

The Lambda protocol

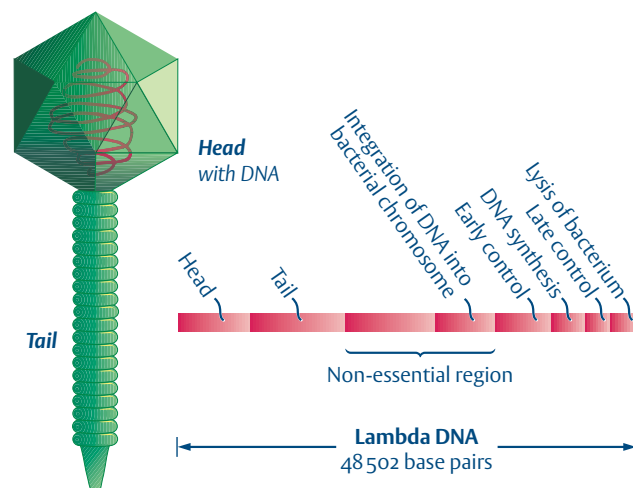
Student's guide

Bacteriophage lambda

Bacteriophages (literally: 'bacteria-eaters') are viruses that invade bacteria. To reproduce, bacteriophages (or phages) must take over the molecular machinery of their bacterial hosts. The phage lambda (λ) preys upon *Escherichia coli*. Lambda enjoys a choice of lifestyles — it can either multiply within its host and destroy it (the lytic cycle), or the λ DNA can be inserted into the bacterial chromosome and remain dormant there for several generations (the lysogenic cycle). An environmental trigger *e.g.*, ultraviolet light, triggers the lytic cycle and activates the phage once more.

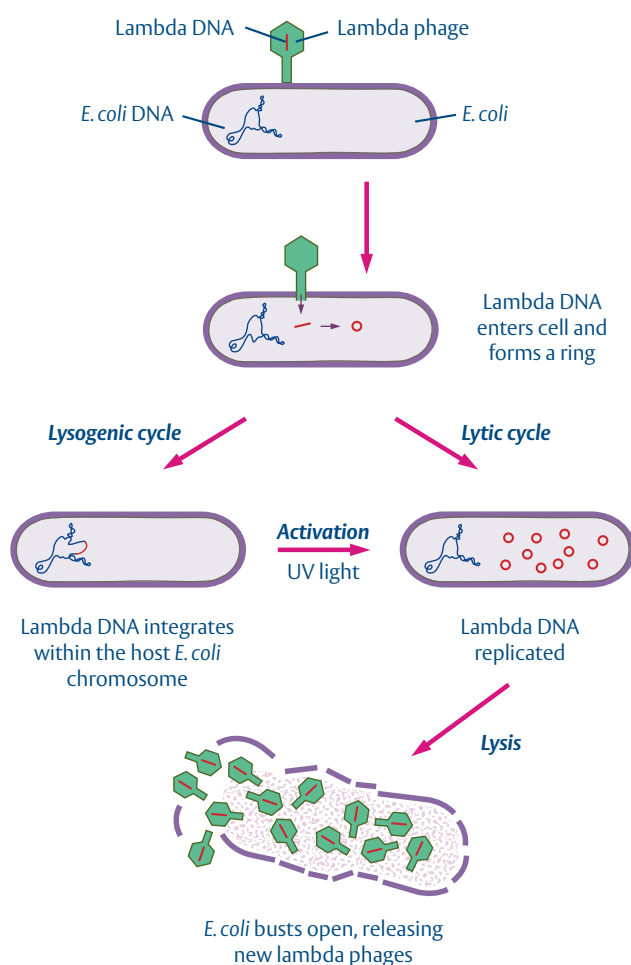
Phage λ is a relatively simple organism. It consists of a double-stranded length of DNA wrapped around a core of protein and encapsulated in a protein coat.

Lambda's entire genome has been sequenced and it is 48 502 base pairs long. Within this genome are genes that code for the virus's protein coat, bursting (lysis) of the bacterial cell, integration of λ DNA into the host's chromosome and so on. The order in which these genes are activated is important. For example, it would be of no benefit to the virus if the host bacterial cell was broken open (lysed) before new virus particles had been assembled. Consequently, λ has evolved an elaborate system of gene regulation, which has now been studied in great detail.



Bacteriophage lambda and some of the main groups of the 60 or so genes in its genome. The DNA can be linear or it can form a ring.

Relatively little of the λ genome is required to package DNA and deliver it into bacterial cells. About 20 000 base pairs can be deleted from its central region and replaced with DNA from another organism, without affecting the phage's viability. Several specially-constructed forms of λ can be used by molecular biologists to ferry new genes into bacteria.



*Infection of the bacterium *E. coli* by bacteriophage lambda, showing the lytic and lysogenic (latent) cycles.*

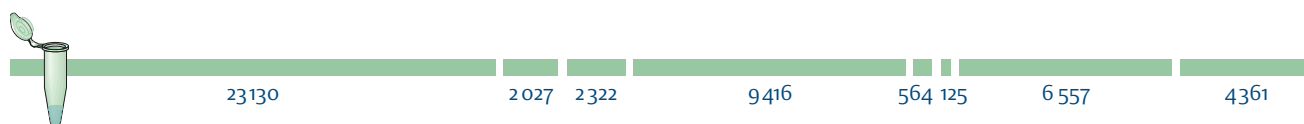
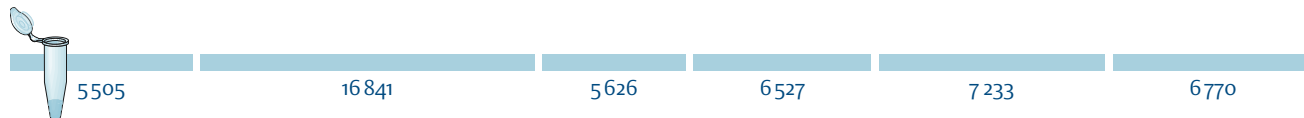
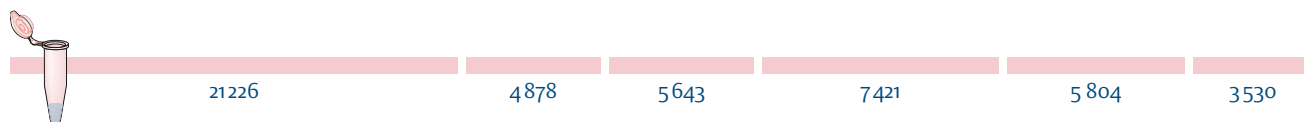
Restriction enzymes

Restriction endonucleases are enzymes that cut DNA. They are so called because they are made by bacteria to *restrict* the proliferation of invading viruses (such as phage λ). Different restriction enzymes cut at specific sequences of bases in the DNA. The bacterium's own DNA is protected by the addition of methyl ($-\text{CH}_3$) groups to adenine (A) or cytosine (C) bases at the sites that normally are 'recognised' by the enzymes.

Restriction enzymes take their names from the bacteria that produce them. *EcoRI* (the first such enzyme to be discovered) comes from *Escherichia coli*, strain RY13. The numeral I in the enzyme's name indicates that this was the first restriction enzyme found in *E. coli*. *BamHI* is obtained from *Bacillus amyloliquefaciens* H, (with the numeral I again showing that this was the first restriction enzyme found in this bacterium). *HindIII* is made by *Haemophilus influenzae* R_d. Here the III indicates that this was the third restriction enzyme isolated from *H. influenzae*.

Many restriction enzymes come from species that would be difficult or dangerous to cultivate. Safer, high-yielding strains of bacteria that have had genes encoding restriction enzymes introduced into them are therefore used for commercial production.

Enzyme name Source microorganism STRAIN	DNA base pair 'recognition' site (5'→3')
BamHI <i>Bacillus amyloliquefaciens</i> H	G↓GATCC
EcoRI <i>Escherichia coli</i> RY13	G↓AATTC
HindIII <i>Haemophilus influenzae</i> R _d	A↓AGCTT

HindIII**BamHI****EcoRI**

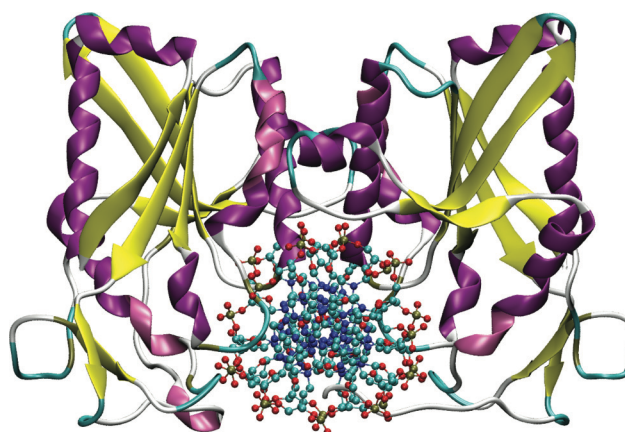
These restriction maps show where *EcoRI*, *BamHI* and *HindIII* cut lambda DNA. The numbers are the sizes of the fragments, in base pairs (bp). The dried enzymes in the kit are in colour-coded tubes.

DNA gel electrophoresis

Gel electrophoresis can be used to separate DNA fragments of different sizes. First, a gel is cast from agarose — a very pure form of agar, which is obtained from seaweed. At one end of the slab of gel are several small wells, made by the teeth of a comb that was placed in the gel before it set. A buffer solution is poured over the gel, so that it fills the wells and makes contact with electrodes at each end of the gel. Ions in the buffer solution conduct electricity. The buffer also stops the gel from drying out.

The invisible DNA fragments are mixed with a small volume of loading dye. This dye is dissolved in a dense sugar solution, so that when it is added to the wells, it sinks to the bottom, taking the DNA with it.

An electrical current is applied to the electrodes, setting up an electrical field across the gel. Phosphate groups give DNA fragments a negative electrical charge, so that the DNA migrates through the gel towards the positive electrode. Small fragments move quickly through the porous gel — larger fragments travel more slowly. In this way the



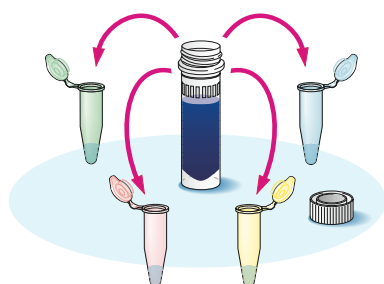
The restriction enzyme *BamHI* attached to a length of DNA, seen down the axis of the DNA. In the protein structure, β -pleated sheets are shown in yellow; α -helices in magenta.

pieces of DNA are separated by size. The loading dye also moves through the gel, slightly ahead of the DNA, so that the progress of the electrophoresis can be seen.

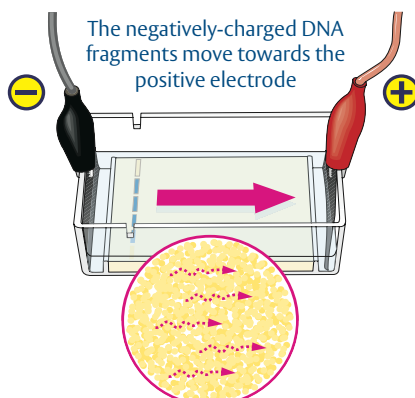
After electrophoresis, the gel is stained to reveal the DNA. In this investigation, each band is comprised of numerous fragments of λ DNA of the same size.

Summary of the protocol

Cut the lambda DNA with different restriction enzymes, then mix it with a heavy dye solution

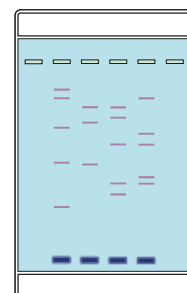


Load the DNA samples into 'wells' in an agarose gel



Large fragments move slowly through the gel, but small fragments move faster

Stain the gel to reveal the DNA fragments

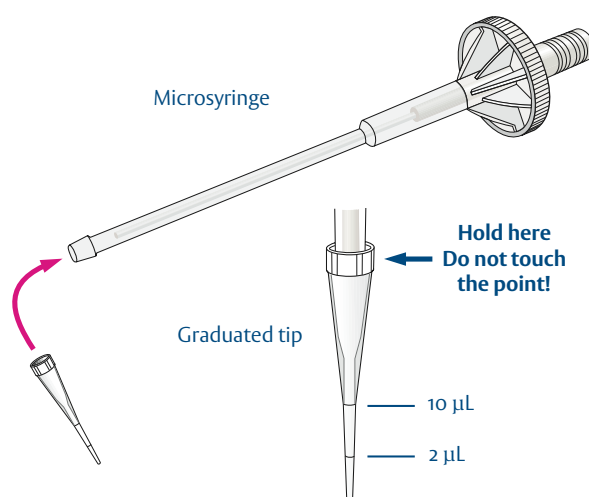


Different enzymes give different patterns of bands

1 Using the microsyringes

The microsyringes are designed to be fitted with disposable tips. Each tip should be used once only, then thrown away. The tips are marked at 2 and 10 microlitres (μL), allowing small volumes of liquid to be dispensed with precision. The best results will be obtained if you observe the following precautions:

- Never pull the plunger right out of the microsyringe — when the plunger is re-inserted the seal may become damaged;
- Before you load the microsyringe, pull the plunger out a little (1–2 mm). This will give you some extra air with which to expel the last drop of liquid from the tip;
- When you dispense liquids, hold the microsyringe as near to vertical as possible, and at eye level so that you can see what you are doing;
- Remove the liquid from the point of the microsyringe tip by holding it against the inner wall of the tube into which you are transferring the liquid;
- Do not touch the point of the microsyringe tip with your fingers. There are proteases and DNases in your sweat which may contaminate and degrade the reactants.



Mass

A microgram is one millionth of a gram
 $1000 \text{ micrograms } (\mu\text{g}) = 1 \text{ milligram (mg)}$
 $1000 \text{ milligrams} = 1 \text{ gram (g)}$

Volume

A microlitre is one millionth of a litre
 $1000 \text{ microlitres } (\mu\text{L}) = 1 \text{ millilitre (mL)}$
 $1000 \text{ millilitres} = 1 \text{ litre (L)}$

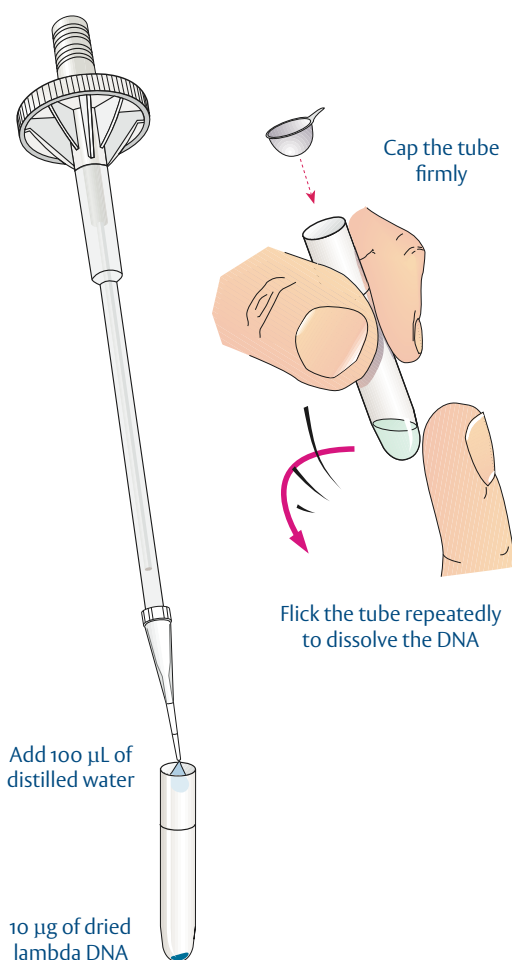
2 Rehydrating the dried DNA

- Add 100 μL of distilled water to the λ DNA tube (the narrow white tube). Use the microsyringe and the 10 μL graduation on the tip 10 times to do this.
- Cap the tube lightly and allow it to stand for 5 minutes.
- Hold the closed tube firmly at the top, then flick the side repeatedly with a finger to mix the contents. Do this for one full minute. *If, by accident, you drop the tube or find that drops of liquid are scattered inside the tube, tap it firmly on the bench several times to return the liquid to the bottom.*
- Allow the tube to stand for a further 5 minutes. The λ DNA solution should look slightly opaque. *It is essential that the DNA is thoroughly mixed with the water. To ensure that this is done, always draw the DNA solution up and down in the microsyringe tip a few times.*

Notes

DNA sticks to glass. For this reason, disposable polypropylene tubes (to which DNA does not adhere) are used in this kit. The white tube contains 10 μg of dried lambda DNA. A buffer in the tube helps to maintain the stability of the DNA once it is in solution. Blue dye has also been added to help you judge when the DNA has dissolved. It is very important to rehydrate the DNA using the correct volume of water so that the final concentration of the buffer is appropriate for the activity of the enzymes.

A common cause of failure when using dried DNA arises when the DNA is not adequately mixed with water. A 'blank' gel usually indicates that the DNA has not been rehydrated at all. Lanes that are overloaded alongside empty ones are usually due to uneven mixing of the solution (here, most of the DNA will have been at the bottom of the tube, so nearly all of it will have been dispensed into the last enzyme tube, and very little or none of it into the other tubes).



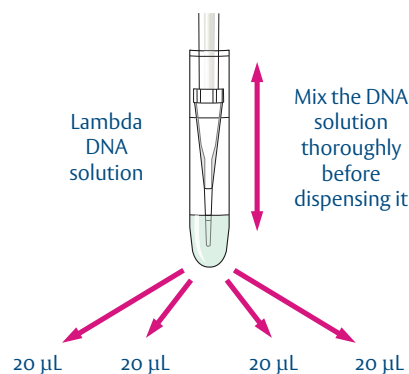
3 Cutting the DNA

- Add a fresh tip to the microsyringe. Put 20 μL of λ DNA solution into an enzyme tube of your choice. Mix the liquid and the dried enzyme by carefully drawing the liquid up and down in the tip a few times. *The liquid in the enzyme tubes should have a distinct blue hue, but there should be no concentration of dye at the bottom of the tube.*
- Repeat this for each enzyme tube and an empty yellow 'control' tube, using a fresh tip each time to prevent cross-contamination between the tubes.
- Close each tube tightly.
- Place the tubes in a foam holder and incubate them at 37°C, in a water bath or incubator, for between 30 and 45 minutes.

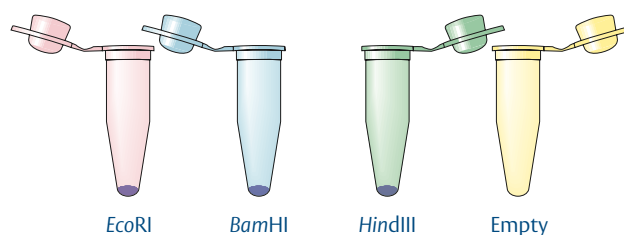
Notes

Each tube contains at least 10 'units' of restriction enzyme. A 'unit' of enzyme will cut 1 μg of lambda DNA in one hour at 37°C. The enzyme tubes are colour coded (see picture). Like the dried DNA, the enzymes have been preserved with an appropriate buffer and a blue dye. It is essential to add the correct volume of liquid to the tubes and to ensure that their contents are thoroughly mixed. This is because *EcoRI* and *BamHI* can be less specific if used in excess or with the wrong buffer concentration. For instance, in an inappropriate buffer concentration *EcoRI* may cut at the sequence AATT instead of the longer sequence, GAATTC.

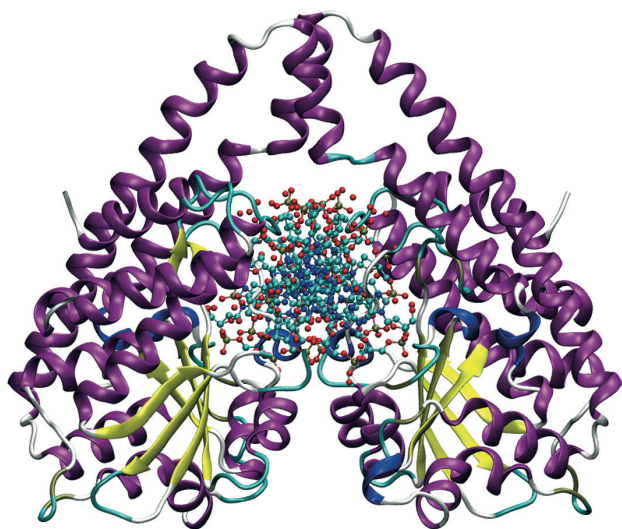
The restriction enzymes provided in this kit work best at 37°C. Most restriction enzymes are denatured readily on heating and once rehydrated they should be used immediately. After several hours enzyme activity will cease.



Use a new tip to add the DNA solution to each tube, to prevent contamination



As you add the DNA solution to each tube, mix it gently so that the dried restriction enzyme dissolves completely



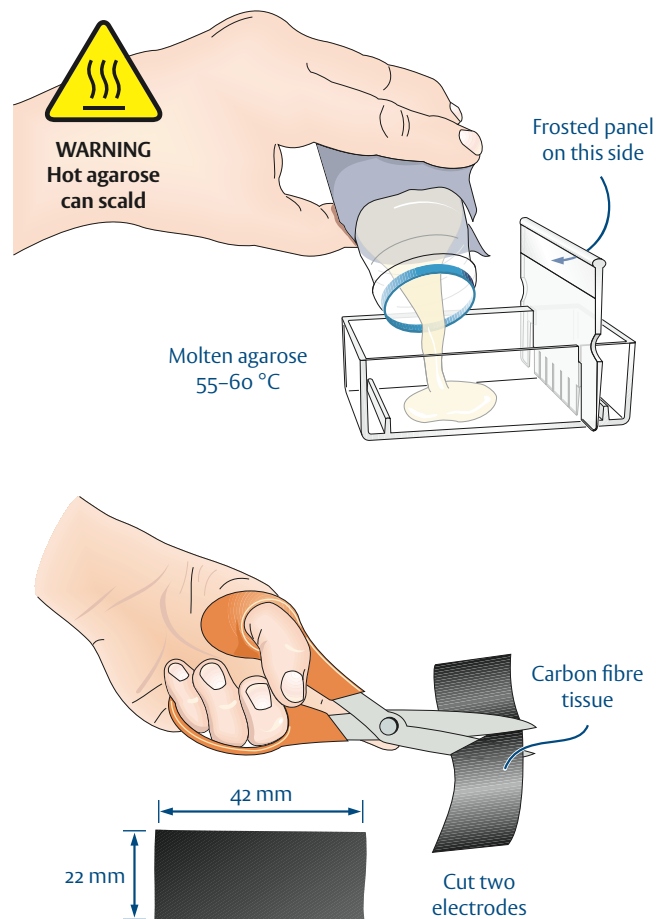
A computer-generated model of the enzyme *HindIII* attached to a length of DNA, seen down the axis of the DNA. In the protein structure, β -pleated sheets are shown in yellow; α -helices in magenta.

Data for this model came from: Watanabe, N. et al (to be published) Crystal structures of restriction endonuclease *HindIII* complex with its cognate DNA and divalent cations. [Protein Data Bank ID: 3A4K]



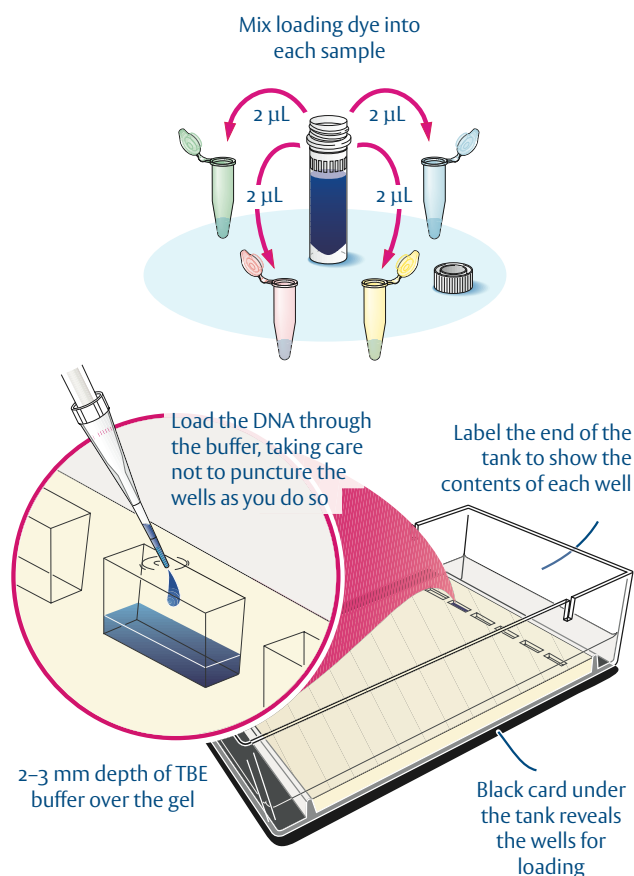
4 Preparing the agarose gel

- Use a microwave oven or a boiling water bath to melt some agarose gel (0.8% made up in TBE buffer). *Ensure that no lumps or fibres remain in the molten agarose — you can see these by holding the bottle up to the light.* Once it has melted, stand the molten agarose in a sealed container in water bath at 55–60 °C until it is needed.
- Place the electrophoresis tank on a level surface, where you can leave it undisturbed for the next 20–30 minutes. *This is necessary because if the gel sets at an angle, the DNA fragments will not run evenly through the gel.* Slot a 4- or 6-toothed comb in place at one end of the tank. If you use the 6-toothed comb, ensure that the frosted panel faces the far end of the tank (see picture).
- Pour 10–12 mL of molten agarose into the tank so that it fills the central cavity and flows under and between the teeth of the comb. Add just enough agarose so that the liquid is level with the top of the two ridges in the tank, and does not bow upwards. If you accidentally spill liquid into the areas outside the two ridges, do not worry — you can remove the agarose later, when it has set.
- Leave the tank undisturbed until the liquid has set. *Agarose is opaque and looks slightly milky when the gel is set.*
- While the gel is setting, cut two pieces of carbon fibre tissue, each about 42 mm x 22 mm. These will be the electrodes at either end of the electrophoresis tank. Put the electrodes to one side until you have loaded the gel.



5 Loading the gel

- Pour slightly more than 10 mL of TBE buffer solution into the electrophoresis tank. The liquid should cover the surface of the gel to a depth of 2–3 mm and flood into the areas that will hold the electrodes.
- Very gently* ease the comb from the gel. As you do so, the buffer solution will fill the wells that formed. Take care not to tear the wells as you remove the comb.
- Put the tank where you are going to ‘run’ it, where it will remain undisturbed.
- It is easier to see what you are doing next if the tank is placed on a dark surface, such as a piece of black card. Alternatively, a strip of black tape can be stuck onto the bottom of the tank beneath the wells. Put a clean tip on the microsyringe. Add 2 µL of loading dye to the tube containing the DNA you wish to load. Mix the dye into the DNA sample thoroughly, by drawing the mixture up and down in the microsyringe tip.
- Pipette the loading dye and DNA mixture into one of the wells, holding the tip above the well but under the buffer solution (see picture). *Take great care not to puncture the bottom of the well with the microsyringe tip.* Wells produced by the 6-toothed comb may not hold all of the mixture.
- Make a note of which DNA you have put into the well (e.g., write on the side of the tank with a marker pen).
- Repeat steps d–f with each DNA digest and the ‘control’ tube with no restriction enzyme. *Remember to use a new tip for each sample, to avoid cross-contamination.*



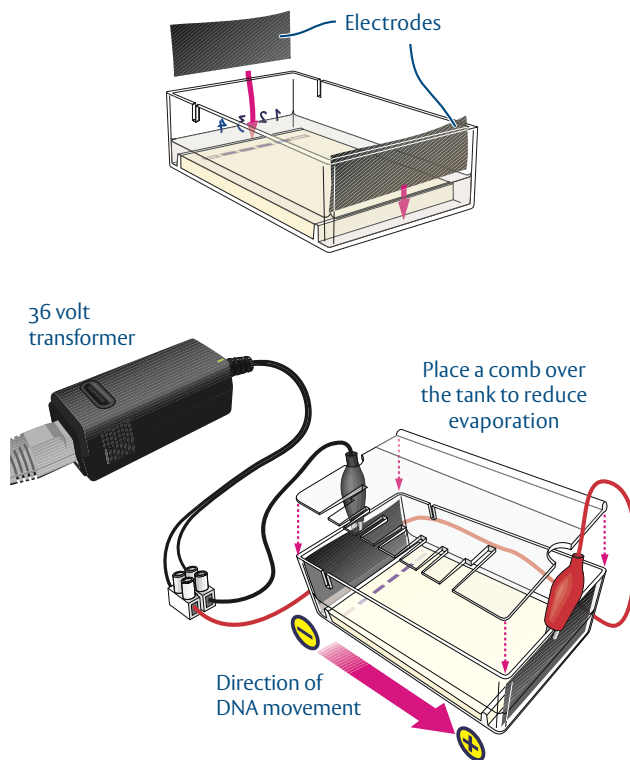
6 'Running' the gel

- Fit one electrode at each end of the tank as shown in the illustration. Attach crocodile clips to the top of the electrodes. A 36 volt transformer or sufficient batteries (in series) should be used, giving a total voltage of no more than 36 volts. *Ensure that the positive terminal of the battery is connected to the electrode furthest from the wells.*
- Check that contact is made between the buffer solution and the electrodes (with care, you can add more buffer if necessary). Place the comb over the tank to reduce evaporation during the electrophoresis. Leave the gel to 'run', undisturbed, for several hours. At 36 volts, it will take about two hours to run a gel.
- Disconnect the power supply once the blue loading dye has reached the end of the gel. (If you leave the batteries connected, the DNA will run off the end of the gel!)
- Rinse the crocodile clips in tap water and dry them thoroughly after use to prevent corrosion.

Notes

Ions in the TBE buffer used in and above the gel conduct electricity. This buffer is alkaline. Under alkaline conditions the phosphate groups of the DNA are negatively charged and therefore the DNA moves towards the positive electrode (anode) when a current is applied. EDTA in the buffer 'mops up' or chelates divalent cations. This helps to prevent damage to the DNA as such ions are required (as co-factors) by DNA-degrading enzymes.

The loading dye does not affect the DNA, but moves through the gel slightly ahead of all but the smallest of DNA fragments. The loading dye moves at about the same rate as DNA fragments of 300 base pairs.



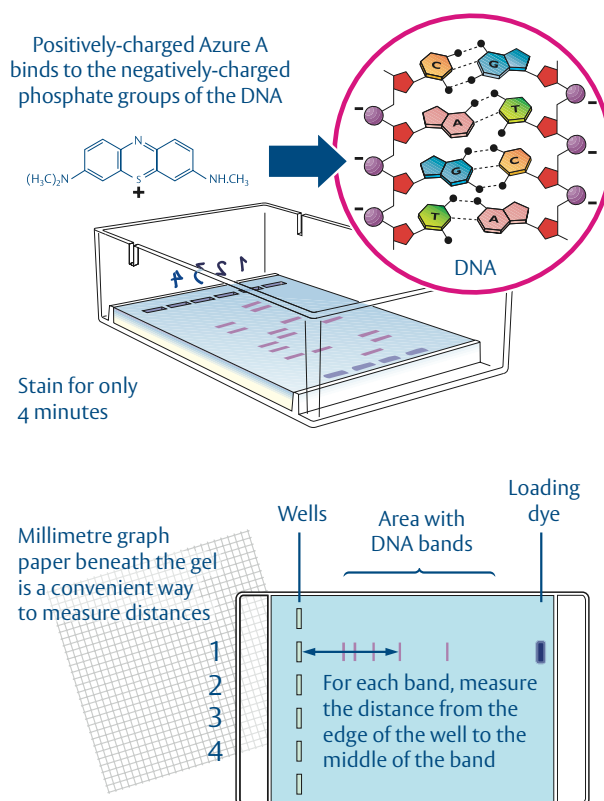
APPLY NO MORE THAN 36 VOLTS!



Serious or lethal electrical shock may occur if you connect the equipment directly to a mains electricity supply.

7 Staining the DNA

- Remove and dispose of the electrodes. Pour off the buffer solution. The buffer may be retained and re-used several times, but it will not last indefinitely.
- Put on some plastic gloves to prevent the stain from touching your skin.
- Pour about 10 mL of staining solution (0.04% Azure A in 20% ethanol) onto the surface of the gel.
- Leave it for *exactly* 4 minutes, then return the stain to a bottle for re-use. (Like the buffer, the DNA stain can be re-used several times before you need to replace it. However, you may find that older stain needs to be left on the gel for a little longer than 4 minutes.)
- Wash surplus stain from the gel surface with about 5 mL of 70% ethanol for a few seconds (industrial denatured alcohol, IDA, can be used for this task).
- Pour away the alcohol, then rinse the surface of the gel with water 3 or 4 times, *but do not leave any water on the gel, as this will remove stain from the top of the gel.* The remaining stain will gradually move down through the gel, staining the DNA as it does so. Faint bands should start to appear after 10 minutes. The best results will be seen if the gel is left to 'develop' overnight. (Put the tank in a plastic bag if you do this, to prevent the gel from drying out.) Stained gels may be stored for many months in a sealed plastic bag, kept in a dark, cool place such as a refrigerator.



Safety guidelines

Lambda DNA and restriction enzymes

The λ DNA and restriction enzymes provided with this kit are safe for use in schools. No living organisms are used, so there is no need to follow strict aseptic techniques. However, cleanliness is important to prevent cross-contamination and ensure success. Used plastic (polypropylene) tubes and microsyringe tips can be disposed of in the normal waste.

Agarose gel

If a microwave oven is used to melt the agarose gel, ensure that the gel is placed in an *unsealed* container. A boiling water bath or hotplate may be used instead, but the gel must be swirled as it melts to prevent charring. The use of a Bunsen burner to melt agarose is *not* recommended.



Molten agarose can scald and it must be handled with care, especially just after it has been heated in a microwave oven.

TBE buffer (Tris-Borate-EDTA)

When used as directed, this buffer presents no serious safety hazards. Spent buffer can be washed down the drain.

Loading dye (Bromophenol blue)

When used as directed, this loading dye presents no hazard. Used loading dye can be washed down the drain.

Electrode tissue

The carbon fibre electrode tissue may release small fibres, which can cause skin irritation if you handle the tissue a lot. Wear protective gloves if you find the tissue unpleasant to handle. The fibres are too large to enter the lungs however, so it is not necessary to wear a face mask. The fibres are soluble in body fluids and are completely biodegradable.

Electrical supply

The gel electrophoresis equipment was designed to be used with direct current at low voltages (≤ 36 volts) from batteries or a transformer such as that supplied by the NCBE. ***Under no circumstances should this voltage be exceeded, as the live electrical components are not isolated from the user.***



Serious or lethal electrical shock may occur if you connect the equipment directly to a mains electricity supply.

DNA stain (Azure A)

The *concentrated* DNA stain solution is flammable and it must be kept away from naked flames. The stain is Azure A, which when diluted as directed, forms a 0.04% solution in 20% ethanol. At this concentration it is not flammable and presents no serious safety hazard, although care should be taken to prevent splashes on the skin or eyes *e.g.*, wear protective gloves and safety glasses. Used solution can be diluted with water and washed down the drain.

Further information

Printed publications

- Delpech, R. and Madden, D. (2001) 'Working with DNA' in: *Topics in Safety* (Third Edition) Association for Science Education. ISBN: 0 86357 3169.
- Brown, N. (1995) Electrophoresis for the visually impaired: the modification of the Lambda protocol and its use with visually impaired A-level students. *Journal of Biological Education* **29**, 166–169.
- Illuminating DNA* by Dean Madden (2000) National Centre for Biotechnology Education. ISBN: 0 7049 1370 4.
- A genetic switch. Phage lambda revisited* by Mark Ptashne (2004) Third edition. Cold Spring Harbor Laboratory Press. ISBN: 0 87969 716 4.
- DNA Science. A first course* by David Micklos and Greg Freyer (2003) Second edition. Cold Spring Harbor Laboratory Press. ISBN: 0 87969 636 2.

Molecular structure data

The computer-generated images in this booklet were created using structural data from the *Protein Data Bank*: www.rcsb.org/pdb

The cover image is of the enzyme *EcoRI* bound to DNA, using data from: Kim, Y.C., *et al* (1990) Refinement of *EcoRI* endonuclease crystal structure: a revised protein chain tracing. *Science* **249**, 1307–1309 [Protein Data Bank ID: 1ERI].

The image of *BamHI* on page three uses data from: Newman, M., *et al* (1995) Structure of *BamHI* endonuclease bound to DNA: partial folding and unfolding on DNA binding. *Science* **269**, 656–663 [Protein Data Bank ID: 1BHM].

The software used was UCSF Chimera and VMD, which can be obtained from: www.cgl.ucsf.edu/chimera/ and: www.ks.uiuc.edu/Research/vmd/ respectively.