discuss denaturing DNA then annealing the fluorescently-labelled DNA probe to a complementary base pair sequence (one of the VNTRs).

10. DNA profiles are stored in National DNA databases. There are many controversial issues about these: Who is responsible? Are they secure? Whose DNA profile is held? How long for? Who can access the information? You may explore these issues further.

    Some of the additional resources may be of help to you if you wish to pursue some of these issues further with your students.

Additional resources

There are a number of additional resources to expand upon the concepts and practice of DNA profiling including a paper-based exercise. 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
Investigation report sheet	R.3
Paper-based DNA profiling	R.9
Questions and possible answers about DNA profiling	R.31
Video resources for DNA profiling	R.32
Articulate for DNA profiling	R.33
Extension: DNA profiling and crime (reading and references)	R.34
Extension: National DNA databases (reading and questions)	R.37

Chapter 10: Serial dilutions

Overview

Chapter 10 builds on the use of accurate micropipetting skills and extends this to performing serial dilutions. It permits students to construct concentration curves from serial dilution data and uses these concentration curves to determine the concentration of an unknown solution.

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 10.1 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
10.1: Performing serial dilutions	Aliquot solutions: • Microfuge tube containing 1 ml of distilled water (**dH_2O**) • Microfuge tube containing 500 µl of 1 M glucose solution (**G**)*2 • Microfuge tube containing 200 µl of 0.2 M glucose solution (**M**) *2 Ensure that there is Benedict's reagent made up*2 Prepare pieces of tin foil*2	Prepare benches with: • P-200 micropipettes*2 • Pipette tips*2 • Empty microfuge tubes • Microfuge tube rack*2 • Waterproof marker pens*2 • Microfuge tubes of glucose solutions*2 • Waste container*2 • Benedict's reagent*2 • 2 Pasteur pipettes*2 • Piece of tin foil*2 • Vortex*C • Boiling water bath*C • Dimple tile*2	

Table 10.1 Preparation for laboratory 10.

You will need to supply the consumables for this practical activity. Table 10.2 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)
Distilled water	dH_2O	1000	800
1 M glucose solution	G	500	400
0.2 M glucose solution	M	200	200

Table 10.2 Aliquotting reagents for laboratory 10.

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 10.3.

Substance / procedure	Hazard	Precautions
Glucose solution	None	Use good laboratory practice.
Benedict's reagent	Low hazard, contains low concentration of copper sulphate	Wear eye protection. Use good laboratory practice to avoid contact with skin.
Boiling water bath	Low hazard	Use good laboratory practice to avoid burns / scalds.

Table 10.3 Health and Safety information for laboratory 10.

Learning objectives

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

- Perform serial dilutions
- Use serial dilutions to determine the concentration of an unknown solution

These learning objectives address a basic technique used in Biology. It is particularly applicable to students following the A level curricula that in which serial dilution is one of the Required Practical techniques.

Prior knowledge and skills

It is helpful if students already know:

- How to use a micropipette for accurate volumetric measurements
- How to draw and use concentration curves to determine the concentration of an unknown sample
- The causes of diabetes, if you wish to use this as an example to demonstrate the relevance of serial dilution

Expected question responses

The question sections include: (i) questions appropriate for after the reading and practical work are complete, and; (ii) questions that are intended to extend understanding of serial dilution. 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 10.1: Performing serial dilutions

1. If you had a glucose solution with an initial concentration of 8 M, what would be the dilution factor after each of 6 doubling dilutions? What would be the concentration of the solutions after each of 6 doubling dilutions?

Doubling dilution	*Dilution factor*	*Solution concentration (M)*
0	1	8
1	*1/2*	*4*
2	*1/4*	*2*
3	*1/8*	*1*
4	*1/16*	*0.5*
5	*1/32*	*0.25*
6	*1/64*	*0.125*

2. As the concentration of glucose increased what happened to the intensities of each of the colours (red, green and blue)?

   The red intensity increased.

   The blue and green intensities decreased.

3. How reliable was your graph for determining the concentration of the mystery sample?

   This will vary between classes and groups. Answers should reflect how close estimations of the mystery sample concentration were.

4. What factors could affect the reliability of your results?

   Answers will vary again, but may include:

   - *Micropipetting errors **or** lack of mixing of samples during serial dilution before successive serial dilutions were made.*
   - *Not mixing samples with Benedict's reagent prior to incubation in waterbath*
   - *Temperature of the waterbath (if it is not hot enough there will be less obvious reaction with Benedict's reagent)*
   - *The light intensity on the dimple tray where results were recorded (we have found the best results are obtained near windows, in good, natural light)*
   - *How much of the precipitate was transferred into the dimple tray with the samples (we have found that the best results come from transferring the sample, **including** precipitate and measuring the light intensity rapidly)*
   - *How long the samples were left to stand (and how much of the precipitate had settled)*

Laboratory 10.1: Performing serial dilutions. Further questions

1. What is the dilution factor from adding a 0.1 ml aliquot of a specimen to 9.9 ml of diluent?

   This is a one in a hundred dilution (1/100).

2. If a 1/8 dilution of the stock solution is made followed by a 1/6 dilution what is the final dilution?

   To work this out you multiply the 2 dilution factors together, so:

   1/8 x 1/6 = ***1/48***

   This gives a 1 in 48 dilution.

3. You have a microfuge tube containing 1 ml of a solution with 4.3×10^4 cells/ml and you are to produce a solution that contains 43 cells/ml. How many 1/10 dilutions must you perform?

   There are different ways to approach this problem. This is one way.

   4.3×10^4 cells/ml = 43,000 cells/ml

   43,000 cells/ml ÷ 43 cells/ml = 1000 fold dilution

   1000 ÷ 10 = 3

   So, ***3 successive 1/10 dilutions*** *are needed to reduce 4.3×10^4 cells/ml to 43 cells/ml.*

4. You have decided to determine how many microbes are living on the lettuce in the salad bar at your favourite restaurant. You place 1 gram of lettuce and 99 ml of water in a blender and blend the mixture. This is sample A. You then transfer 1 ml of this dilution into to another that contains 9 ml of water. This becomes sample B. You next transfer 1 ml of sample B into a separate container that contains 9 ml of water. This is sample C. Next you transfer 1 ml each from samples B & C onto separate nutrient rich agar plates, swirl, let harden and incubate at 37°C. When you examine the plates after 48 hours you find 110 colonies growing on plate C.

   (For your information 1 gram lettuce = 1 ml).

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

So from the question we know the following:

Sample	*Dilution factor*	*Number of colonies (bacteria)*
A	***1/100***	***110 x 100 = 11,000***
B	*1/100 x 1/10 =* ***1/1,000***	***110 x 10 = 1,100***
C	*1/1,000 x 1/10 =* ***1/10,000***	*110*

We can work out that sample A is 100-fold less dilute than sample C. Therefore the number of bacteria (colonies) that you would expect to find in sample A would be: 110 x 100 = ***11,000 bacteria.***

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

Sample B is less dilute than sample C, so you would expect more bacteria on the plate from sample B.

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

If there were 11,000 bacteria in sample A and that was a 100-fold dilution of the original lettuce then the original 1 gram of lettuce had: 11,000 x 100 = ***1,100,000 bacteria*** *(and you may wish to consider whether you wishes to get your salad from there again in the future!)*

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

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

0.1 g/ml = 100,0000 µg/ml

100,000 µg/ml ÷ 10 µg/ml = 10,000 (= 10 x 10 x 10 x 10)

*So a 10,000-fold dilution would need to be made, which is **4 successive, 10-fold dilutions**.*

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

*An order of magnitude is 10 x difference. The difference in the working concentration and stock concentration is 10,000-fold. So there are **4 orders of magnitude difference**.*

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; a sheet that can be used for reporting on the DNA profiling investigation on page R.3; the paper-based 'clone that gene' activity on page R.4, and; the paper-based DNA profiling activity on page R.9.

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

INVESTIGATION REPORT:	
NAME:	DATE:

Results from the experiment that you've just completed in DNA Profiling are to be presented in court. You need to complete the report below to inform the court about the work you've carried out and your conclusions.

Briefly outline the scientific techniques that you have used to analyse the samples

Present your results, fully labelled and explained so that anyone can understand what is shown

State your conclusions from the evidence that you have found

How would it be possible to extend this investigation?

PLEASE SUBMIT YOUR REPORT TO _____ BY _____.

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

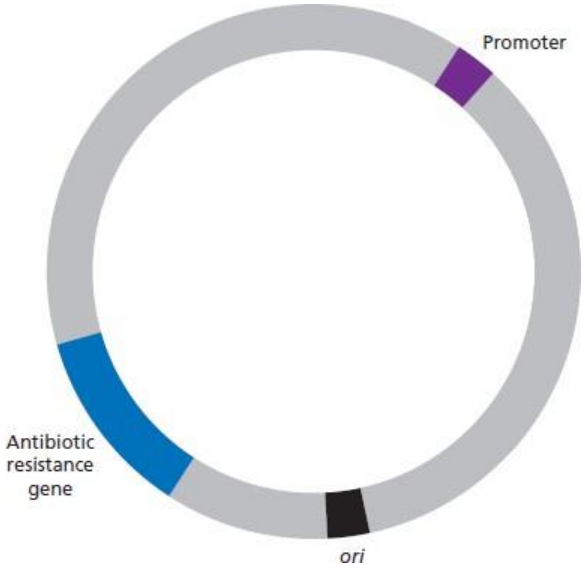


Figure C.1 A plasmid vector with 3 of the desirable characteristics shown.

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	*Hind*III	↓ 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

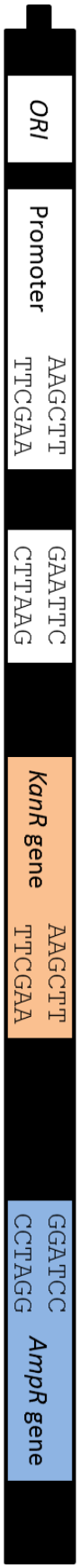
This ‘clone that gene’ activity gives you the opportunity to make a paper model of a recombinant plasmid that contains an insulin gene by completing these 3 stages of the procedure.

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 sellotape, 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.

Paper-based DNA profiling

What is DNA profiling?

In 1984, Alec Jeffreys described a method for DNA profiling using Variable Number Tandem Repeats (VNTRs), whilst working at the University of Leicester. The possibility of producing DNA profiles that could be used in criminal investigations was quickly recognised and the UK National DNA database was set up. It started by examining the length of 6 VNTRs and X/Y chromosome information, but in 1999 it expanded to include the length of 10 VNTRs and X/Y chromosome information.

As more samples began to be analysed more rapid techniques for analysis than Southern blotting were developed. Now when DNA is obtained for analysis, multiple copies of the VNTR regions of an individual's DNA are made using the polymerase chain reaction (PCR). The amplified VNTR regions are separated by agarose gel electrophoresis to determine their length (in base pairs). This length is divided by the length of one VNTR, to determine how many copies of the VNTR an individual has. The number of each specific VNTR analysed that an individual has is stored as their DNA profile. In reality, this information is stored as a series of numbers, an example of which is in figure D.1.

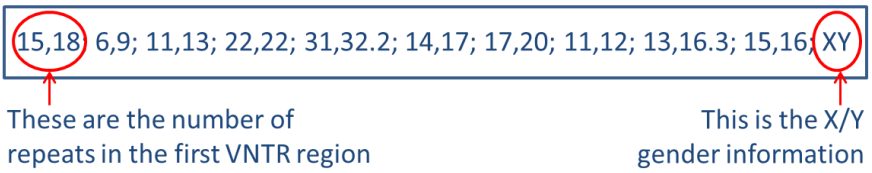


Figure D.1 An example of a DNA profile using 10 VNTRs and X/Y information

How are DNA profiles used?

The DNA profiles from the National DNA database can be matched to DNA left at a crime scene to help find who has been there. This is exactly what you will do in this exercise. You will need to:

1. Use the number of repeats of 4 VNTRS (FGA, VWA, D18551 and D19S433) and the known length of the repeats to calculate the length of the 8 VNTR regions (in base pairs).
2. Plot the 8 bands that would be produced for your suspect onto an agarose gel showing a DNA ladder with bands of known size.
3. Compare the DNA profile that you generate with the DNA profile obtained from the crime scene to work out whether the suspect that you are analysing was at the crime scene.

Information about the 4 VNTR regions you will use is given in table D.1 below.

VNTR name	Chromosome	VNTR length (bp)	Dye used
FGA	4	4	NED (red)
VWA	12	8	5-FAM (pink)
D18S51	18	4	JOE (green)
D19S433	19	4	NED (red)

Table D.1 Information on the 4 VNTRs being used to give a preliminary identification of a suspect

Suspect 1

Information on the suspect's DNA:

	Number of repeats for VNTR region			
Suspect	FGA	VWA	D18S51	D19S433
1	17 , 41	23 , 11	18 , 13	12 , 15

Your task is firstly to calculate the size of the VNTR fragments by multiplying the VNTR length (in base pairs) by the number of repeats (the first one is done for you).

Suspect	FGA repeats	Total size (bp)	VWA repeats	Total size (bp)	D18S51 repeats	Total size (bp)	D19S433 repeats	Total size (bp)
1	17 41	68	23 11		18 13		12 15	

Now plot the 8 bands onto the paper copy of an agarose gel using the DNA ladder to determine where DNA bands of different sizes would migrate to.

Suspect 2

Information on the suspect's DNA:

	Number of repeats for VNTR region			
Suspect	FGA	VWA	D18S51	D19S433
2	36 , 33	10 , 14	30 , 20	12 , 11

Your task is firstly to calculate the size of the VNTR fragments by multiplying the VNTR length (in base pairs) by the number of repeats (the first one is done for you).

Suspect	FGA repeats	Total size (bp)	VWA repeats	Total size (bp)	D18S51 repeats	Total size (bp)	D19S433 repeats	Total size (bp)
2	36 33	144	10 14		30 20		12 11	

Now plot the 8 bands onto the paper copy of an agarose gel using the DNA ladder to determine where DNA bands of different sizes would migrate to.

Suspect 3

Information on the suspect's DNA:

	Number of repeats for VNTR region			
Suspect	FGA	VWA	D18S51	D19S433
3	22 , 39	23 , 18	37 , 14	11 , 15

Your task is firstly to calculate the size of the VNTR fragments by multiplying the VNTR length (in base pairs) by the number of repeats (the first one is done for you).

Suspect	FGA repeats	Total size (bp)	VWA repeats	Total size (bp)	D18S51 repeats	Total size (bp)	D19S433 repeats	Total size (bp)
3	22 39	88	23 18		37 14		11 15	

Now plot the 8 bands onto the paper copy of an agarose gel using the DNA ladder to determine where DNA bands of different sizes would migrate to.

Suspect 4

Information on the suspect's DNA:

	Number of repeats for VNTR region			
Suspect	FGA	VWA	D18S51	D19S433
4	17 , 37	18 , 16	28 , 23	11 , 16

Your task is firstly to calculate the size of the VNTR fragments by multiplying the VNTR length (in base pairs) by the number of repeats (the first one is done for you).

Suspect	FGA repeats	Total size (bp)	VWA repeats	Total size (bp)	D18S51 repeats	Total size (bp)	D19S433 repeats	Total size (bp)
4	17 37	68	18 16		28 23		11 16	

Now plot the 8 bands onto the paper copy of an agarose gel using the DNA ladder to determine where DNA bands of different sizes would migrate to.

Suspect 5

Information on the suspect's DNA:

	Number of repeats for VNTR region			
Suspect	FGA	VWA	D18S51	D19S433
5	22 , 29	21 , 16	10 , 27	9 , 13

Your task is firstly to calculate the size of the VNTR fragments by multiplying the VNTR length (in base pairs) by the number of repeats (the first one is done for you).

Suspect	FGA repeats	Total size (bp)	VWA repeats	Total size (bp)	D18S51 repeats	Total size (bp)	D19S433 repeats	Total size (bp)
5	22 29	88	21 16		10 27		9 13	

Now plot the 8 bands onto the paper copy of an agarose gel using the DNA ladder to determine where DNA bands of different sizes would migrate to.

Suspect 6

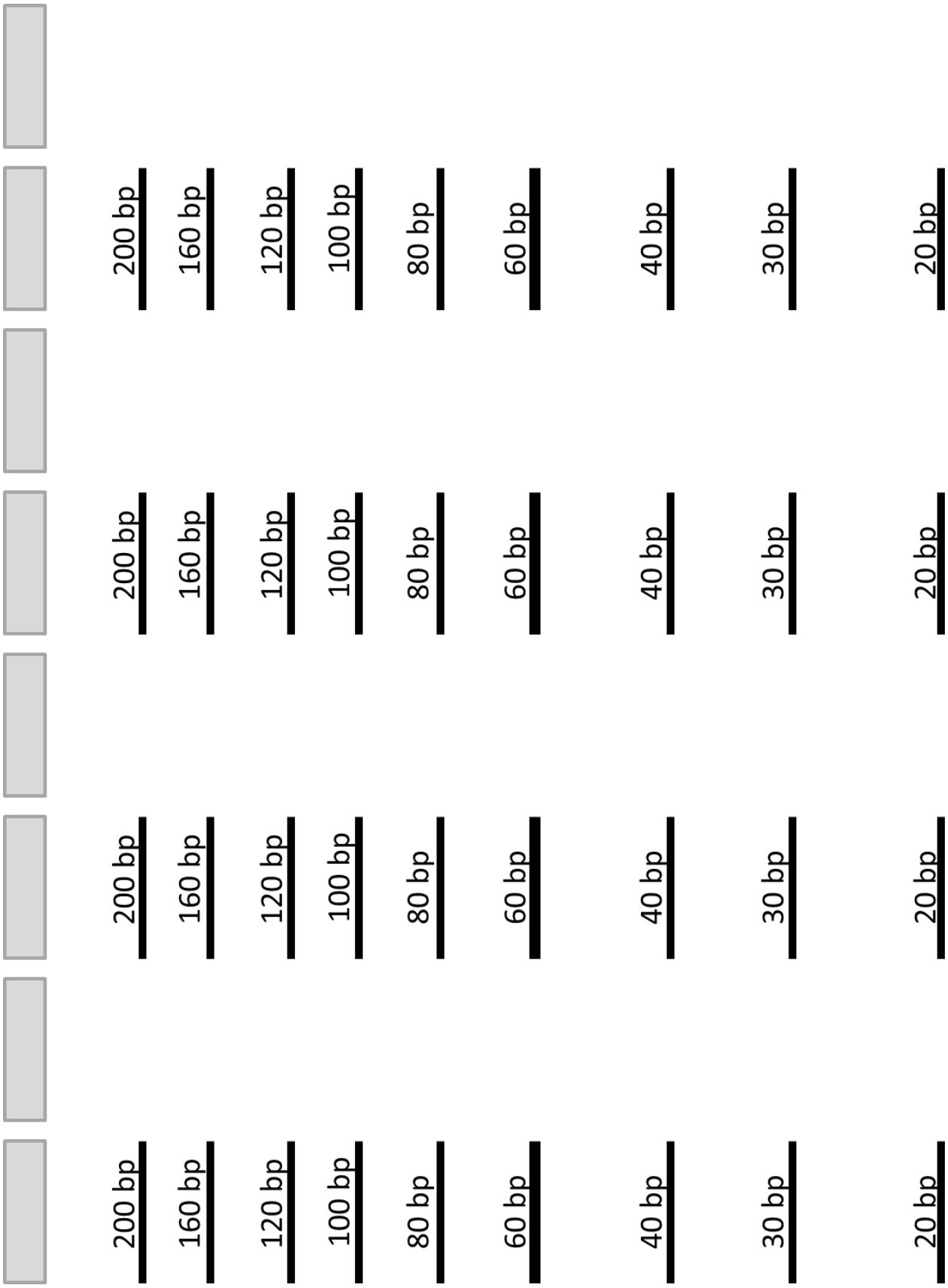
Information on the suspect's DNA:

	Number of repeats for VNTR region			
Suspect	FGA	VWA	D18S51	D19S433
6	42 , 31	14 , 20	33 , 16	10 , 14

Your task is firstly to calculate the size of the VNTR fragments by multiplying the VNTR length (in base pairs) by the number of repeats (the first one is done for you).

Suspect	FGA repeats	Total size (bp)	VWA repeats	Total size (bp)	D18S51 repeats	Total size (bp)	D19S433 repeats	Total size (bp)
6	42 31	168	14 20		33 16		10 14	

Now plot the 8 bands onto the paper copy of an agarose gel using the DNA ladder to determine where DNA bands of different sizes would migrate to.


200 bp
160 bp
120 bp
100 bp
80 bp
60 bp
40 bp
30 bp
20 bp

Teacher notes

To complete this exercise each student will require:

- The sheet with information on 'what is DNA profiling?' and 'How is DNA profiling used?'
- Data on the DNA profile of one of the six suspects
- An OHT with the mock agarose gel wells and DNA ladder on it
- OHT pen and calculator

After reading the instructions, the student should calculate the length of the VNTR regions for the suspect they have got. Answers to the calculations are given in table D.2 below.

Suspect	FGA repeats	Total size (bp)	VWA repeats	Total size (bp)	D18S51 repeats	Total size (bp)	D19S433 repeats	Total size (bp)
1	17 41	**68** **164**	23 11	**184** **88**	18 13	**72** **52**	12 15	**48** **60**
2	36 33	**144** **132**	10 14	**80** **112**	30 20	**120** **80**	12 11	**48** **44**
3	22 39	**88** **156**	23 18	**184** **144**	37 14	**148** **56**	11 15	**44** **60**
4	17 37	**68** **148**	18 16	**144** **128**	28 23	**112** **92**	11 16	**44** **64**
5	22 29	**88** **116**	21 16	**168** **128**	10 27	**40** **108**	9 13	**36** **52**
6	42 31	**168** **124**	14 20	**112** **160**	33 16	**132** **64**	10 14	**40** **56**

Table D.2 Length of the VNTR regions for the 6 suspects.

Students will then plot bands of these sizes onto their mock agarose gel OHT. If they then work in groups of 6, with each group having suspects 1-6, they should be able to produce an OHT, stuck together with sellotape that shows the DNA profiles of the 6 possible suspects. The expected results are shown in figure D.2.

You should then produce a DNA profile of the crime scene DNA on an OHT (figure D.3) and the group can match the DNA profile to find out which of their suspects could have been present at the crime scene. When compared to the crime scene DNA, students should find that suspect 6 matches for the 4 VNTR region sizes tested.

Figure D.2 Expected results for the 6 DNA profiles on the mock agarose gel.

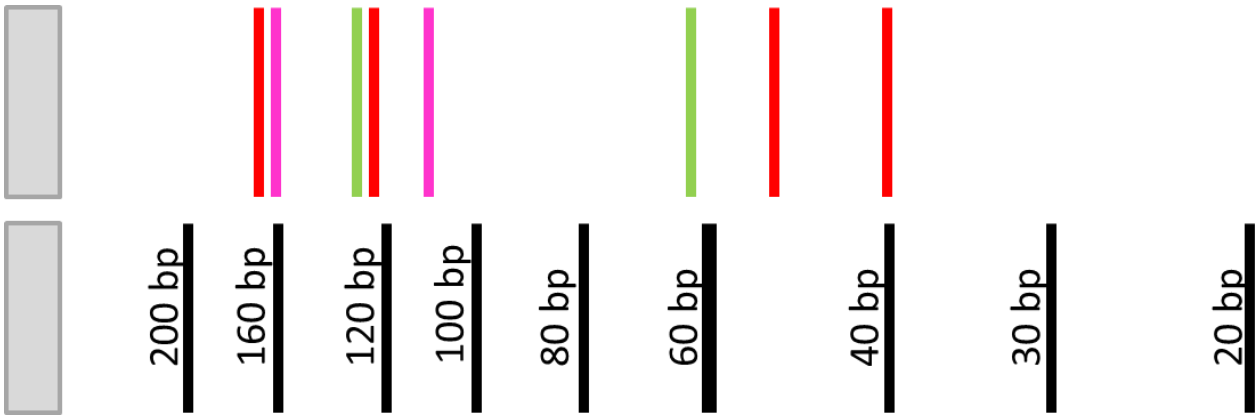


Figure D.3 The crime scene DNA

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).

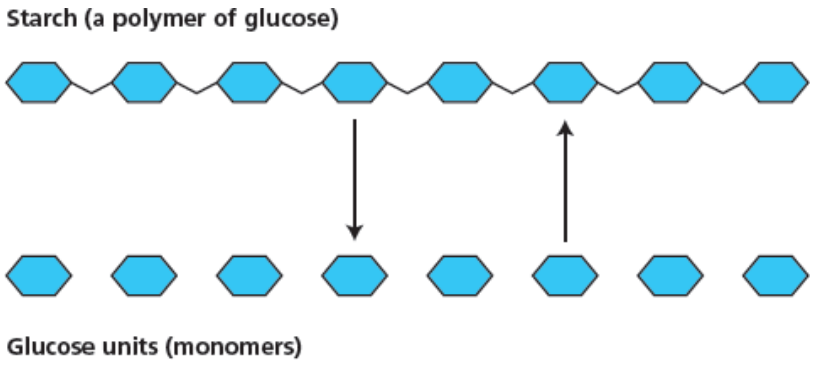


Figure R.1a: Starch is made up of subunits of glucose bonded together.

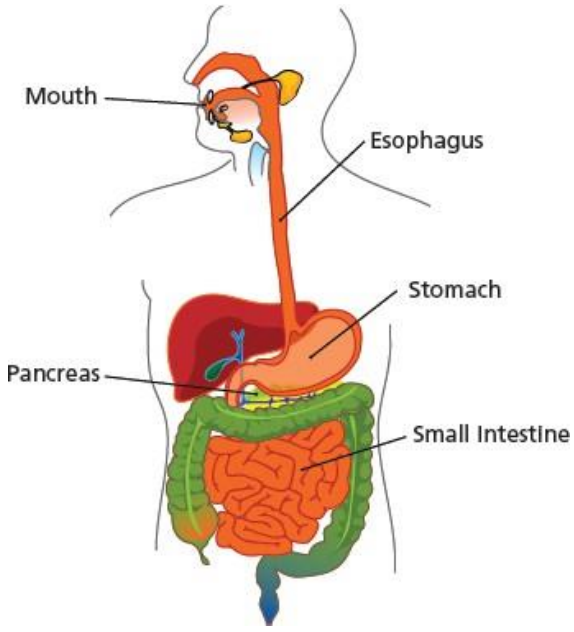


Figure R.1b: Nutrients such as starch are broken down into smaller molecules during digestion in the mouth, oesophagus, and stomach.

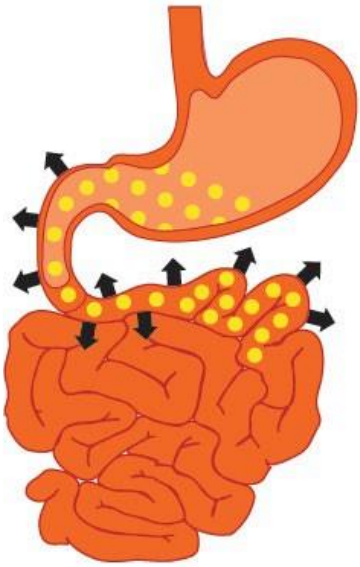


Figure R.1c: Glucose molecules pass through the small intestinal wall into the bloodstream, which delivers the glucose to cells in the body.

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.

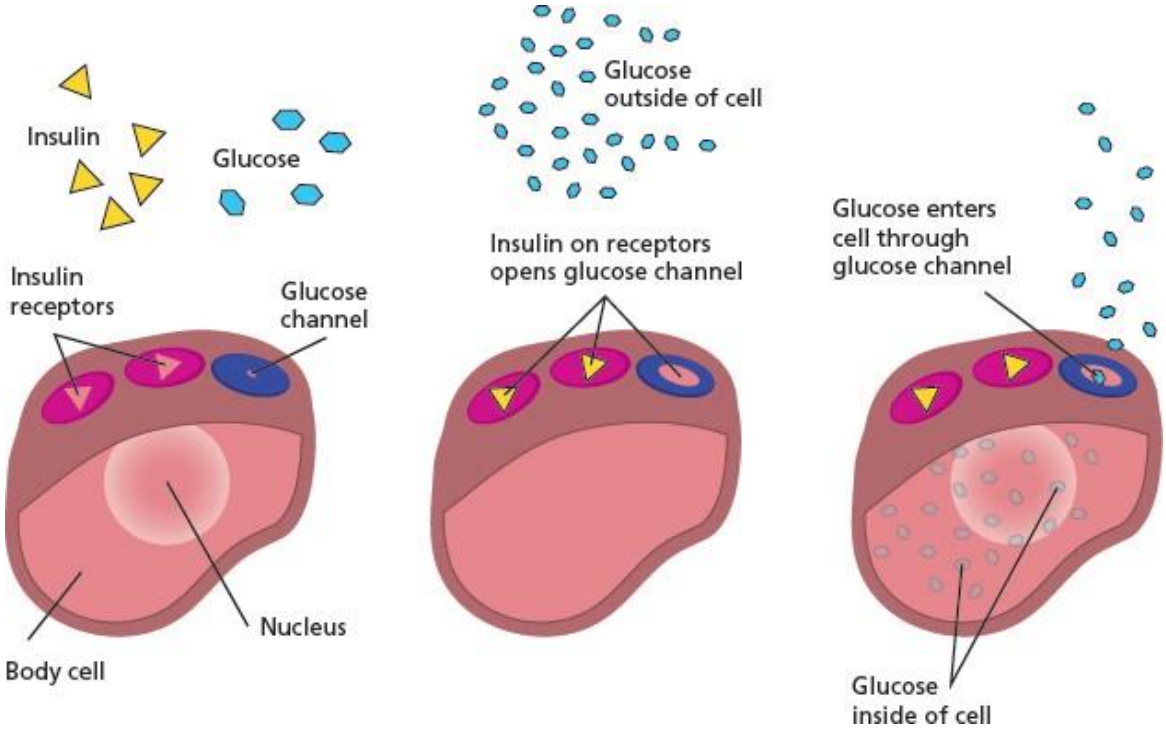


Figure R.2: How glucose crosses the cell membrane. Insulin in the blood binds to specific receptors on the cell. This binding alters the conformation of the cell membrane, resulting in the formation of a glucose channel. Glucose in the blood can now enter the cell through these channels.

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.

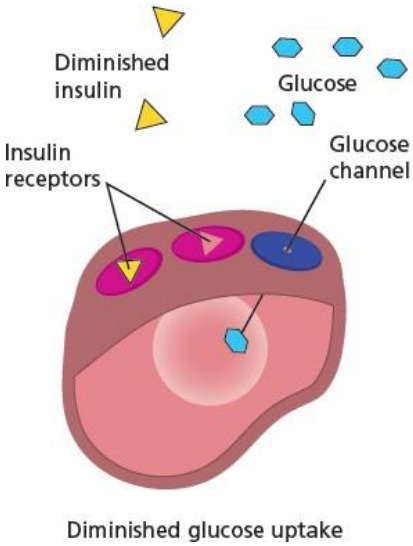


Figure R.3a: In type 1 diabetes, there is a lack of insulin in the body.

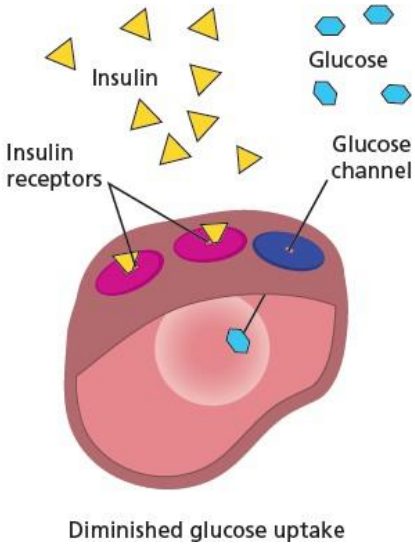


Figure R.3b: In type 2 diabetes, cells cannot bind the insulin.

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.)

Red fluorescent protein in sea anemones: why glow?

Red fluorescent protein is derived from a protein found in sea anemones. While sea anemones are sedentary, remaining attached to rocks, they are also predatory animals, using their stinging tentacles to catch their prey. The protein glows because it can absorb one colour of light and then emit light of a different colour—a process known as *fluorescence*. But why is it important for sea anemones to fluoresce? Our best guess is that fluorescent proteins somehow help sea anemones survive, but the role these proteins play is not yet well understood. Fluorescent molecules may serve as a sunblock, turning harmful UV light into light that is less damaging to the anemone's tissues. Another possibility is that while humans can't detect the fluorescence in bright sunlight, some animals may be able to, causing prey to be attracted to the glow.

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?

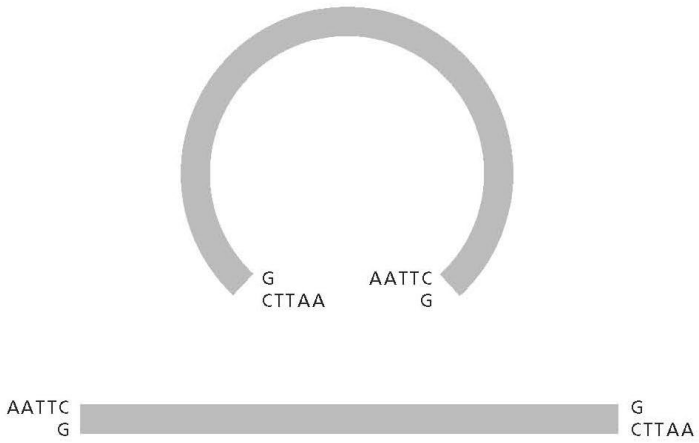
   The bacteria might have a way to protect their own DNA in sequences where it could be cut by the restriction enzyme, or their DNA might not have the sequence that is cut by the restriction enzyme.

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

The components of the pARA-R plasmid

The recombinant plasmid that is used in this program to clone the *rfp* gene is the pARA plasmid. Many different types of plasmid vectors have been developed to clone genes. All have the basic components needed to clone and express genes in bacteria, including a sequence for initiating DNA replication (the *ori* site), a promoter sequence for initiating transcription, a selectable marker, and a restriction site or sites near the promoter to insert the gene of interest. The pARA-R plasmid has been constructed such that the *rfp* gene will be inserted using *Bam*HI and *Hin*dIII. The use of two different restriction enzymes ensures that the *rfp* gene will be inserted in only one orientation, the one that is appropriate for transcribing the sense strand of the DNA. You may want to review the idea of "sense" and "anti-sense" strands with the students.

The pARA-R plasmid has been constructed to include components of the arabinose operon, which allow the expression of the *rfp* gene to be regulated. All living organisms, including bacteria, have the ability to regulate the expression of their genes. The most obvious example of this regulation is in cellular differentiation in multicellular organisms. Most cells contain the full complement of DNA, but during differentiation only certain genes are expressed as cells become skin, muscle, or root cells. Much of this regulation occurs at the level of initiation of transcription. Initiation of transcription can be turned on by proteins called *activators*, or turned off by proteins called *repressors*. While bacteria do not differentiate, they do respond to environmental conditions, such as the presence or absence of a sugar, including arabinose. The arabinose operon is a classic example of gene regulation in bacteria (see figure R.4). The operon comprises three genes, *araB*, *araA*, and *araD*, which code for proteins responsible for the transport and breakdown of arabinose. It also includes a promoter and a DNA sequence 5' proximal to the promoter sequence, which binds the AraC protein. The AraC protein regulates the expression of the arabinose genes by either allowing their transcription in the presence of arabinose or turning off gene expression in its absence.

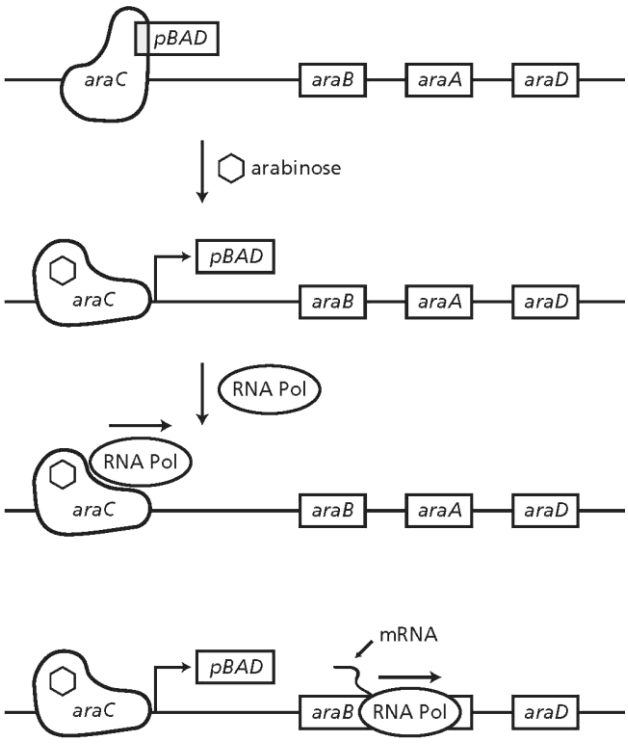


Figure R.4 The arabinose operon

In the absence of arabinose, the AraC protein blocks the binding of RNA polymerase to the promoter; initiation of transcription therefore cannot occur, and the three genes—*araB, araA,* and *araD*—are not expressed. When arabinose is present in the environment, the sugar binds to the AraC protein, which alters the shape of the DNA in such a way that RNA polymerase can bind to the promoter and the *araB, araA,* and *araD* genes are expressed. The plasmid pARA-R used in this program has the *pBAD* promoter and the *araC* gene, as well as the genes for resistance to ampicillin and kanamycin. The *araB, araA,* and *araD* genes have been removed and replaced by the *rfp* gene, putting the *rfp* gene under the control of the arabinose promoter. Colonies of bacteria harboring this plasmid will be red in the presence of arabinose and white in its absence.

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	*Hind*III	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.

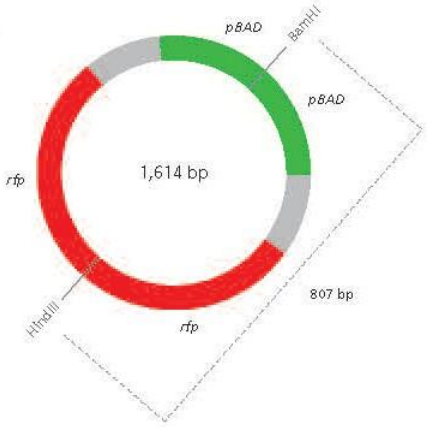
1. What role do DNA ligases have in nature?

   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).

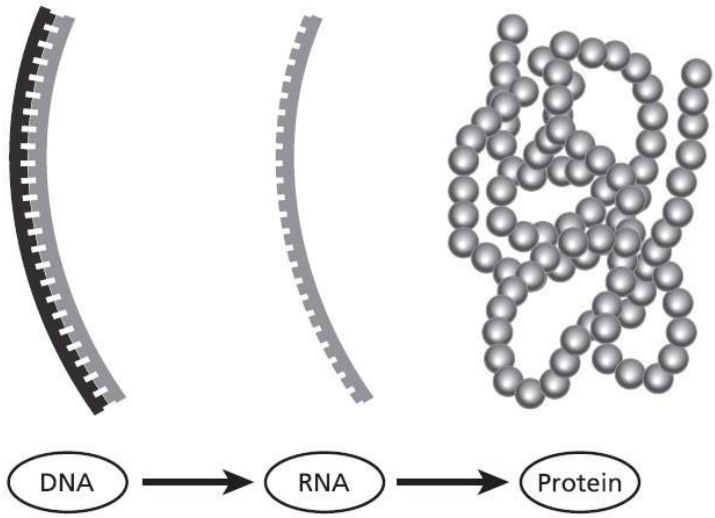


Figure R.5 The central dogma

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).

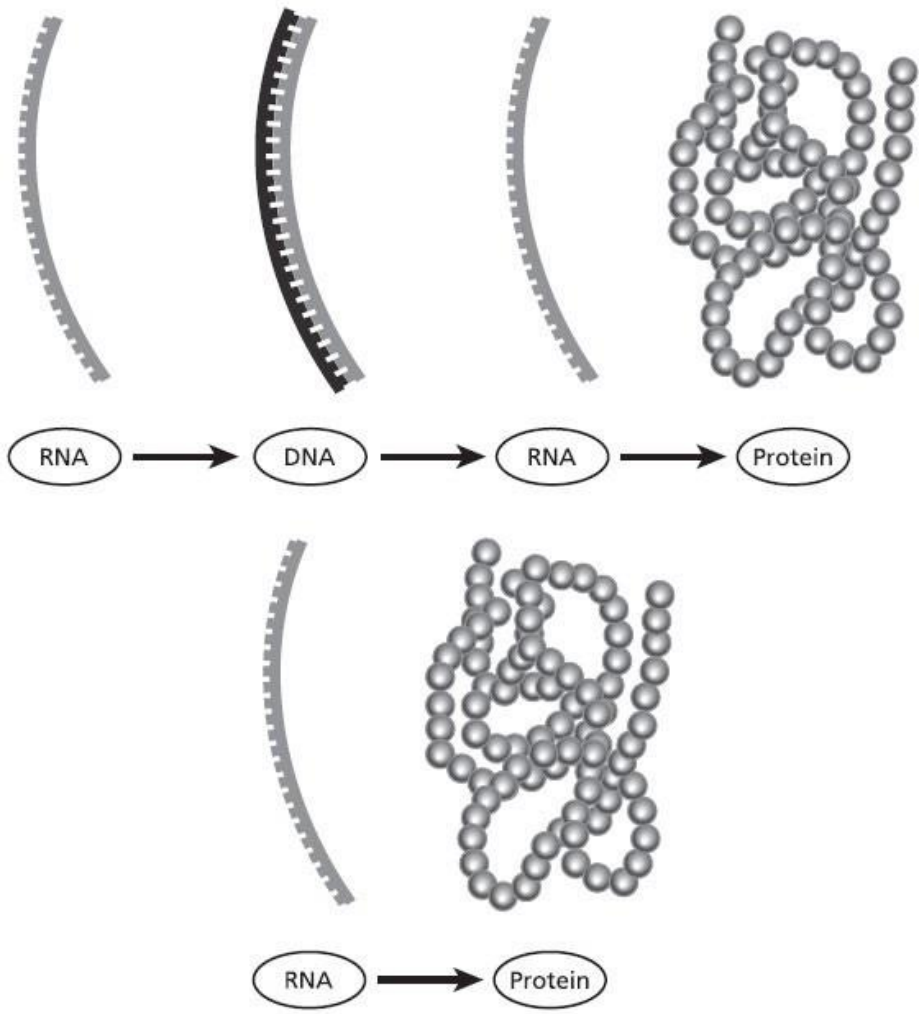


Figure R.6 Alternative pathways for the central dogma

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

Video resources for PCR and more

Some video resources are suggested below, which may help you with explanations about either the technical skills or scientific knowledge included in this laboratory.

Subject content	Length	Location
A nice overview of PCR from Youtube. Made by the DNA Learning Centre in New York it shows the temperature on a thermometer alongside the animation of PCR.	1 min 28 sec	https://youtu.be/2KoLnIwoZKU?si=LMWOxddi9Ko0qhOl
More PCR resources from the DNA learning Centre in New York: about Kary Mullis and the number of DNA copies produced after each PCR cycle.	Worth a few minutes exploring	https://dnalc.cshl.edu/resources/3d/19-polymerase-chain-reaction.html
The 2D and 3D animations are well worth a view	Worth a few minutes exploring	2d – https://dnalc.cshl.edu/view/17044-Polymerase-chain-reaction-PCR-.html 3d - https://dnalc.cshl.edu/view/15625-Polymerase-chain-reaction-PCR-.html
PCR virtual lab. A description of what happens in a PCR and a chance to try it yourself on the computer in the Utah Universities virtual lab!	9 min 30 sec	https://learn.genetics.utah.edu/content/labs/pcr/
The Biorad PCR song (as featured during training).	2 min 13 sec	https://youtu.be/FpxwJNNufko?si=iBF2QzwOw1TIYwFp
Videos to support all the ABE Labs …		
Playlist of useful videos linked closely to the **ABE labs** to support the programme including How to use a micropipette, preparation of agarose gels and several animations	6 videos	https://www.youtube.com/playlist?list=PLUDxmv1yB6zk0AjxKUnFPb59c13433aT9

Articulate for PCR

To encourage peer-learning of vocabulary and new scientific concepts you can use the game of Articulate. Print and cut out the word cards below. Put them into an envelope. Arrange the students into groups. Get them to pick out a word, without showing it to their group. Without saying the word, they must describe it so that another member of their group can guess it.

The words below relate to this practical activity, but you can increase the number of words to suit the time available, or scope of learning for the lesson.

Agarose gel	Deoxynucleotide triphosphates	Lyse
Alleles	DNA (deoxyribonucleic acid)	Micropipette
Amplification	DNA polymerase	Nuclease
Annealing	Extension	Nucleotide
Buccal epithelial cells	Electrophoresis	Primer
Buffer	Gene	Protein
Clone	Heterozygous	Supernatant
Co-factor	Homozygous	Thermal cycling
Complementary DNA	*In vitro*	Thermophilic
Denaturation	Intron	*Thermus aquaticus (Taq)*
Dimorphic	Locus	

Questions and possible answers about DNA profiling

These 'prior knowledge' questions are intended to find out students' understanding prior to teaching of this episode on DNA profiling. After producing written answers, students can share their thinking. Peer-learning is a powerful tool in formulating understanding, and all students should be encouraged to participate in a supportive environment. You may wish to gently address misconceptions or note areas of interest to the students for further discussion in the future.

1. **What is a DNA profile?**

   Answers will vary but may describe a DNA profile as a banding pattern of DNA fragments, which is unique to each individual, like a genetic fingerprint.

2. **How do scientists create a DNA profile?**

   Answers may include a description of how DNA is cut into fragments (using restriction enzymes), differently sized fragments are separated (by agarose gel electrophoresis) then either visualised using a DNA stain (a simplified version of the procedure), or the DNA fragments containing Variable Number Tandem Repeats (VNTRs) are detected using fluorescently-labelled DNA probes.

   Some students may know about how the amount of DNA for profiling is amplified using PCR. Some may have read about how capillary electrophoresis can be used to separate the differently-sized fragments, as an alternative to agarose gel electrophoresis.

3. **DNA profiles are stored in National DNA databases. Who oversees National DNA databases and why can storing DNA profiles in them be controversial?**

   Consider issues such as whose DNA should be held, how long for, who the DNA belongs to, who has access to the DNA profiles and how secure is the information?

   This is intended to begin to elicit opinions on the implications of holding DNA profiles in the National DNA database. Some delicacy may be required as it is quite likely that the students will know someone whose DNA profile is held (about 10% of the population of England and Wales have DNA profiles in the National DNA database, largely due to the ability of the Police in these areas to take DNA samples, without consent, from anyone arrested on suspicion of any recordable offence (like begging or being drunk and disorderly) between April 2004 and December 2008).

   A greater percentage of the population in this country have their DNA profiles stored in our National DNA database, than in any other. After a ruling in December 2008 by the European Court of Human Rights, that DNA profiles of innocent people should not be retained on the UK National DNA database, more than 1 million DNA profiles are being removed. Otherwise DNA profiles are deleted from the database when the person to whom they belong reaches 100 years old.

   The Home Office took over running of the National DNA database from the National Policing Improvement Agency (NPIA) in October 2012, and is now responsible for safeguarding the information and providing information to the police forces to aid in criminal investigations. Attempts have been made to access data for personal gain (see: http://www.dailymail.co.uk/news/article-445902/Five-civil-servants-suspended-DNA-espionage.html) so there are justified concerns about security and access.

Video resources for DNA profiling

Some video resources are suggested below, which may help you with explanations about either the technical skills or scientific knowledge included in this laboratory.

Subject content	Length	Location
A nice basic summary of DNA profiling from the Naked Scientists on Youtube	5 min 26sec	https://youtu.be/ZxWXCT9wVoI?si=zzJBdb-iNK-VMUOW
Restriction enzymes: an animation of restriction enzymes operating, produced by the DNA Learning Center in New York.	Advanced by clicking	https://dnalc.cshl.edu/resources/animations/restriction.html
DNA Profiling overview from Bozeman Science	6 min 09sec	https://youtu.be/DbR9xMXuK7c?si=BG-XDbFgehj8GLWA

Articulate for DNA profiling

To encourage peer-learning of vocabulary and new scientific concepts you can use the game of Articulate. P**rint** and cut out the word cards below. Put them into an envelope. Arrange the students into groups. Get them to pick out a word, without showing it to their group. Without saying the word, they must describe it so that another member of their group can guess it.

The words below relate to this practical activity, but you can increase the number of words to suit the time available, or scope of learning for the lesson.

Agarose gel	DNA probe	National DNA database
Chromosome	DNA profile	Restriction enzyme
Diploid	Electrophoresis	Southern blot
DNA (**d**eoxyribo**n**ucleic **a**cid)	Micropipette	Variable Number Tandem Repeat (VNTR)

EXTENSION: DNA PROFILING AND CRIME

The possibility of using the DNA profiling technique described by Alec Jeffreys in 1984[1], to produce DNA profiles that could be used in criminal investigations, was quickly recognised[2]. After trying several different VNTR markers, the UK National DNA database was set up in April 1995[3]. Other DNA databases were subsequently set up in other countries. Some are shown in table 1 below.

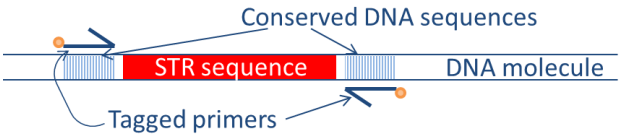
Country	Name of database	Start date
United Kingdom	UK National Criminal Intelligence DNA Database[3]	April 1995
New Zealand	National DNA Databank[6]	1996
France	Fichier National Automatisé des Empreintes Génétiques (This translates as the, 'Automated National File of Genetic Prints')[7]	June 1998
United States of America	Combined DNA Index System (CODIS)[8]	October 1998

Table 1. National DNA databases.

Initially the UK National DNA database examined the length of 6 VNTRs and X/Y chromosome information[3], but in 1999 it expanded to include the length of 10 VNTRs and X/Y chromosome information[4]. Although monozygotic twins will never be able to be distinguished by DNA fingerprinting, the chance of 2 unrelated individuals having the same DNA profile at all 10 VNTR sites is estimated at 1 in 1 billion[5].

Due to the high usage of DNA profiling in criminal investigations, the methods used have advanced beyond Southern blotting. More rapid techniques for generating DNA profiles from crime scenes have been developed. The standard method is outlined below.

1. Sections of DNA recovered from a crime scene are amplified (copied) using the Polymerase Chain Reaction (PCR). A PCR is set up with tagged primers that bind to specific, well-conserved sequences either side of a Short Tandem Repeat (STR) sequence. The PCR will create many copies of the sequence between the primers.



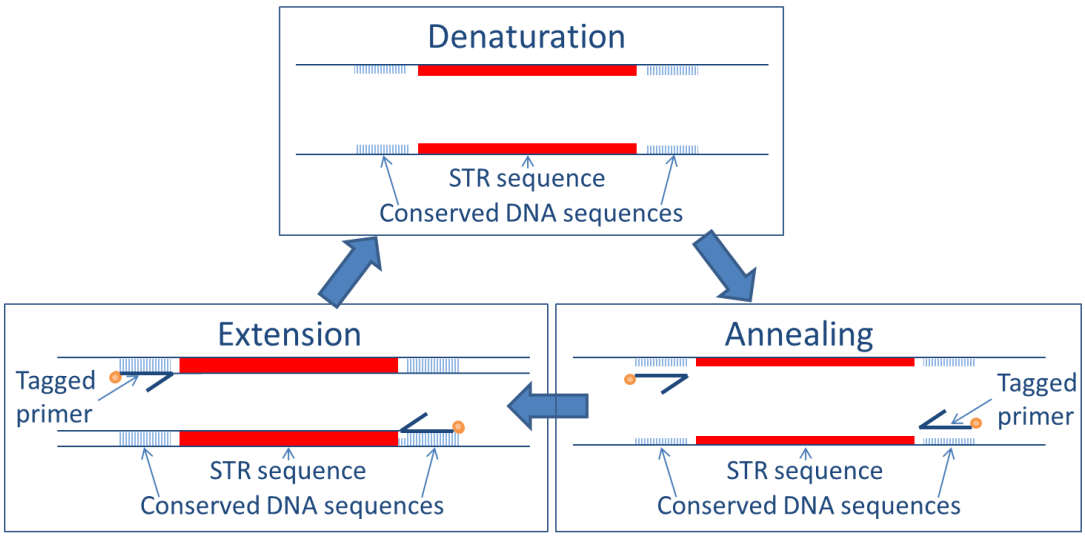
A Short Tandem Repeat (STR) is a common type of Variable Number Tandem Repeat (VNTR). In fact, about 3% of the human genome consists of STR sequences[10].

Ten regions of DNA containing STR sequences are amplified by PCR. These ten regions are shown in table 2. Three different chemical tags are used on the primers. These are also shown in table 2.

Name of VNTR	Chromosome no.	Sequence of repeat	Tag on primer	No. of repeats
FGA	4	CTTT	NED	12.2–51.2
TH01	11	TCAT	NED	3–14
VWA	12	[TCTG][TCTA]	5-FAM	10–25
D2S1338	2	[TGCC][TTCC]	5-FAM	15-28
D3S1358	3	[TCTG][TCTA]	5-FAM	8-21
D8S1179	8	[TCTA][TCTG]	JOE	7 - 20
D16S539	16	GATA	5-FAM	5 - 16
D18S51	18	AGAA	JOE	7–39.2
D19S433	19	AAGG	NED	9 – 17.2
D21S11	21	Complex [TCTA][TCTG]	JOE	12 – 41.2
Amelogenin	X / Y		JOE	

Table 2. The VNTR sequences used for the UK National Criminal Intelligence DNA Database[9, 13].

The PCR uses multiple cycles of; (i) denaturing DNA at temperatures between 94 and 98°C, to separate the two DNA strands, (ii) annealing (binding) of primers to the single-stranded DNA at temperatures between 50 and 65°C, then (iii) extending the short double-stranded length of DNA using a DNA polymerase from a thermophilic bacteria at 72°C. An illustration of PCR is shown below.

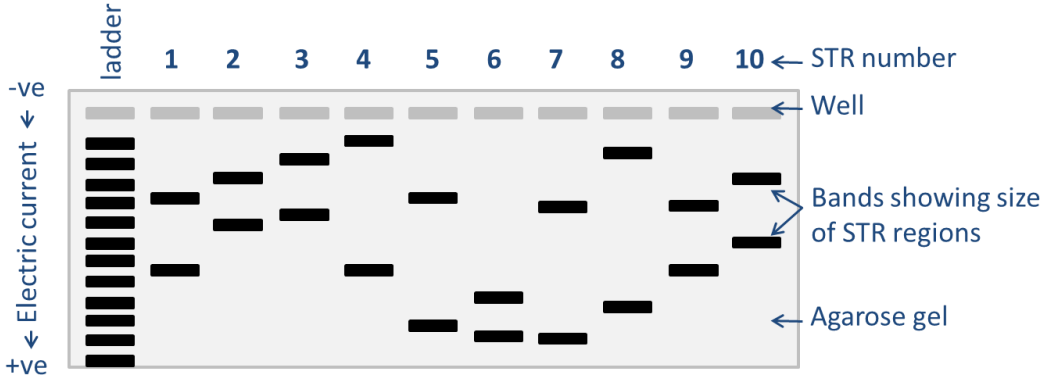


2. Agarose gel electrophoresis is used to determine the size of the STR region.

   Two differently-sized STR regions will be present in diploid somatic cells. These will show up as two bands by agarose gel electrophoresis. One STR region will be inherited from each parent. This is why more closely related individuals will have more similar DNA profiles.

   Agarose gel electrophoresis involves loading DNA into the wells of (holes in) an agarose gel. When an electric current is put through the gel, the DNA (which is negatively charged) moves toward the anode. Smaller molecules move faster and larger molecules move more slowly through the honeycomb-like agarose gel, therefore the DNA molecules become separated by size.

   The relative size of different STR regions can be found by comparison to a 'DNA ladder', which has bands of known size. An example of what this looks like is shown below.



3. In automated systems lasers detect the position of the 'tags', and therefore the size of the DNA. These generate a DNA profile, which consists of 20 numbers, each relating to the number of STR sequences at one of the ten STR loci used[4].

   This information, along with information from the amelogenin sex-typing test[11], is stored on the UK National DNA database. The STR region data is always placed in the same order within a DNA profile[12].

An example of a DNA profile created using SGM Plus

15,18; 6,9; 11,13; 22,22; 31,32.2; 14,17; 17,20; 11,12; 13,16.3; 15,16; XY

These are the number of alleles for the first STR region, worked out from the size of the bands on the agarose gel

Gender information from the amelogenin sex-typing test

4. DNA profiles are stored in a database (see Table 1). The majority of work on criminal DNA profiling in the UK is now performed using a system called SGM Plus to complete this work. It is manufactured by Applied Biosystems[13].

References

1 Jeffreys, A. J., Wilson, V. and Thein, S. W. (1984) Hypervariable 'minisatellite' regions in human DNA. *Nature 314*; 67-73.

2 Kimpton, C. P., Gill, P., Walton, A. *et al.* (1993) Automated DNA profiling employing multiplex amplification of short tandem repeat loci. *PCR Methods and Applications 3*; 13–22.

3 Werrett, D. J. (1997) The national DNA database. *Forensic Science International 88*; 33–42.

4 Cotton, E. A., Allsop, R. F., Guest, J. L. *et al.* (2000) Validation of the AMPFlSTR SGM plus system for use in forensic casework. *Forensic Science International 112*; 151–161.

5 http://www.parliament.uk/documents/post/postpn258.pdf , accessed on 11th March 2013

6 http://www.esr.cri.nz/competencies/forensicscience/dna/Pages/DNAdatabank.aspx , accessed on 28th March 2013

7 http://www.assemblee-nationale.fr/12/rap-info/i0504.asp , accessed on 28th March 2013

8 http://www.fbi.gov/about-us/lab/biometric-analysis/codis/codis-and-ndis-fact-sheet , accessed on 28th March 2013

9 Butler, J. M. (2006) Genetics and Genomics of Core Short Tandem Repeat Loci Used in Human Identity Testing. *Journal of Forensic Science 51*; 253-265.

10 Lander, E. S., Linton, L. M., Birren, B. *et al.* (2001) Initial sequencing and analysis of the human genome. *Nature 409*; 860–921.

11 Sullivan, K. M., Mannucci, A., Kimpton, C. P. *et al.* (1993) A rapid and quantitative DNA sex test: fluorescence-based PCR analysis of X–Y homologous gene amelogenin. *BioTechniques 15*; 637–641.

12 http://www.npia.police.uk/en/13340.htm , accessed on 11th March 2013

13 http://www3.appliedbiosystems.com/cms/groups/applied_markets_support/documents/generaldocuments/cms_041049.pdf , accessed on 29th March 2013

EXTENSION: NATIONAL DNA DATABASES

What are National DNA databases?

Banks of information about individuals' DNA, usually held electronically by the Government and used by law enforcement agencies to identify suspects of crimes. In the UK, the database is now known as The National DNA Database (**NDNAD**)

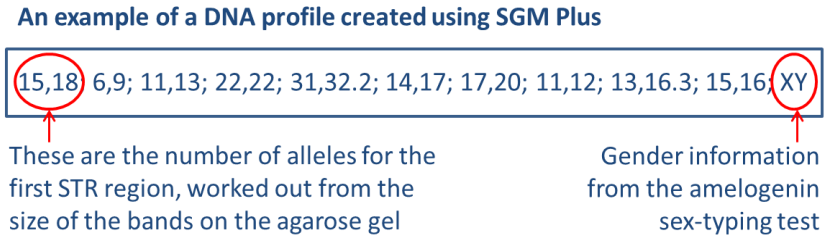
How many samples are held on the UK National Criminal Intelligence DNA Database?

As at 31st March 2023, NDNAD held 7,045,155 subject profile records. (on 30th September 2012, there was DNA profiling information from an estimated 6,070,673 individuals). More data available at Forensic Information Databases Strategy Board Annual Report April 2022 - March 2023

What information is kept in the UK National Criminal Intelligence DNA Database?

Records of the allele lengths at 10 loci and a sex determination marker.

What does a DNA profile on the UK National Criminal Intelligence DNA Database look like?



What are the chances of 2 unrelated people having the same DNA profile?

The chance of two unrelated individuals having matching full SGM Plus profiles is less than 1 in a billion (1,000,000,000). To date, no two unrelated people have been found with the same SGM Plus DNA profile on the UK National Criminal Intelligence DNA Database.

Whose samples are held in the National DNA database in the UK?

In England and Wales anyone arrested and detained in a Police station on a recordable offence can be asked to provide a DNA sample. Recordable offences include begging, being drunk and disorderly and taking part in an illegal demonstration. DNA profiles have been kept even if the person arrested was never charged. This has led to more DNA samples being put onto the database than in any other country (nearly 10% of the population is on the UK National Criminal Intelligence DNA Database, compared to Austria, which has the next highest percentage of just over 1%).

This issue has begun to be addressed with the Protection of Freedoms Act, adopted by the Coalition Government on 1st May 2012. This will remove DNA profiles of over 1 million innocent people from police databases.

What are people's concerns about privacy?

DNA profiles and DNA samples are held by a private company on behalf of the Home Office, who took responsibility for the UK National Criminal Intelligence DNA Database from the National Policing Improvement Agency (NPIA) on the 1st October 2012. There have been concerns about privacy (http://www.guardian.co.uk/politics/2009/jul/19/dna-database-crime-privacy-discrimination, accessed on 27th April 2013) and corrupt individuals removing valuable genetic data without consent (http://www.dailymail.co.uk/news/article-445902/Five-civil-servants-suspended-DNA-espionage.html#axzz2K1V0Chaq, accessed on 27th April 2012).

Questions to explore

1. In March 2007, when the UK/English the National DNA database was run by the National Policing Improvement Agency (NPIA) (its running was taken over in October 2012 by the Home Office), five NPIA employees were accused of copying and storing DNA profiles, with the aim of setting up their own rival company.

   See: http://www.dailymail.co.uk/news/article-445902/Five-civil-servants-suspended-DNA-espionage.html

   Who should hold information on peoples' DNA profiles? How should the information be safeguarded?

2. Monozygotic twins would show the same DNA profile.

   How may Police Officers investigating a crime scene be able to distinguish the perpetrator?

3. Sterile technique and avoidance of contamination are serious issues in the use of DNA technology. Police in Germany hunted a woman, nicknamed 'The Phantom of Heilbronn', whose DNA was found at 40 crime scenes between 1993 and 2009. When the DNA of this woman matched the DNA sample from a male asylum seeker in France, detectives began to doubt the evidence. It was later found that the DNA had come from a worker in a factory in Austria where they made the cotton swabs used for DNA sampling. Police had accidentally purchased swabs that were guaranteed to be sterile, but not DNA-free.

   See: http://news.bbc.co.uk/1/hi/world/europe/7966641.stm

   Who should have responsibility for purchasing, processing DNA samples and avoiding such incorrect analysis?

4. The STR regions used are some of the original ones found when they were described as tools for human identity testing in the early 1990s[9]. They continue to be used as they provide a link to historic DNA profile data.

   Should a new array of STR regions, offering better coverage of the genome and more accepting of DNA degradation be developed?

5. A greater population in this country have their DNA profiles stored in our National DNA database, than in any other. This is largely because, since April 2004, the Police in England have the right to take DNA samples without consent from anyone arrested on suspicion of any recordable offence (like begging or drunk and disorderly). In December 2008 the European Court of Human Rights ruled that samples of innocent people should not be retained on the UK National DNA database. Now more than 1 million DNA profiles are being removed from the UK National DNA database. Sir Alec Jeffreys believes this is happening too slowly.

   See: http://www.guardian.co.uk/politics/2009/may/07/dna-database-plans-condemned

   Whose DNA profile should be stored? Does is breach Human Rights to have your DNA sequence taken and held on the National database? How long should your DNA profile be held?