

06 | DNA sequencing



Equipment list

Laboratory work station

- P20 micropipette
- Box of micropipette tips
- Waste container
- Microfuge tube rack
- Microfuge tube of **GG** (loading dye with GelGreen®)
- Microfuge tube of **H₂O** (nuclease-free water)
- PCR tube with initials (PCR product from last session)
- Permanent marker

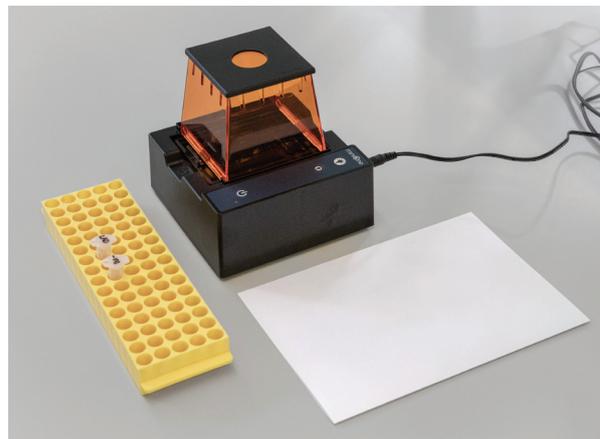
Class equipment

- Vortex
- Microcentrifuge
- Fridge (4°C)
- Gloves
- Safety glasses

Electrophoresis station

The 4 electrophoresis stations will each have:

- MiniOne electrophoresis gel tank with a 9-well, 2% agarose gel in 1 x TAE buffer
- Microfuge tube of **-ve** (PCR negative control pre-mixed with loading dye containing GelGreen®)
- Microfuge tube of **LAD** (100 bp DNA ladder, premixed with loading dye containing GelGreen®)



Health and safety

All students working in a science lab are expected to follow good laboratory practice, including: not eating or drinking in the lab, tying back long hair, 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.

Equipment

Care should be taken when using a micropipette to eject tips downwards into a waste disposal container.

There will be an electrical potential of 42 V across the MiniOne gel and through the buffer during electrophoresis. To prevent access to liquids carrying a 42V current, the orange top covers the buffer chamber. The electrical supply will not flow if:

- The photo hood is removed
- The tank is not properly placed inside the base unit, and electrodes are not making contact
- There is no, or insufficient, buffer in the buffer chamber
- The buffer is too dilute

Reagents

Substance	Hazard	Precautions
Loading dye with GelGreen®	The 6 x loading dye from ThermoFisher Scientific is classified as non-hazardous. GelGreen® is classified as non-hazardous, however it does stain DNA, which we have a lot of, so safety precautions are recommended.	When handling, wear eye protection (safety glasses are sufficient) and chemical-resistant gloves.
PCR product	Biological materials: very low risk of contaminating living cells	As a precaution students should wear eye protection (safety glasses are sufficient) and gloves when handling.
100 bp DNA ladder premixed with loading dye containing GelGreen®	This 100 bp DNA ladder from NEB is classed as non-hazardous. Contains biological materials: very low risk of contaminating living cells.	Use in a controlled way to prevent contamination, mirroring aseptic techniques. Autoclave materials that come into contact with DNA before disposal.
TAE buffer (Tris, EDTA, Acetic acid)	A 1 x concentration of TAE gel electrophoresis buffer contains no substances at a concentration that is considered to be hazardous to health.	As a precaution students should wear eye protection (safety glasses are sufficient) and gloves when handling.
Agarose gel	Agarose and the 1 x TAE buffer it is melted in are not hazardous substances.	As a precaution students should wear eye protection (safety glasses are sufficient) and gloves when handling.

Instructions | DNA discovery

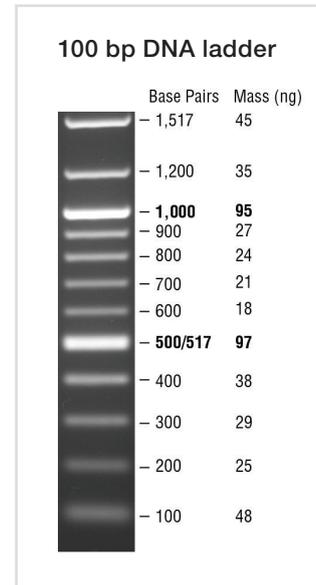
Checking PCR product with gel electrophoresis

You will be observing your DNA samples using a DNA stain and gel electrophoresis to separate DNA of different sizes. This will allow you to discover whether you did successfully extract DNA and amplify the DNA barcode by PCR. It involves a number of steps:

1. Preparing the PCR product
2. Loading samples onto the gel
3. Carrying out electrophoresis
4. Visualising and photographing the DNA in the gel after electrophoresis

A DNA ladder, with DNA bands of known size is loaded onto the gel alongside PCR samples. This allows the size of PCR products to be determined by comparison to the DNA ladder.

▲ Remember to wear nitrile gloves and safety glasses when working with the loading dye solution or electrophoresis buffer.



Credit: New England Biolabs

To load and visualize your PCR products, you will need to mix them with loading dye containing a DNA stain before loading them into the wells of the gel. The loading dye has 2 functions. Firstly, it is more dense than the electrophoresis buffer and will ‘pull’ the DNA sample with it as it sinks into the well of the gel. Secondly, it contains a stain, which will bind to the DNA and fluoresce under blue light, allowing the DNA to be visualised.

1. To prepare the PCR product for loading:

- Label the microfuge tube of **GG** (2 µl of the loading dye with DNA stain) with your initials.
- Transfer 4.0 µl of your PCR product, from the PCR tube with your initials, into this microfuge tube (with the loading dye containing DNA stain), using a p20 micropipette.
- Using a new pipette tip, transfer 4.0 µl of **H₂O** (nuclease-free water) into the same microfuge tube, using a p20 micropipette.

This gives a total volume of 10.0 µl for loading into one well of the gel.

Summary of microfuge tube contents for loading onto the gel

Label	Reagent	Volume in tube
GG	Loading dye with GelGreen® DNA stain	2.0 µl
Initials	PCR product	4.0 µl
H ₂ O	Nuclease-free water	4.0 µl
Total volume		10.0 µl

- Gently pipette the reaction up and down 3 or 4 times to mix as otherwise the GelGreen® DNA stain will settle in this mixture. You can vortex gently instead, but need to load all 10 µl into a well of the gel, so take care not to splatter the sample up the sides of the tube.

◆ **Top tip:** *If the sample gets splattered all up the sides of the tube, spin for 10 seconds in the microcentrifuge to collect in the bottom of the tube, then vortex gently again to mix.*

- 2. In the gel electrophoresis station, locate the microfuge tube of **-ve** (PCR negative control) and the microfuge tube of **LAD** (DNA ladder). These have already been prepared for loading by combining them with loading dye containing the GelGreen® DNA stain.

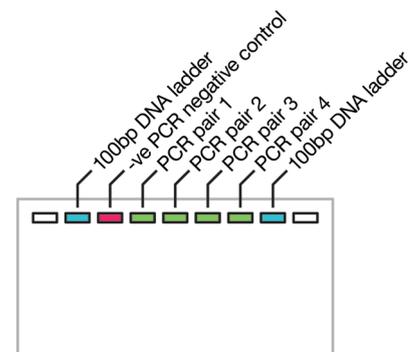
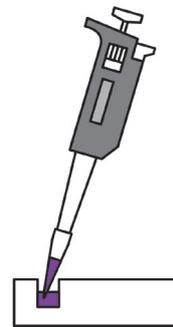
- 3. To load the wells of the gel, ready for electrophoresis:

- Set the P20 micropipette to dispense 10 µl.
- Each gel has been made with 9 wells, 7 of which you will fill (a suggested loading order is given in the diagram).
- Take turns to load 10 µl of sample into a well of the gel.

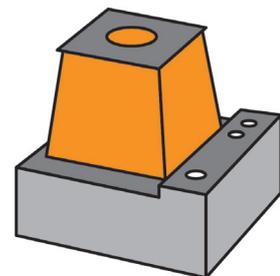
▲ *Remember to position the tip **just above** the well to load your sample. It is important not to put the tip too far into the well, as it can tear the agarose between two wells, or between one well and the bottom of the gel. If this happens your sample will either spread into more than one well or be lost out of the well altogether.*

- Your gel should have a DNA ladder on either side of the samples and a negative control loaded, as well as PCR samples.

Make sure that you note which well you load your sample into.



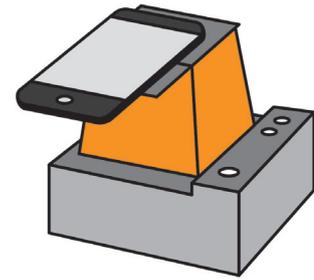
- 4. When all wells are loaded, put the orange lid on securely and press the power button to start electrophoresis. This will give a 42 V potential difference across the gel and through the electrophoresis buffer. Small bubbles will form near the electrodes.



- 5. Allow to electrophorese for 20 minutes.

6. Turn on the blue light and observe the gel through the window in the orange filter. Place a smart device camera or digital camera lens, directly on the photo hood top. Focus on the DNA bands in the gel, then take an image.

▲ *Do not zoom in as this will result in blurry pictures. The photo hood is already at the optimal focal length for a smart device.*



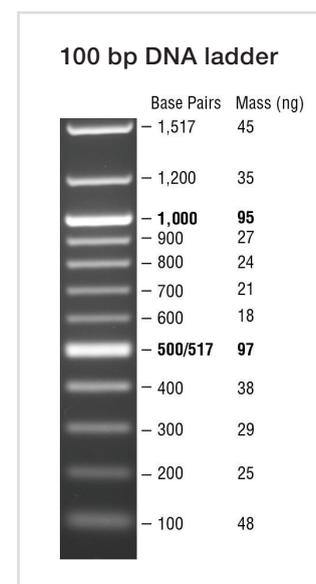
This image will be used to interpret whether you have successfully extracted DNA and amplified the barcode gene using PCR.

7. Turn off the current and unplug the electrophoresis unit at the socket.

8. One person, wearing gloves, should remove the lid from the electrophoresis tank, carefully lift the agarose gel out of the buffer and place it into an autoclave bag, for autoclaving before disposal. The gel electrophoresis buffer can be disposed of down the sink with copious amounts of water. Both gel tray and buffer tank should be rinsed with water, gently dried and stored until next needed.

9. Interpreting your gel:

- Orientate the gel so that the wells are away from you.
- Check that you can see and identify the differently-sized bands from the DNA ladder. Remember the smaller bands will have travelled further from the wells than the larger bands. Note where the more intense 500 bp and 1000 bp bands are.
- No DNA band is expected from the negative control PCR. Sometimes there is a weak / fuzzy band at about 100 bp. This is a 'primer dimer' – an artefact produced by primers overlapping each other and amplifying themselves.
- Observe whether there is a single DNA band of about 710 bp in the lanes containing PCR samples. This is the size of the barcode gene that is expected to be amplified by the PCR.
- Make sure that there is enough DNA in your PCR sample to send off for sequencing. If there is a band clearly visible at (or near) 710 bp, then amplification has been successful and your sample should be sent for sequencing.



Credit: New England Biolabs

□ 10. Preparing samples to send for sequencing:

If your DNA extraction and PCR were successful you will have seen a DNA band on your gel of about 710 bp. The next step is to find the DNA sequence of this barcode gene. Once you have the sequence, it can be compared to other DNA sequences in a database to find if it is from a known invertebrate.

We will send your samples for Sanger sequencing, to obtain the nucleotide sequence.

To provide your DNA sample for sequencing you need to:

- Take a clean 1.5 ml microfuge tube.
- Write the 7 letter code of your sample (from the sample record sheet) onto the top and side of the tube using a permanent (Sharpie) marker.
- Transfer 20 µl of your PCR product, from the PCR tube labelled with your initials, into this labelled microfuge tube.
- Place your sample into the Isofreeze box.
- Store the samples in the Isofreeze box at 4°C (in the fridge), until you are ready to deliver them.

Bring the Isofreeze box containing your samples and your completed sample record sheet with you when you visit the Wellcome Genome Campus and we will submit the samples for Sanger sequencing. The results will be emailed to you within a week.

Explanation | DNA sequencing

How does sequencing work?

Your DNA sample will be purified, then sequenced using capillary gel electrophoresis, which is based on the Sanger sequencing method developed by Fred Sanger.

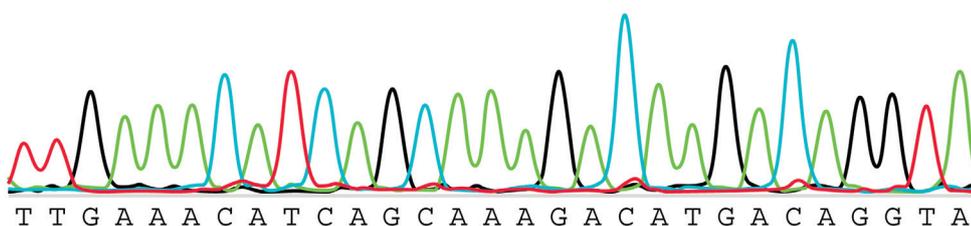
You can read about Sanger sequencing here:

yourgenome.org/theme/what-is-sanger-sequencing

You can read about capillary gel electrophoresis here:

yourgenome.org/theme/what-is-capillary-sequencing

When the DNA is sequenced, you will receive a chromatogram, giving an idea of the accuracy of the DNA sequence, and a text file, containing the DNA sequence.



>Sequence

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TTGAAACATC
AGCAAAGACA
GACAGGTA...
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