National Centre for
Biotechnology Education

University of Reading



The Lambda protocol

5.1 Teacher's and technician's notes

Equipment and materials

Required by each student or working group

Use straight from the kit

- A copy of the Student's guide
- 1 tube of dried lambda DNA (long white tube)
- 1 tube of dried restriction enzyme *Eco*RI (red tube)
- 1 tube of dried restriction enzyme *Bam*HI (blue tube)
- 1 tube of dried restriction enzyme *Hin*dIII (green tube)
- 1 empty microtube (coloured yellow)
- 1 piece of carbon fibre tissue, from which two electrodes, each approximately 42 mm x 22 mm, can be cut
- 9 white pipette tips, graduated at 2 and 10 µL
- Bromophenol blue loading dye (~8 µL is needed)
- Foam floater for holding the tubes in a water bath (shared between groups)

Supplied in the kit, but prepared in advance

- ~20 mL Toluidine blue O solution (prepared by diluting the 2x concentrate from the kit with an equal volume of distilled or deionised water)
- 10–12 mL of 0.8% (w/v) DNA electrophoresis-grade agarose, made up in 0.5X TBE buffer (that is, the TBE concentrate diluted as stated above). ***IMPORTANT: Do not use water to make up the agarose gel. The ions in the buffer solution are required to conduct the electrical current.***
- 0.5 X TBE buffer can be made up in advance by diluting the 50ml of 10X TBE up to 1 litre of distilled or deionised water.

Not in the kit: supplied by you

- An NCBE electrophoresis tank[1]
- A six- or four-toothed comb for the electrophoresis tank[1]
- A microsyringe[1]
- Two electrical leads with crocodile clips[1]
- A 36 V mains transformer[2] or new dry cell batteries with a combined voltage of 36 V *e.g.*, 4 x type *6LR61* (9 volt)
- Scissors for cutting the electrode tissue
- A permanent marker pen for marking the tubes and the lanes on the gel tank
- Access to an incubator or water bath set at 37 °C
- Black card or paper (at least 50 mm wide) to go under the tank so that the wells can be seen
- Distilled or deionised water, for rehydrating the dried lambda DNA
- Protective gloves and eye protection (to protect skin and eyes from the DNA stain)

Notes

1. These non-consumable items can be purchased from the NCBE as a *Base Unit*, which contains eight gel tanks, eight of each type of comb, eight pairs of electrical leads and eight microsyringes. The protocol may be performed with other electrophoresis apparatus, but the volumes of agarose, TBE buffer and other reagents needed will vary. The results will differ too, as the intensity of the DNA bands will be affected by the exact sizes of the wells.
2. A suitable 36 V mains transformer can be purchased from the NCBE. A single transformer of this type can run at least four NCBE gel tanks.

Use of the reagents in the kit

Tris-Borate-EDTA (TBE) buffer

The TBE buffer concentrate (10X) should be stored at room temperature. We now recommend using a 0.5X TBE buffer to ensure the boric acid concentration is below the hazard level as recommended by CLEAPSS. 10X TBE contains 0.9M boric acid, once diluted to 0.5X this would be 0.045M, which is below the 0.05M hazard level. To make this, dilute the 50ml of 10X TBE up to 1 litre with distilled or deionised water.

Diluted buffer solution can be stored indefinitely in a refrigerator at about 4 °C in a sealed container. During storage, crystals of EDTA may come out of solution in the concentrate. The loss of a small amount of EDTA should not affect the electrophoresis — simply filter the crystals out of the solution. If the buffer concentrate crystallises significantly, however, you should obtain a new supply.

The TBE buffer can be re-used a couple of times if desired. Eventually it becomes 'tired' due to electrolysis and evaporation and should be replaced with a fresh solution. Spent buffer should be washed down the drain with water.

Agarose gel

To prepare the agarose gel, the agarose powder must be dissolved in diluted (0.5x) TBE buffer. The concentration of agarose required for analysis of lambda DNA fragments is 0.8% (w/v). The liquid must be boiled so that all of the powder dissolves, and that no flecks or lumps can be seen when the solution is held up to the light. The best way of preparing the gel is to use a microwave oven.

IMPORTANT: Do not heat sealed containers of agarose gel in a microwave oven — they are likely to explode! Always loosen the tops on bottles, or heat the agarose solution in a beaker or flask covered with plastic film that has been punctured several times to let any steam escape.

Also note that hot, molten agarose can scald, and it must be handled with care, especially just after it has been heated in a microwave oven, when it can froth up without warning. Heat-proof gloves should be worn when handling containers of the hot, molten gel.

Once it has been boiled, the agarose solution can be kept molten in a water bath at 55–60 °C until you are ready to cast the electrophoresis gels. Alternatively, the gel can be poured into a container such as a screw-capped bottle, sealed, allowed to set and kept until required. Wide-mouthed glass bottles with plastic tops are ideal, such as *Duran*®-style bottles.

Unlike nutrient agar, agarose gel will not support the growth of microorganisms, so it can be stored in sealed containers, set, for many months at room temperature. To re-melt the gel, use a 60 °C water bath or a microwave oven.

It is a good idea to make up all the agarose gel from the kit in one go, rather than trying to prepare small volumes. If you do the latter, the chances are that the gel will be of the wrong concentration, which can significantly affect the time taken to run the gel and the quality of the results obtained.

Agarose is expensive. Damaged, unused, gel *e.g.*, from a poorly-cast gel, may be re-melted and cast again. Care should be taken not to allow excessive evaporation from the gel as it is re-heated, however, as this will alter the concentration of the agarose, which will slow down the movement of the DNA fragments. Do not attempt to re-use agarose that has already been used for electrophoresis.

Used agarose gel is not hazardous (provided it has been stained only with Toluidine blue O or a similar non-hazardous stain) and may be disposed of in the normal waste.

Toluidine blue O stain (Replaces Azure A stain)

The *concentrated* DNA stain solution is 0.08% Toluidine blue O dissolved in water. This solution replaces Azure A from previous versions of the kit and is safer to use as it is not flammable. The stain also produces clearer bands on the gel than Azure A.

The concentrate should be diluted with an equal volume of water before use. Both the concentrate and the diluted stain should be stored at room temperature.

Many chemicals that bind to DNA are mutagens, because they intercalate within the double helix like an extra pair of bases, causing frame-shift mutations. In contrast, Toluidine blue O and similar stains (such as Azure A, Azure B and Methylene blue) are not thought to be mutagens.

The concentrate should be diluted before use with an equal volume of distilled or deionised water. At this concentration it presents no serious safety hazard, although care should be taken to prevent splashes onto the skin and eyes *e.g.*, wear protective gloves and eye protection (see the accompanying Safety Data Sheet).

Exposure of the solution to strong sunlight may weaken the stain. The diluted Toluidine blue O solution may be re-used several times. After many uses its effectiveness will be reduced as it is gradually diluted with buffer solution washed from the gel. With elderly solution it may be necessary to stain the gel for longer than the four minutes that is suggested in the *Lambda protocol Student's guide*.

Used or unwanted Toluidine blue O solution may be washed down the drain with plenty of water.

DNA and restriction enzymes, tubes and pipette tips

The DNA and restriction enzymes provided in this kit are safe for use in schools. No living organisms are used, so there is no need to follow strict aseptic techniques. Cleanliness is important, however, to prevent cross-contamination and ensure success.

The dried DNA and restriction enzymes MUST be stored at room temperature, with silica gel desiccant in the foil pouch provided. The pouch should be tightly-sealed to prevent the dried enzymes and DNA from absorbing moisture from the air. In dry conditions, the DNA and enzymes will remain stable for many years.

Under no circumstances should the pouch be stored in a fridge or freezer as moisture will condense inside, damaging the dried DNA and enzymes.

Used plastic (polypropylene) tubes and microsyringe tips together with any remaining DNA or enzyme solutions can be disposed of in the normal waste.

Bromophenol blue loading dye

When used as directed, this loading dye presents no hazard. It should be stored at room temperature. Used loading dye can be washed down the drain.

Carbon fibre electrode tissue

The electrode tissue is essentially carbonised cotton. The fibres comprising the tissue are bound together with PVAc (polyvinyl acetate), which is also known as 'wood glue', 'carpenter's glue' or 'school glue'.

The tissue may release small fibres, which can cause skin irritation if you handle it 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.

Used electrode tissue should be disposed of in the normal waste.

Electrical safety

The NCBE 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.

Hints and tips

Using microsyringes

Students' ability to dispense precise volumes using the microsyringes will improve with experience. It is therefore a good idea to practise transferring liquids (*e.g.* food colouring) before starting work with expensive DNA, enzymes and other reagents. Some extra microsyringe tips are provided in the kit for this purpose.

Loading the gel

A steady hand is required to load gels. Practice gels may be cast from cheaper agar (use a 1% solution) rather than agarose. When practising, use water rather than buffer over the gel. Sufficient loading dye has been included in the kit for such attempts. Practice gels can be washed out and re-used several times. *Note: agarose, dissolved in diluted buffer, not water, must be used for the 'real' gels with DNA.*

Melting agarose

Ensure that the container used to prepare the agarose gel is clean. Tiny flecks of dust will not affect the way the gel runs, but they can prove a nuisance when you are trying to see faint bands in the gel.

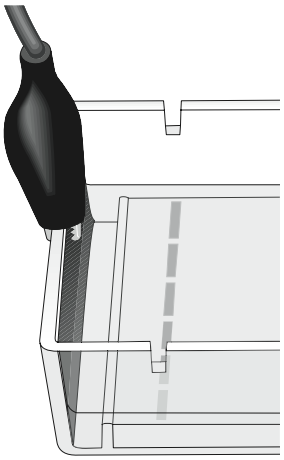
For convenience, dissolve and melt the agarose using a microwave oven. Less than a minute at full power in a 940 watt oven is sufficient for 100 mL of gel. The container (flask or beaker) used to hold the molten agarose must not be sealed, but covered lightly with plastic film that has been punctured with one of two small holes. Swirl the gel half way through the heating cycle to ensure that it is mixed thoroughly.

The gel takes too long to run

At 36 V, the loading dye should move about 5 mm per hour. At a lower voltage the loading dye will move more slowly. If, after the first 10 minutes, the loading dye does not seem to have moved and bubbles are not visible at the cathode, check the electrical contacts between the batteries and the electrodes. Ensure that there is enough buffer above the gel to cover the plastic ridges at each end of the tank, but not so much that most of the current passes through the buffer solution rather than the gel. In a warm environment liquid may evaporate from the buffer. The tank should be covered with the comb to reduce such evaporation.

Corrosion of crocodile clips

The crocodile clip used at the anode will slowly corrode due to electrolysis. This can discolour the buffer solution and gel, but will not interfere with the electrophoresis. The chances of corrosion can be reduced by ensuring that only the first two teeth of the clip are in contact with the electrode tissue, as shown here:



Eventually, you will need to replace corroded clips. Gold-plated crocodile clips are not subject to such corrosion. These are available at relatively low cost from high street electronic component retailers as well as the usual school suppliers.

Viewing the stained gel

The bands are seen most easily by holding the gel at an angle against a well-lit surface. A light box is ideal, but if one is not available, you can put a sheet of white paper on an overhead projector, and put the stained tanks on that for direct observation. Several authors suggest that a yellow or orange filter improves the contrast between stained bands and the background. While this may be true if you are taking photographs, filters can make it more difficult to spot very faint bands.

A magnifying lens can be useful; flat fresnel lenses the size of a credit card (sold for use with atlases) are ideal.

If the contrast between the background and the DNA bands is strong, an image of the gel may be projected onto a screen using an overhead projector.

There's no DNA on the gel

The most common cause of failure when using dried DNA arises when the DNA is not adequately rehydrated and mixed with water. A 'blank' gel usually indicates that the DNA has not been rehydrated at all. 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. Proper mixing is also essential when the loading dye is added to the DNA samples.

There's a large amount of indistinct DNA on the gel

If some of the wells look empty, or have very faint bands, and most of the DNA has ended up in a single (overloaded) well, this is caused by inadequate mixing. 'Streaking' of the bands is a clear sign of overloading. The bands should be relatively sharp and distinct. Follow the instructions above to ensure that the DNA solution is mixed sufficiently before it is dispensed into the enzyme tubes.

The DNA bands are very faint

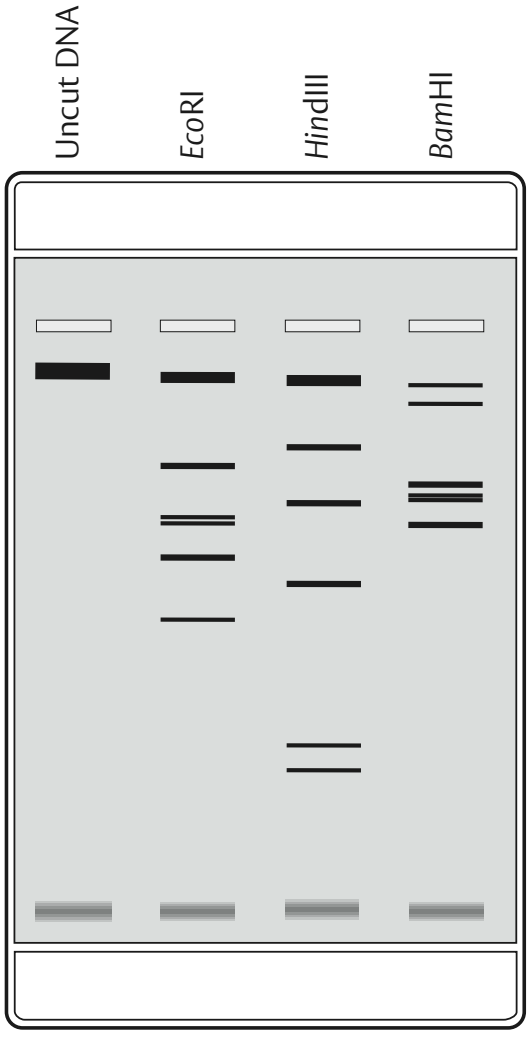
Are you using the stain correctly? Once you have applied the Toluidine blue O solution to the gel, poured off the stain, then rinsed off the excess, take care not to leave any water on the gel. If necessary, wipe over the gel gently with a finger tip to disperse beads of water. The blue stain that starts in the upper layer of the gel can re-dissolve even in small drops of water left on the surface. 'Soaking' away the stain into water drops on the surface prevents it from passing down through the gel to stain the nucleic acid.

You should not attempt to de-stain the gel. Although this is necessary with methylene blue (to remove 'background' stain), the method using Toluidine blue O has been specifically devised to avoid the need for de-staining. Unlike the bands on gels stained with methylene blue, those revealed by Toluidine blue O should not fade for several months.

The bands on the gel are very close together

There are several possible causes of this. Provided the buffer has not evaporated excessively while the gel was running (slowing the electrophoresis down), it may be that the concentration of agarose you are using is wrong. Always make up more gel than you need to ensure that its concentration is accurate. There is a very significant difference between say, a 1% and a 0.8% agarose gel — only a slight error in weighing a small amount of agarose powder can produce inaccuracies of this scale. The concentration of agarose you should use with this kit is 0.8% (mass to volume).

Specimen results



Sizes of DNA fragments in base pairs

*Eco*RI	*Hind*III	*Bam*HI	
			Wells
21 226	23 130	16 841	
7 421	9 416	7 233	
5 643 + 5 804	6 557	6 700 + 6 527	
4 878	4 361	5 626 + 5 505	
3 530	2 322		
	2 027		
	564	(not visible on gel)	
			Loading dye
	125	(runs off the gel)	

Electrophoresis calculation

The distance moved by the DNA fragments on a gel is inversely proportional to the log of the molecular mass of the fragment:

$$\text{Distance moved} = \frac{1}{\text{Molecular mass}^{\text{Log}}}$$

The molecular mass of a particular DNA fragment is roughly proportional to its length, in base pairs.

Instructions

a) Place the stained gel, in its tank, over a piece of graph paper ruled with millimetre squares. Carefully measure the distance moved by each group of DNA fragments, taking the leading edge of the well as the origin.

b) Complete the following table, showing the sizes of fragments produced by restriction digests of Lambda DNA (refer to the *Lambda Protocol Student's Guide* for the sizes of the fragments produced.

Restriction enzyme	**Sizes of fragments** (base pairs)	**Log of size**	**Distance moved** (mm)
*Eco*RI			
*Hin*dIII			
*Bam*HI			

c) Plot on a sheet of graph paper the distance migrated by each group of fragments (x axis) against the logarithm of the size of the fragments in base pairs (y axis).

d) Join the points with a line of best fit.

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