



MARPLE  
DIAGNOSTICS

**MARPLE training booklet**

**August 2021**

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## Introduction to MARPLE

MARPLE, Mobile And Real-time PLant disEase diagnostics, is a technique for quickly identifying individual strains of the wheat yellow rust pathogens. Strains that are new to an area have the potential to be much more damaging than strains that have been present in the past, so identifying them as fast as possible is key to protecting wheat crops.

There are several stages to the MARPLE technique. To identify the strains present in your samples, you will need to:

- Collect samples from the field
- Extract DNA
- Amplify highly variable genes to be able to distinguish strains
- Prepare the genes for sequencing
- Sequence the genes
- Analyse the sequence data

The MARPLE diagnostics kit contains all the equipment and materials required to carry out the MARPLE technique, and this booklet describes in detail how to carry out each stage of the process.

Together, the kit and this booklet contain everything you need to carry out strain-level identification of wheat yellow rust.



## MARPLE diagnostics Step by step

### **A**      **Sample collection**

The first step in any genetic analysis is to collect the samples you want to analyse. These samples need to be stored in a way that preserves the DNA in the best possible condition. We do this by storing samples in RNAlater solution that preserves nucleic acid integrity.

### **B**      **DNA extraction**

The next step is to extract DNA from these samples. This is the process of taking tissue from an organism and breaking it down, so that we can pull out the DNA and throw away everything else. We do this by first manually grinding up or squashing the tissue, breaking open the cells with a lysis solution, then somehow taking the DNA out of the solution. The MARPLE diagnostics protocol purifies the DNA from the solution using magnetic beads (read more about how the magnetic beads work and how to use them on page 40).

### **C**      **DNA amplification**

Instead of sequencing the whole genome, in MARPLE diagnostics we focus on sequencing just a small portion, using a set of pre-defined genes that vary in sequence between individual strains. Once you have extracted DNA from your sample, you need to select just the parts that contain the genes you want to sequence. We do this using a process called PCR (polymerase chain reaction), where an enzyme called polymerase copies the DNA strands. Pairs of specially designed molecules called primers, match the DNA sequence at each end of the genes and tell the polymerase which sections to copy.

### **D**      **DNA quantification**

Now we have the gene sequences, we need to know how much DNA we have extracted to prepare the DNA for sequencing - this is what the quantification step does. There are machines that are designed to measure the amount of DNA in a sample, but if you do not have access to one of these then you can estimate the amount of DNA by attaching a dye to the DNA, running it through a gel to separate the DNA strands into their different lengths. Then the DNA of each length can be visualised using the dye. By comparing the brightness of different samples, the amount of DNA can be estimated.

### **E**      **Library preparation**

The next step is to prepare the DNA we want to sequence into a form that is called a library. A DNA library is just the DNA fragments we want to sequence and any additions we add to these DNA fragments during the library preparation step. MARPLE diagnostics uses a type of sequencing called nanopore sequencing and the portable MinION sequencer that was created by Oxford Nanopore. Whilst preparing our DNA libraries, we need to add molecules to our DNA to help with the sequencing process. We might also have many samples that we need to put into the sequencer at the same time, so we need to be able to tell them apart, we do this by adding barcodes. A barcode is a unique sequence that will attach to each strand of DNA in the sample and be read by the sequencing machine along with the DNA. By attaching a different barcode to each sample, and then looking for the barcodes in the sequence data, we can tell which sequences came from which sample.

***F*****MinION sequencing**

Sequencing is ‘reading’ the code of the DNA. There are many ways to do this, but MARPLE diagnostics uses nanopore sequencing and the MinION sequencer.

Inside the MinION are hundreds of tiny holes called nanopores with electricity running through them. The DNA strands need to pass through a pore to be sequenced, but they can’t find their way into the holes on their own, so the molecules that are attached during the library preparation help this to happen. As a strand of DNA passes through a pore, the MinION measures how each position in the code (base) affects the voltage of the electricity in the pore.

Once you have prepared your library you can plug your MinION sequencer into the computer and put your library solution into the MinION. Use the MinKNOW software to manage the setup for your sequencing run - and start sequencing.

***G*****Basecalling**

The sequencing run will produce a file of the voltage measured at each position in your DNA sequences. Because we know about the chemistry of the molecules that make up DNA, you can then use MinKNOW to convert each of those voltage measurements into a base (A,T,G or C), this process is called ‘base calling’.

During basecalling the MinKNOW software also recognises the different barcodes that were added to the sequences during library preparation. Using this information it can then sort the sequence data into separate folders for each sample.

***H*****Bioinformatics**

Once you have generated DNA sequences, bioinformatic tools are used to analyse the data to find the useful information that it contains. The first step of this process is to take all the sequences for each sample and identify which gene they come from. We can then compare the gene sequences from the samples to the same gene sequences in known yellow rust strains. This allows us to see how similar or different they are, and to fit the samples into a phylogenetic tree, which shows the genetic relationships between strains.

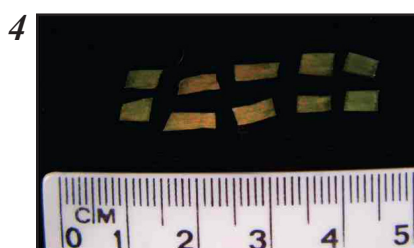
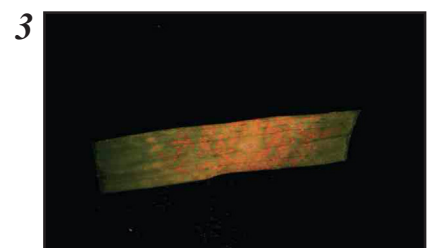
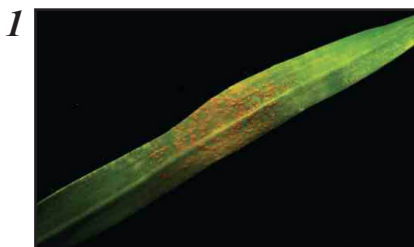
MARPLE contains an automated bioinformatics pipeline, which allows these complex analyses to be performed easily with just a few simple instructions from the user.

## **MARPLE protocol**

Before you begin the protocol, please check the materials section (page 38) to make you sure have the equipment and reagents required.

## **A Sample collection**

- 1** Select a leaf with obvious yellow rust pustules (see pictures below).
- 2** Use scissors to cut out a region around the area with the pustules.
- 3** Trim the piece you have cut out so you have only the area with the pustules.
- 4** Cut the section of leaf into smaller pieces (about **5mm x 5mm**).
- 5** Put all the small pieces of infected leaf into 1 tube of RNeasy lysis solution. Shake the tube to soak the leaf pieces in the solution. Tightly secure the lid.
- 6** Fill out the sampling form with all the details of the sample. Write on the tube label:  
Your name; date of collection; location of collection; host variety

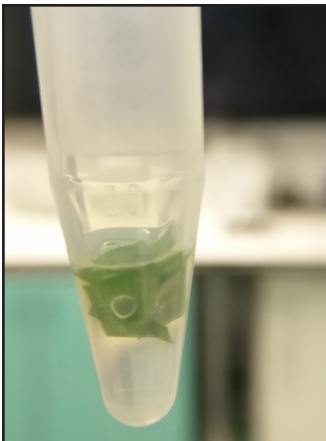


## **B** DNA extraction

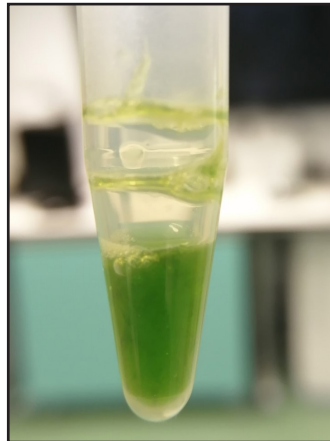
This section uses magnetic beads. Magnetic beads are explained on page 40.

- 1** Remove pieces of leaf from the tube using tweezers. Gently remove liquid from the surface of the leaf fragments with tissue and put them in to a fresh 1.5mL microfuge tube. Be mindful not to let the fragments dry out completely, so move onto the next step immediately.
- 2** Add **200µL** lysis buffer to the sample tube (how to make lysis buffer is explained on page 31).
- 3** Disrupt the tissue using a clean micro-pestle. Squash the tissue between the micro-pestle and the side of the tube, making sure the sample does not get stuck at the bottom (see images).

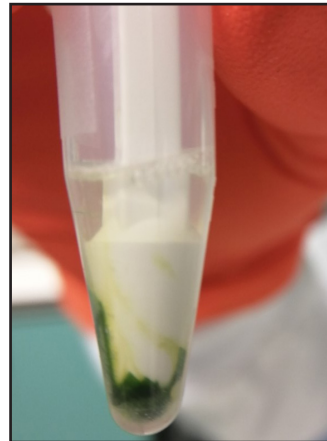
**Before disrupting**



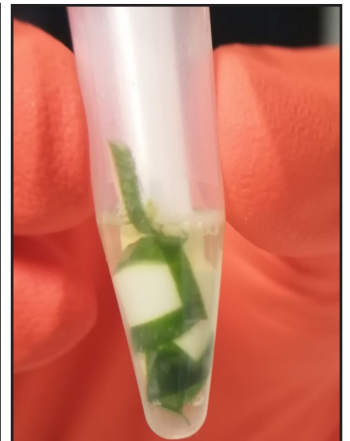
**After disrupting**



**Bad technique**



**Good technique**



- 4** If possible, briefly centrifuge in a bench top micro-centrifuge to gather the cell debris at the bottom of the microfuge tube (or let the contents settle for 2m00s). Transfer the supernatant to a new 1.5 mL microfuge tube, leaving the cell debris behind.
- 5** Prepare the AMPure beads by bringing them up to room temperature and shaking them well, or vortexing.  
Add an equal amount of AMPure XP beads to the sample (For example, if 150 µL of supernatant was transferred to a new microfuge tube, 150 µl AMPure XP beads should be added). Mix the sample by pipetting and/or shaking. Wait for **5m00s**.

While you are waiting, prepare **500µL** 80% ethanol for each sample.

- 6** Transfer the microfuge tube to a magnetic rack and open the lid. Wait roughly **2m30s** for the beads to pellet and the supernatant to clear - if it is not clear in this time then wait another **0m30s**. **Do not** remove the microfuge tube from the magnetic rack until stated.
- 7** Remove and discard as much supernatant as you can without disturbing the pellet. This can be difficult, so carefully slide the pipette tip down the side of the tube (away from the beads) and draw up the liquid very slowly. Do not remove any beads. If beads are accidentally pipetted, return supernatant to the microfuge tube and allow to re-pellet before trying again.
- 8** Add **200µL** 80% ethanol without disturbing the pellet and leave to stand for **0m30s** , then remove and discard the ethanol very carefully, without disturbing the pellet of beads.
- 9** Repeat step 8. Leave the tube containing the beads on the rack to dry for **0m30s**, or until no drops of ethanol are left.
- 10** Remove the microfuge tube from the magnetic rack and add **30µL** nuclease-free water. Gently re-suspend beads in solution by pipetting water up and down, over the pellet. You are releasing the DNA from the magnetic beads, so it is important to wash the water over the beads multiple times to collect all of the DNA in the solution.
- 11** Incubate at Room temperature with the lid closed for **2m00s**.
- 12** Transfer the microfuge tube back to the magnetic rack and open the lid. Wait for the beads to pellet, roughly **2m00s**.
- 13** Transfer supernatant to a new microfuge tube, but avoid disturbing the bead pellet. If beads are accidentally pipetted, replace supernatant and allow to re-pellet before trying again.  
  
This tube contains DNA from your sample.

## C DNA amplification using PCR, and PCR clean-up

This section uses magnetic beads. Magnetic beads are explained on page 40.

### Primer pools

Primers for different genes often interact with each other. Because we have many genes to PCR amplify, we have separated the primers into 4 pools (A-D), where primers have been grouped to make sure they do not stop each other from working. Each primer pool (A-D) contains different primers for a subset of the genes we need to amplify for the MARPLE diagnostics method and this means that you will be running 4 PCR reactions for every rust sample you have.

### Note

This protocol shows instructions for 1 sample, which can be repeated for higher numbers of samples. However, if you have many samples it may be faster to prepare a master mix for each primer pool, then use these with each of your samples. To do this, multiply the volumes from **step 1** by your number of samples plus one, using a different primer pool for each, and add these to 4 fresh 1.5mL tubes. Then, take 4 fresh 0.2mL tubes per sample. Add **20µL** of each master mix (A-D) to one of the four tubes. Add **5µL** of DNA from each sample to a tube containing each master mix. This will produce 4x[number of samples] reaction tubes to be put into PCR. This will replace **steps 1 and 2** from this section of the protocol.

**1** Take 4 fresh 0.2mL microfuge tubes. Add the following to each of the four tubes with a different primer pool (A-D). The volumes for 1 reaction are:

- **12.5µL** AmpliTaq Gold™ 360 Master Mix
- **5µL** Primer pool (A, B, C or D)
- **2.5µL** Nuclease-free water

**2** Add **5µL** of sample DNA to each of the four reaction tubes containing the different primer pools (A-D). If possible, briefly micro-centrifuge to spin down the reagents. Gently tap the microfuge tubes onto a bench-top to eliminate bubbles.

**3** For each sample, load your 4 reactions (A-D) into the PCR machine and run your PCR using the following conditions. This should take roughly **3h15m**.

A	B	C
95°C	10 mins	x1
95°C	15 s	x40
63°C	30 s	
72°C	3 mins	
72°C	7 mins	x1
4°C	Hold	x1

**4** In a 1.5mL microfuge tube, combine the **4 x 25µL** reactions (one from each pool, A-D) of each sample to create a “combined PCR product”. This will result in a total volume of **100µL**.

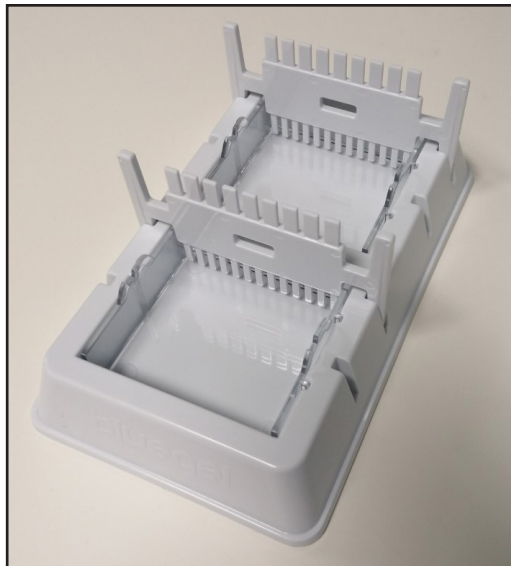
- 5** You can now clean up the DNA in your “combined PCR product”. Prepare the AMPure beads by bringing them up to room temperature and shaking them well, or vortexing. To the combined PCR product, add **100µL** of AMPure XP beads. Mix the sample via pipetting and/or shaking then wait **5m00s**.
- While you are waiting, prepare **500µL** 80% ethanol for each sample.
- 6** Transfer the microfuge tubes to a magnetic rack and open the lids. Wait **2m30s** or until the beads have formed a pellet and the supernatant is clear. Do not remove the microfuge tube from the magnetic rack until stated.
- 7** Remove and discard as much supernatant as possible without disturbing the pellet. Do not remove any beads. If beads are accidentally pipetted, return supernatant to the microfuge tube and allow to re-pellet before trying again.
- 8** Add **200µL** 80% ethanol without disturbing the pellet and leave to stand for **0m30s** then remove and discard the ethanol.
- 9** Repeat **step 8**.
- 10** Remove the microfuge tube from the magnetic rack and add **30µL** nuclease-free water, gently re-suspend beads in solution by pipetting water up and down, over the pellet.
- 11** Incubate at Room temperature with the lid closed for **2m00s**.
- 12** Transfer the microfuge tube back to the magnetic rack and open the lid. Wait for the beads to pellet, roughly **2m00s**.
- 13** Transfer supernatant to a new microfuge tube, avoiding disturbing the bead pellet. If beads are accidentally pipetted, replace supernatant and allow to re-pellet before trying again.

## **D** DNA quantification - run a gel

This section uses the Bluegel tank and the computer programs Fiji and Microsoft Excel.

In the previous section you have used PCR to produce a set of amplified gene sequences for each of your samples. PCR is a delicate process and how well each reaction amplifies the DNA will vary. However, in order to use the correct amount of each PCR product during library preparation we need to know the relative amounts of DNA in each one. We can do this by dyeing the PCR products then visualising them on a gel. We can then use computer programs Fiji and Excel to measure and calculate the relative brightnesses.

- 1** Add combs to the gel tray. Each section of the comb will give you 1 well, you need enough wells for 1 per sample plus 1 blank well in each half of the tray to measure background.



- 2** Add 2 x **0.5g** agarose tablets to **100mL** of TBE 0.5X solution. This will create a 1% agarose gel.

Heat the agarose solution in a microwave, or on a stove, until the agarose has dissolved into solution. Once dissolved, if the solution has evaporated considerably then add more TBE until you reach a total volume of **100mL**.

- 3** Allow the solution to cool to approximately **50 °C** (hand hot).

- 4** Check if your agarose tablet contained dye, if yes, pour into a gel tray and leave until set (at least **10m00s**). If no, add **10µL** GelGreen DNA stain to the agarose solution (1 µl per 10ml of TBE) before pouring into the gel tray.

- 5** For each of the combined PCR products that you purified above, pipette **5 $\mu$ L** of DNA into a new 0.2mL microfuge tube.
- 6** Prepare **1 $\mu$ L** DNA ladder in a new 0.2mL microfuge tube, then add **4 $\mu$ L** water.
- 7** Add **1 $\mu$ L** loading dye to each 5 $\mu$ L sample, and to the DNA ladder from above.
- 8** Create a control solution by adding **5 $\mu$ L** water and **1 $\mu$ L** loading dye to a fresh tube.
- 8** Load the DNA ladder and each sample into a separate well of the gel. Load the control solution into another well, we will use this row later on to get a background measurement when comparing the brightness of the gel bands.
- 9** Put the lid on the gel tank. Switch on and run until the bands have separated. Check every 10 minutes.
- 10** Take an image of the gel using your phone. Transfer the image to a computer which has the program Fiji. You can do this in whichever way is easiest for you (email, USB stick, etc).

## DNA quantification continued - measuring and comparing concentrations using Fiji and MS Excel

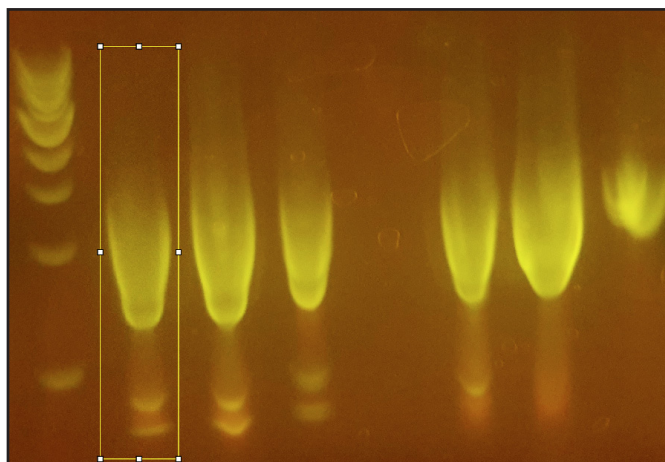
**1** Open the program Fiji (if this is not installed on your MARPLE laptop, it can be downloaded from <https://imagej.net/software/fiji/>).

**2** Open the gel image that you produced above.

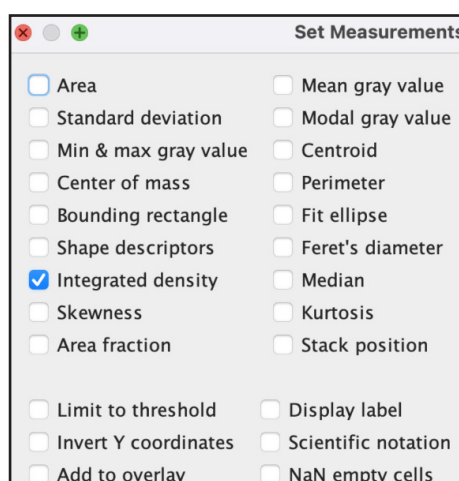
**3** Select the 'rectangle' icon.



**4** Highlight the first lane of the ones you want to measure, by clicking and dragging to draw a box around the whole lane.



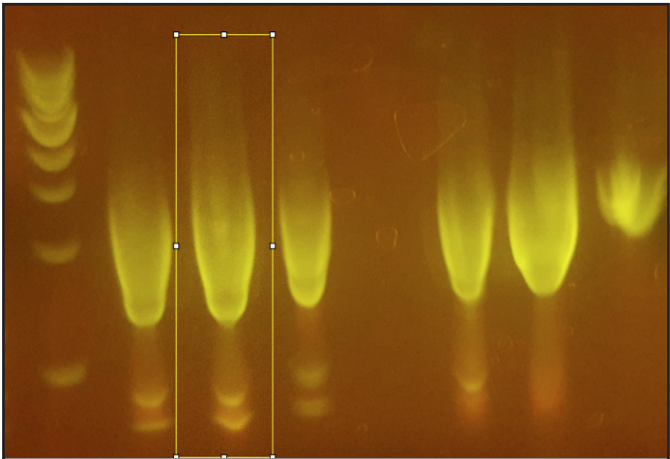
**5** Click **analyse > set measurements**. Make sure 'Integrated density' is ticked. Click 'OK'.



6 Click **analyse > measure**. A results box will appear with the density measurement inside.

Results		
	IntDen	RawIntDen
1	26605590	26605590

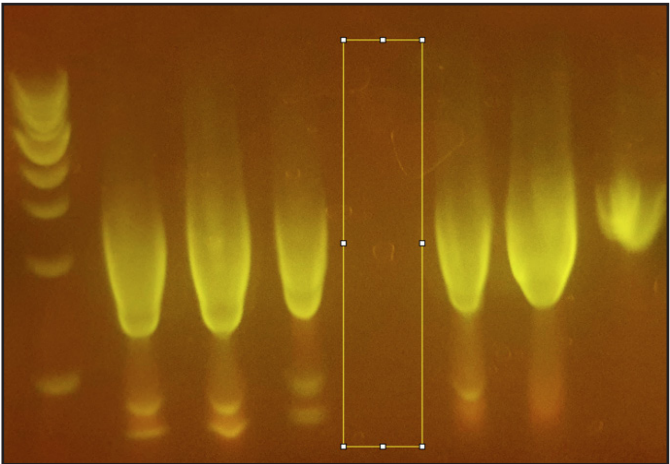
7 Click and drag to pick up the box you drew before and move it over to the next lane you want to measure.



8 Click **analyse > measure**.

9 Repeat steps 7 and 8 for each lane with bands, ignoring the ladder lane and blank lane.

10 Measure the blank lane in the same way. We will use this to measure a background brightness value to subtract from the other lanes, leaving us with just the brightness of the bands.



Results		
	IntDen	RawIntDen
1	26605590	26605590
2	30323880	30323880
3	28545633	28545633
4	30052759	30052759
5	31468234	31468234
6	27376482.000	27376482.000
7	25115133.000	25115133.000

- 11** Create an excel spreadsheet with 6 columns labelled: Lane, IntDen, RawIntDen, IntDen-Background, Total IntDen, Relative Density.

	A	B	C	D	E	F	G
1	Lane	IntDen	RawIntDer	IntDen - B:	Total IntDe	Relative Density	
2							
3							

- 12** Copy and paste each line from the results box into a new row of your spreadsheet under the columns: Lane, IntDen, RawIntDen. Change the lane number of the final measurement to 'background'. You can now close Fiji.

	A	B	C	D
1	Lane	IntDen	RawIntDer	IntDen
2		1 26605590	26605590	
3		2 30323880	30323880	
4		3 28545633	28545633	
5		4 30052759	30052759	
6		5 31468234	31468234	
7		6 27376482	27376482	
8	Backgroun	25115133	25115133	
9				

- 13** In cell D2 (under the IntDen-Background column heading) type the formula `=B2-$B$N`, but replace the 'N' with the row number of your background measurement. The '\$' in the formula is very important as it fixes the cell name in the formula, this means it will not change when you drag the formula into other cells.

In this example the background measurement is in row 8, so the formula would be '`=B2-$B$8`'. This subtracts the background measurement (taken from the blank lane) from all the other lane measurements.

	A	B	C	D	E
1	Lane	IntDen	RawIntDer	IntDen - B:	Total In
2		1 26605590	26605590	<code>=B2-\$B\$8</code>	
3		2 30323880	30323880		
4		3 28545633	28545633		
5		4 30052759	30052759		
6		5 31468234	31468234		
7		6 27376482	27376482		
8	Backgroun	25115133	25115133		
9					

- 14** Press Enter, then select cell D2 again. Click and hold the square that appears in the bottom right corner of the cell, then drag it down to highlight all the column D cells for rows that have recorded measurements in them, except for the background (see image).

	D
IntDen - B: Total	
590	1490457
880	

	A	B	C	D	E
1	Lane	IntDen	RawIntDen	IntDen - B: Total IntDen	
2	1	26605590	26605590	1490457	
3	2	30323880	30323880	5208747	
4	3	28545633	28545633	3430500	
5	4	30052759	30052759	4937626	
6	5	31468234	31468234	6353101	
7	6	27376482	27376482	2261349	
8	Background	25115133	25115133		
9					

- 15** In cell E2 (under the Total IntDen heading) type ‘=sum(’ then, without pressing Enter, click and drag to highlight all your IntDen - Background columns with data in them (these cells will then appear in the formula as a range), type a close bracket ‘)’ and press Enter.

	A	B	C	D	E	F
1	Lane	IntDen	RawIntDen	IntDen - B: Total IntDen		Relative Density
2	1	26605590	26605590	1490457	=sum(D2:D7)	
3	2	30323880	30323880	5208747		
4	3	28545633	28545633	3430500		
5	4	30052759	30052759	4937626		
6	5	31468234	31468234	6353101		
7	6	27376482	27376482	2261349		
8	Background	25115133	25115133			
9						

- 16** In cell F2 (under the header Relative Density), type the formula ‘=D2/\$E\$2’. Press Enter.

	A	B	C	D	E	F	G
1	Lane	IntDen	RawIntDen	IntDen - B: Total IntDen		Relative Density	
2	1	26605590	26605590	1490457	23681780	=D2/\$E\$2	
3	2	30323880	30323880	5208747			
4	3	28545633	28545633	3430500			
5	4	30052759	30052759	4937626			
6	5	31468234	31468234	6353101			
7	6	27376482	27376482	2261349			
8	Background	25115133	25115133				
9							

- 17** Click on cell F2, then click and hold the square that appears in the bottom right corner of the cell, then drag it down to highlight all the column D cells for rows that have recorded measurements in them, except for the background (see image).

	A	B	C	D	E	F	G
1	Lane	IntDen	RawIntDer	IntDen - B: Total IntDe	Relative Density		
2	1	26605590	26605590	1490457	23681780	0.062937	
3	2	30323880	30323880	5208747		0.219947	
4	3	28545633	28545633	3430500		0.144858	
5	4	30052759	30052759	4937626		0.208499	
6	5	31468234	31468234	6353101		0.26827	
7	6	27376482	27376482	2261349		0.095489	
8	Background	25115133	25115133				
9							

- 18** In cell G2, type the formula  $=\$F\$2/F2*10$ , but replace the 'N' with the row number of the smallest value in column F (which in this example also happens to be F2, but yours might be any row number). Press Enter.

	A	B	C	D	E	F	G	H	I	J
1	Lane	IntDen	RawIntDer	IntDen - B: Total IntDe	Relative D	Amount to add to library loading solution				
2	1	26605590	26605590	1490457	23681780	0.062937	$=\$F\$2/F2*10$			
3	2	30323880	30323880	5208747		0.219947				
4	3	28545633	28545633	3430500		0.144858				
5	4	30052759	30052759	4937626		0.208499				
6	5	31468234	31468234	6353101		0.26827				
7	6	27376482	27376482	2261349		0.095489				
8	Background	25115133	25115133							
9										

- 18** Click on cell G2. Then click on the square in the bottom right hand corner of the cell, and drag down to highlight all the column G cells in rows that have measured values, except the background.

	A	B	C	D	E	F	G	H	I	J
1	Lane	IntDen	RawIntDer	IntDen - B: Total IntDe	Relative D	Amount to add to library loading solution				
2	1	26605590	26605590	1490457	23681780	0.062937	10			
3	2	30323880	30323880	5208747		0.219947	2.86145			
4	3	28545633	28545633	3430500		0.144858	4.344722			
5	4	30052759	30052759	4937626		0.208499	3.01857			
6	5	31468234	31468234	6353101		0.26827	2.346031			
7	6	27376482	27376482	2261349		0.095489	6.591008			
8	Background	25115133	25115133							
9										

**Note** Column G now contains the volume of each sample (in  $\mu\text{L}$ ) that you will use to make the library loading solution during the library preparation section (below). One volume for the lowest concentration sample will be 10. If the others are all  $<2$  this indicates that the lowest concentration sample does not contain enough DNA. You can extract and/or amplify this sample again before repeating the quantification protocol, or remove this sample from the quantification analysis and begin again from **step 13**.

## **E Library preparation**

This section follows Oxford Nanopore Technologies' Rapid Barcoding Kit (SQK-RBK004), check to make sure kit matches ID and/or if the protocol has been updated.

**1** Thaw/defrost reagents, spin down, and mix according to the table below:

Reagent	1. Thaw/defrost at room temperature	2. Briefly spin down	3. Mix well by pipetting
Fragmentation Mix (RB01-12)	Not Frozen	Yes	Yes
Rapid Adapter (RAP)	Not Frozen	Yes	Yes
Sequencing Buffer (SQB)	Yes	Yes	Vortex, then spin down again
Loading Beads (LB)	Yes	Yes	Yes
Flush Buffer (FLB) - 1 tube	Yes	Yes	Vortex, then spin down again
Flush Tether (FLT)	Yes	Yes	Yes

**2** In a 0.2mL PCR tube, add **7.5µL** DNA sample and **2.5µL** Fragmentation Mix (This contains the barcode, so use a different barcode (e.g. RB01) for each sample).

**3** Mix gently by flicking/tapping the tube and spin down.

**4** Using a PCR or MiniPCR machine, incubate the tube at **30 °C for 1m00s** and then at **80 °C for 1m00s**. Return sample to Room temperature using PCR machine or briefly put the tube on ice to cool it down.

**5** We now need to combine the samples to make a solution containing roughly equal amounts of DNA from each. We can do this by using the volumes that were calculated in the quantification section.  
Add a number of microlitres of each sample equal to the number in the 'Amount to add to library loading solution' column of your spreadsheet (from above). Make a note of the total volume.

- 6** If barcoding 4 or more samples, concentrate the pooled samples using AMPureXP beads, as shown below.  
If barcoding 3 or fewer samples, skip the bead clean-up and continue from **step 16**.
- 7** Prepare AMPure XP beads for use by vortexing or shaking well.  
To the combined PCR product, add **100 $\mu$ L** of AMPure XP beads. Mix the sample via pipetting and/or shaking then wait **5m00s**.  
  
While you wait, prepare **500 $\mu$ L** ethanol for each sample.
- 8** Transfer the microfuge tubes to a magnetic rack, with the lids open, and wait **2m30s** or until the beads have formed a pellet and the supernatant is clear. Do not remove the microfuge tube from the magnetic rack until stated.
- 9** Remove and discard as much supernatant as possible without disturbing the pellet. Do not remove any beads. If beads are accidentally pipetted, return supernatant to the microfuge tube and allow to re-pellet before trying again.
- 10** Add **200 $\mu$ L** 80% ethanol without disturbing the pellet and leave to stand for **0m30s** , then remove and discard the ethanol.
- 11** Repeat **step 10**.
- 12** Remove the microfuge tube from the magnetic rack and add **14.5 $\mu$ L** 10 mM Tris-HCl pH 7.5-8.0 with 50 mM NaCl, gently re-suspend beads in solution by pipetting water up and down, over the pellet.
- 13** Incubate at Room temperature with the lid open for **2m00s**.
- 14** Transfer the microfuge tube back to the magnetic rack and wait for the beads to pellet, roughly **2m00s**.

- 15** Transfer supernatant to a new microfuge tube, avoiding disturbing the bead pellet. If beads are accidentally pipetted, replace supernatant and allow to re-pellet before trying again.
- 16** Add **1 $\mu$ L** of RAP to **14.5 $\mu$ L** of barcoded DNA.
- 17** Mix gently by tapping/flicking the tube, then spin down.
- 18** Incubate the reaction for **5m00s** at Room temperature. Then keep the library cool/on ice, until ready to load into the flow cell.

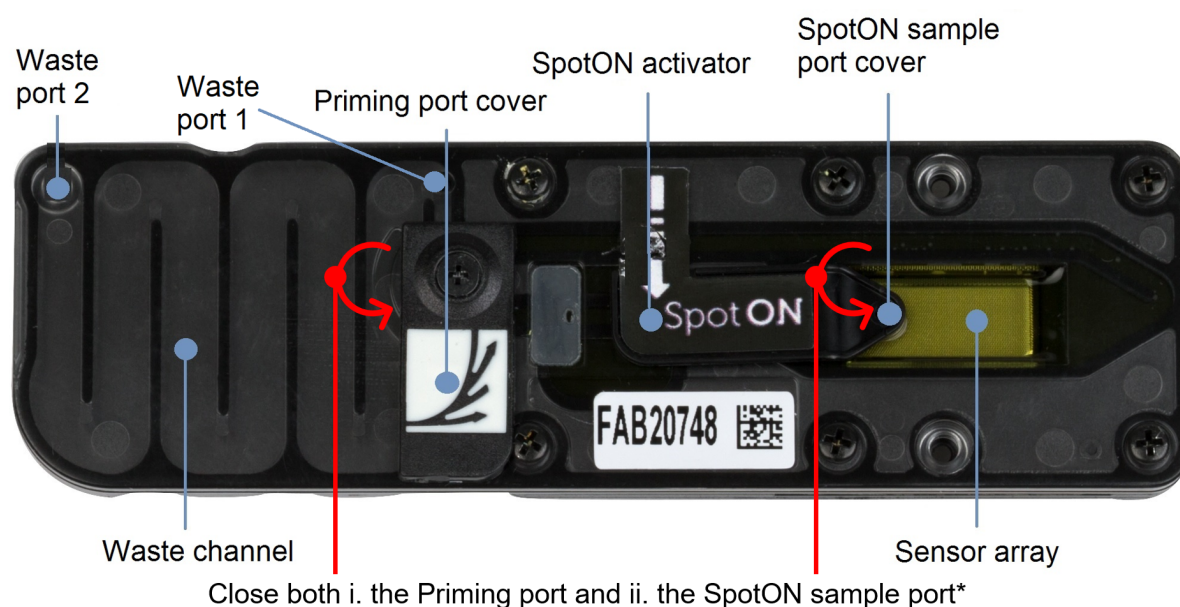
## F MinION sequencing

This section uses the MinION, flowcell and the computer program MinKNOW.

Before starting a sequencing run, perform a hardware check (page 29) and a flowcell check (page 30) to make sure your equipment is working properly.

- 1 Open the MinION and insert the flow cell by sliding it under the clip.

### The MinION flowcell



\*Both ports are shown in a closed position

- 2 Open the priming port by turning it clockwise. To remove any air bubbles you need to pipette out a small amount of the storage buffer. This is done as follows:
  1. Set a P1000 pipette to **200 $\mu$ L**
  2. Insert the tip into the priming port
  3. Adjust the P1000's volume by turning the wheel until it is set to **220-230 $\mu$ L**, or until a small volume of the buffer has entered the pipette tip.

You should be able to see that buffer liquid is filling the tube all the way from the priming port to the sensor array.

- 3 Add **30 $\mu$ L** of Flush Tether (FLT) directly to the tube of Flush Buffer (FB) and mix by vortexing, this is now the flow cell priming mix.

- 4** Load **800µL** of the flow cell priming mix into the flow cell through the flow cell priming port, avoiding the introduction of air bubbles. Wait for **5m00s**.
- While you wait, prepare the library for loading.
- 5** The Loading Beads (LB) tube contains a suspension of beads. These beads settle very quickly. It is very important that they are mixed immediately before use.
- In a new microfuge tube, prepare the library as follows:
- **34µL** Sequencing buffer (SQB)
  - **25.5µL** Loading beads (LB), mixed immediately before use
  - **15.5µL** DNA library
- 6** Complete the flow cell priming:
- Gently lift the SpotON sample port cover to make the sample port accessible
  - Load **200µL** flow cell priming mix into the flow cell through the priming port (not the SpotON sample port), avoiding the introduction of air bubbles.
- 7** Gently mix the prepared library by pipetting up and down. Add **75µL** of the library to the flow cell via the SpotON sample port, drop by drop. Do not touch the pipette tip to the sample port, let the library drop from the pipette tip onto the port. Let each drop flow into the port before adding the next drop.
- 8** Gently close the SpotON sample port, making sure the bung enters the port. Close the priming port by rotating it anticlockwise.
- 9** Open the MinKNOW software on your computer and log in. Select your MinION device from the list of connected devices.
- 10** From the homepage, click on **Start sequencing**.
- 11** Type in a name for your experiment, sample ID and choose your flowcell type from the dropdown menu.
- When ready, click on **Continue to kit selection**.

- 12** Select the name of the kit you have used to barcode your samples for sequencing. This was probably **SQK-RBK004**, but check your kit.  
When ready, click on **Continue to run options**.
- 13** Select a run length - you are aiming for ~ **800,000 reads per sample**, but how long this takes will depend on your number of samples and how many active pores your flowcell has. You can always continue the run, or stop the run early if you need to.  
Select a bias voltage - the default of -180mV is fine for most runs, but you may want to reduce this to -160mV if your run is short and the flowcell is new.  
In the 'advanced user options', make sure adaptive sampling is **off**.  
When ready, click **Continue to basecalling**.
- 14** Switch the 'basecalling', 'barcoding' and 'alignment' options all to **disabled**.  
When ready, click **Continue to output**.
- 15** Select a location to save your data. We are not basecalling at this stage, so the output format should be **FAST5**.  
When ready, click on **Continue to final review**.
- 16** Check the settings that you have selected for your sequencing run, you can make changes by selecting **Edit**.  
When ready, click **Start** to begin your sequencing run.
- 17** You will be taken to the 'Sequencing overview' page, which has many options to monitor the progress of your run.  
If you need to stop the sequencing run, select **Experiments** from the left menu, and click **Stop**.
- 18** When the run is complete, generate a run report by clicking on **Export PDF**. This will be saved to the same folder as your sequence data.

## **G Basecalling**

This section uses the computer program MinKNOW.

While there is an option to perform basecalling during the sequencing run, this can be very slow. Therefore this protocol performs basecalling after the sequencing run has been completed.



- 1** From the MinKNOW homepage, click on **Analysis**, then select **Basecalling**.
- 2** Select the folder containing the **.fast5** files that were generated during the sequencing run that you want to basecall.  
When ready, click **Continue to output**.
- 3** Select where you want your basecall files to be saved. If nothing is changed, the files will be put into a new folder called 'basecalled' inside your input folder.  
The 'Compress .fastq files' options should be turned **off**.  
The 'Output .fast5 files' option should be turned **on**.  
When ready, click **Continue to basecalling**.
- 4** Choose a basecalling model from the dropdown menu.  
You can choose either:  
    FLO-MIN106 / FLO-FLG001 - DNA - Fast  
    or  
    FLO-MIN106 / FLO-FLG001 - DNA - High accuracy  
The **High accuracy** model is recommended unless you need the basecalling to be completed very quickly.  
When ready, click on **Continue to barcoding**.
- 5** Select the name of the kit you have used to barcode your samples for sequencing. This was probably **SQK-RBK004**, but check your kit.  
The 'Trim barcodes' and 'Mid-read barcodes' options should be turned **off**.  
The 'Barcode both ends' option should be turned **on**.  
The 'Minimum barcoding score should be set at **60**.  
Leave both 'override' options turned **off**.  
When ready, click on **Continue to alignment**.
- 6** Turn the 'Alignment reference' option **off**.  
When ready, click **Continue to review**.
- 7** Check your settings and select **Start** to begin post-run basecalling.

## H Bioinformatics

In this section you will be using bash commands in the terminal. If you are new to using the terminal see the ‘Introduction to command line bioinformatics’ section (page 41).

Before you start, you should have a folder containing all the files for your experiment. In this folder should be a folder called fastq, then a folder for each barcode (called barcode01 etc), with the .fastq files inside. If this is not the case, do it now.

- 1 Copy all the files from the MARPLE bioinformatics folder into your experiment folder. You should have the following files:

 marple2_input-5.fasta	 gff2bed.py
 marple2_input-5.gff3	 mpileup_to_fasta.py
 13_23-run24.fastq	 cov-stat.sh
 14_106-run24.fastq	 do_marpl...pipeline.sh
 F18-run24.fastq	 marple_pi...nofilter.sh
 samples_run24.txt	 read_cov...ge_stats.sh
 compsnps...pileup.py	

- 2 Open the Terminal on your computer.  
Navigate to the folder for the current experiment using the bash commands below.

### Bash navigation commands:

<b>pwd</b>	print working directory
<b>cd x</b>	change directory to folder called 'x'
<b>cd ..</b>	go up one directory in the file structure
<b>ls</b>	list files in the current directory

- 3 Type the following command into the terminal and press Enter:  
**cat fastq/barcode01/\*.fastq >> barcode01.fastq**  
 This will take all .fastq files in barcode01 and put them all into new file called barcode01.fastq in your experiment folder.  
 Repeat this for all the other barcodes in your experiment (replace the barcode01 parts of the command).

- 4 Open **text\_file.txt**. Type in the names of the new barcodeXX.fastq files that you just created (barcode01, barcode02, etc). These must each be on a new line, but with no other text or punctuation.

- 5** Type the following command into the terminal and press Enter:  
**source activate marple**  
 This will begin the MARPLE session in your terminal.
- 6** Type the following command into the terminal and press Enter:  
**bash do\_marple\_pipeline.sh text\_file marple2\_input-5.fasta marple2\_input5.gff3**  
 When typing file names, you can press Tab to complete.  
 This will perform the steps of the MARPLE pipeline on all of the files you listed in the text file.  
 This may take a long time to complete, the progress will be displayed on the screen.
- 7** Type the following command into the terminal and press Enter:  
**cat final\_output/barcode01.fastq.marple2\_input-5.fasta.sam.bam.mpileup.snp.\_ratios.txt.output.processed.fasta.sorted.concatenated.renamed >> tree-input.fasta**  
 The 'final' to the 'renamed' contains no spaces.  
 When typing file names, you can press Tab to complete.  
 This will take all of the files that were the output from the last step for each barcode, and put them together in one file called tree-input.fasta.
- 8** Type the following command into the terminal and press Enter:  
**bash submit\_tree.sh \$Inpath \$savename**  
 Replace the underlined section with what you want your output file to be called (ie, the name of your experiment).  
  
 This will create a phylogenetic tree of rust strains containing your samples. The file can be opened in many programs designed to work with phylogenetic trees, eg Dendroscope or MEGA.

## **Extra Instructions**

## Hardware check

This section will tell you how to check that your MinION is working correctly.

- 1** Insert a Configuration Test Cell (CTC) into the MinION and plug it into the computer.
- 2** Open the minKNOW software. From the homepage, click on **Hardware check**.
- 3** Select the green **Start** button.  
The check will begin, and will take about **1m00s** to complete.
- 4** If the check is passed, a green icon will appear. This means your MinION is working properly and is ready to be used.  
  
If the check is failed, an orange icon will appear. If this happens, remove the CTC and start again from **step 1** - if the second check also fails, your MinION is not working properly and you cannot use it. Contact your manager about this problem.

## Flowcell check

This section will tell you how to check that your flowcell is working correctly.

- 1** Insert the flowcell that you want to check into the MinION and plug it into the computer.
- 2** Open the minKNOW software. From the homepage, click on **Flow cell check**.
- 3** Choose **MinION** from the 'flow cell type' dropdown menu, and select **Start** to begin the check.  
You will be taken to the 'sequencing overview page'.
- 4** Once the check is complete, the number of available pores in your flowcell will be shown under the picture of the flowcell.

**Note** You will need a minimum of around 500 pores to use the flowcell, but 800 is desirable, out of a maximum of 2048. The more samples you have to sequence the more pores you will need.

## Preparation of reagents

### Preparation of lysis buffer

To make 500mL lysis buffer

**Reagents you will need:**

- 1M Tris HCl
- 0.5M EDTA
- 10% SDS

**Equipment you will need:**

- Weighing scale

- 1** Take a beaker and add 400mL distilled water.
- 2** Add **5mL** 1M Tris HCl.
- 3** Add **1mL** 0.5M EDTA.
- 4** Add **5ml** 10% SDS solution.
- 5** Add more distilled water to make up the total volume to **500mL**.

## Preparation of SDS (sodium dodecyl sulphate)

To make 1L 10% SDS solution from solid SDS

### Reagents you will need:

- Solid SDS
- Deionised water (or distilled water)

### Equipment you will need:

- Weighing scale
- Thermometer
- Magnetic stirrer

- 1** Dissolve **100g** SDS in **900mL** of H<sub>2</sub>O. Be careful not to breathe in the SDS powder, wear a mask or use a fume cupboard if possible.
- 2** Heat to **68C** and stir with a magnetic stirrer if possible.
- 3** Add H<sub>2</sub>O until the total volume reaches **1L**.

### *Note*

Store at room temperature.

Does not require sterilising.

Do not autoclave.

## Preparation of EDTA

You may have access to pre-prepared EDTA, but if you ever need to make your own you can follow this protocol.

To make 1L 0.5M pH8.0 EDTA

### Reagents you will need:

- Solid EDTA dihydrate (EDTA.2H<sub>2</sub>O)
- Deionised water (or distilled water)
- Sodium hydroxide

### Equipment you will need:

- Weighing scale
- Magnetic stirrer
- pH meter

- 1** Add **186.1g** of sodium EDTA.2H<sub>2</sub>O to **800mL** of deionised water (H<sub>2</sub>O).
- 2** Stir vigorously with a magnetic stirrer.
- 3** While monitoring the pH with a pH meter, slowly add sodium hydroxide (NaOH) until the solution reaches **pH 8.0**.
- 4** Add more deionised water up to total volume of **1L**.
- 5** Sterilise by autoclaving, if possible.
- 6** Store at room temperature.

## Preparation of 5X TBE

If you have agarose tablets that contain TBE, you will not need to make your own, but if you do ever need to make TBE, you can follow this protocol.

The advantage of 5X TBE over 10X TBE is that 5X is less likely to precipitate.

### Reagents you will need:

- Solid Tris base
- Solid boric acid
- 0.5M EDTA solution
- Deionised water

### Equipment you will need:

- Weighing scale
- pH meter

**1** Add **54g** Tris base and **27g** of boric acid to **20mL** of **0.5M** EDTA solution.

**2** Adjust to **pH 8.3** by slowly adding concentrated HCL.

**3** Add more deionised water up to total volume of **1L**.

**4** Store at room temperature.

### *Note*

To use TBE during gel electrophoresis you will need a 0.5X TBE solution. You can dilute your 5X TBE to 0.5X by mixing **1 part** 0.5X TBE with **10 parts** deionised water.

## Preparation of Tris HCl

You may have access to pre-prepared Tris HCl, but if you ever need to make your own you can follow this protocol.

To make 1L 1M Tris HCl

### Reagents you will need:

- Solid Tris base
- Deionised water
- Concentrated hydrochloric acid

### Equipment you will need:

- Weighing scale
- pH meter

- 1** Dissolve **121.14g** tris base into **800mL** of deionised water .
- 2** While monitoring the pH with a pH meter, slowly add concentrated hydrochloric acid (HCl) until the pH meter reads **pH 7.0**.
- 3** Add more deionised water up to total volume of **1L**.
- 4** Store in fridge.

## **Extra Information**

## Glossary

<b>Centrifuge/spin down</b>	Spin a tube, or set of tubes, to force any liquid down to the bottom. This is normally done using a machine, which is also called a centrifuge. Commonly small centrifuges are used called microcentrifuges, or microfuges.
<b>Incubate</b>	Keep at a certain temperature for a certain amount of time
<b>Library preparation</b>	The process used to prepare DNA molecules for sequencing, tags or barcodes are added so that individual samples can be identified in the analysis.
<b>Magnetic beads</b>	In their tube, the tiny magnetic beads are suspended in the liquid and are not visible. When they are added to a solution of DNA, the beads attach to the DNA molecules. When the tube is placed in the magnetic rack, the beads are attracted to the magnet, and form a visible pellet along with the DNA. This allows the supernatant to be carefully removed, without removing the DNA.
<b>Nuclease-free water</b>	Water that does not contain any enzymes that can damage DNA samples.
<b>PCR</b>	Polymerase chain reaction, this process uses primers to copy one section of the DNA many times, controlled by cycles of different temperature steps.
<b>Pellet</b>	A small, rounded mass
<b>Supernatant</b>	The liquid that is left behind when solid elements are removed from a solution
<b>Suspend/re-suspend</b>	Mix solid particles that might have sunk to the bottom of a tube back into the liquid, by shaking or pipetting up and down
<b>Vortex</b>	Shake using a vibrating machine

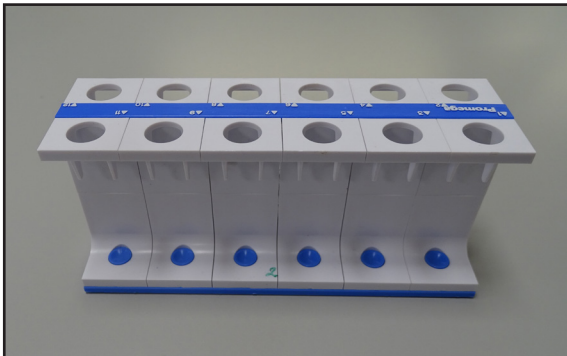
# Materials

## Equipment and consumables

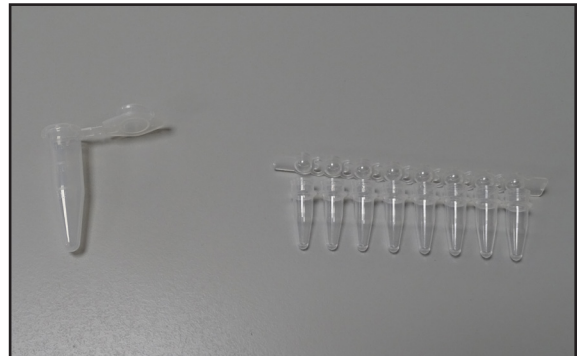
- Pipettes and tips (P1000, P200, P20, P2)
- Timer
- PCR machine \*
- Microfuge tubes (1.5 mL and 0.2mL) \*
- Micropestle/s \*
- Microfuge tube rack
- Gel electrophoresis tank, gel tray and combs\*
- Ice bucket (if possible)
- Magnetic tube rack \*
- Vortex machine (if possible) \*
- Microfuge (if possible, with racks for 1.5mL and 0.2mL tubes) \*
- Stove or microwave
- Computer with minKNOW, Fiji and Microsoft excel
- Flow cell
- MinION

\* Items are pictured below

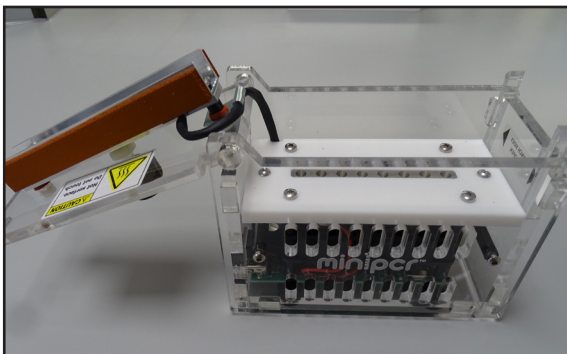
**Magnetic tube rack**



**Microfuge tubes (1.5mL, 0.2mL)**



**Mini PCR machine**



**Micropestle**



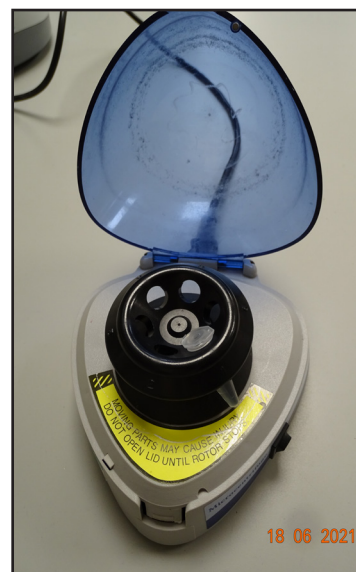
### Gel electrophoresis tank



### Vortex mixer



### Microfuge



## Reagents

- Primer pools
- AMPure XP beads
- Nuclease-free water
- 70% ethanol in nuclease-free water (made fresh)
- Lysis buffer: 0.1 M Tris-HCl pH 7.5, 0.05 M ethylenediaminetetraacetic acid (EDTA\*) pH 8 with 1.25% sodium dodecyl sulphate (SDS) \*
- GelGreen DNA stain
- Rapid barcoding kit
- Flow cell priming kit
- 10 mM Tris-HCl pH 7.5-8, with 50 mM NaCl \*
- AmpliTaq Gold™ 360 Master Mix
- Qiagen DNeasy Plant Mini kit
- TBE buffer \*
- Gel loading dye
- DNA ladder
- Agarose tablets

\* Instructions for making these reagents are given on pages 31-35, but most you will have already prepared.

## Magnetic beads

Parts of the MARPLE protocol use magnetic beads to purify DNA from a solution. Magnetic beads are very small balls of plastic, covered in a magnetic mineral called magnetite. On the outside of the beads are many molecules that can attach to DNA, or detach from DNA, depending on the electric charge of the chemicals in the solution.

Before you use them, the magnetic beads will be in a tube marked AMPure XP beads - this contains the beads in a solution. If the beads have sunk to the bottom, they will look like a brown mass at the bottom of a clear liquid. If the beads are suspended in the solution, the liquid will look brown. The beads must be suspended in the liquid before you use them, this can be done by shaking the tube or vortexing.

### How to use

The beads work best if they are stored in the fridge, but brought up to room temperature before you use them.

To use the beads, add the same volume of beads in solution to the tube containing your DNA solution (ie, if you have 100µl of DNA solution, add 100µl of magnetic bead solution). Mix well by pipetting the mixture up and down, then leave time for the beads to attach to the DNA.

Next, place your tube/s in a magnetic rack. The rack contains a magnet that will attract the beads (with the DNA) to the side of the tube, leaving everything else in the solution. The solution will turn from brown to clear as the beads gather together into a pellet.

Once the liquid is clear you can use your pipette to remove the liquid and throw it away. Leave the tube in the magnetic rack and pipette up as much liquid as you can, leaving behind the pellet of beads with the DNA attached. This must be done very carefully - do not take out any brown beads or you will be throwing away your DNA! It helps to point the tip away from the pellet (see picture). If you get beads in your pipette tip, just push them back into the tube and wait for the liquid to clear again.

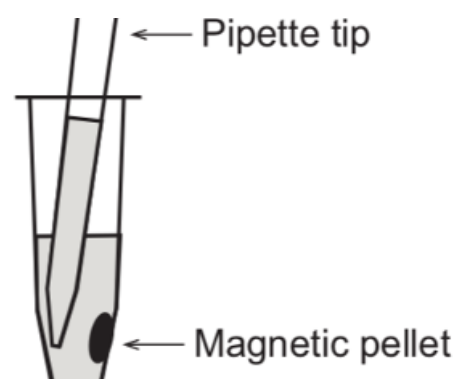
Next you add ethanol to wash the pellet, then wait for the beads to form a pellet again before pipetting out the ethanol. Repeat this step as many times your protocol says.

Once the pellet is clean you can remove the tube from the magnetic rack and add nuclease-free water and mix to resuspend the beads. Now wait to give time for the DNA to detach from the beads.

Now the DNA will be in the liquid and you need to remove the beads, so put the tube back in the magnetic rack. Once the pellet has formed and the solution is clear, use your pipette to remove the liquid and put it into a new tube. You now have a solution of pure DNA in water.

#### Removing supernatant

When removing the supernatant from a tube containing a pellet of magnetic beads, point the pipette tip away from the pellet and pipette slowly. If you pipette beads, return them to the tube and start again.



## Introduction to command line bioinformatics

Most of us are used to interacting with files on our computers through an interface such as Windows or macOS, which are designed to be intuitive and user-friendly. However, for some tasks it is easier to pass instructions directly to the computer's command line. The bioinformatics part of the MARPLE diagnostics protocol uses the **terminal** to pass instructions to the command line of your computer to automate analyses that would otherwise be very slow or impossible. We will type instructions (called **commands**) into the terminal written in a programming language called **bash** and run them by pressing 'Enter'.

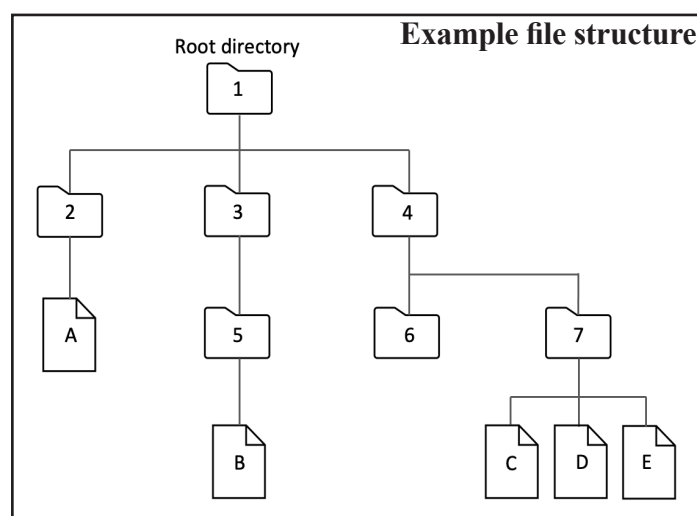
To make the process as simple as possible, most of the commands are already written and saved into files called **scripts**. A script is a long list of commands that will run automatically, but you will need to tell the script which data files to use as its input, and what to call the files that are the output from the analysis. The exact commands to use are given in the bioinformatics section of the MARPLE protocol (page 26), but to use them you must be able to navigate through the file structure of your computer from the terminal.

### Bash commands:

<b>pwd</b>	print working directory
<b>cd x</b>	change directory to folder called 'x'
<b>cd ..</b>	go up one directory in the file structure
<b>ls</b>	list files in the current directory
<b>cat</b>	concatenate, join things together

When you start you will always be in your root directory. Your current 'location' in the file structure is called your **working directory** - you can view your current working directory by running the command **pwd**.

The command to change directory is **cd**. From folder 1 in the example file structure shown above, you could navigate to folder 4 using the command **cd 4**. You can go up one directory in the file structure by using the command **cd ..**.



If you want to navigate to a directory further away in the file structure, but you know its location, you can use **cd** along with the file path of the directory. The file path starts with the `~` symbol, which represents the working directory. For example, to navigate to folder 7 from folder 6 you could use the command **cd ~/4/7** . Alternatively, you could use **cd ..** to navigate from folder 6 to folder 4, then **cd 7** to get to folder 7.

You can view the contents of a directory with the command **ls**. If you run **ls**, it will show you which folders you can enter from your current location or which files you can pass to a command using just the name of the file. To refer to files not in your working directory, use the file path. If you were in folder 5, you could call file B by using just its name 'B', but file C would be called using '`~/4/7/C`' .

While you are typing, press 'Tab' to automatically complete a file name. This prevents typing mistakes.

If you want to call multiple files with a common element in the name, it can be helpful to use **wildcard** symbols. The `?` can be used to take the place of any single character, the `*` can take the place of any number of characters, and `[ ]` can be used to match any 1 character enclosed within the square brackets. As an example, say you had 10 .txt files called file\_1.txt - file\_10.txt. The table below shows which files would be called by different wildcard statements.

Wildcard statement	Files returned
file_?.txt	file_1.txt - file_9.txt
file_*.txt	file_1.txt - file_10.txt
*.txt	All files in directory with .txt extension
file_[135].txt	file_1.txt, file_3.txt, file_5.txt
file_[5-10].txt	file_5.txt - file_10.txt

One of the commands that you will be using in the MARPLE protocol is **cat**, which stands for 'concatenate'. This command joins together the files or elements that are provided with the command.

This has been a short introduction to working with bash in the terminal but there are many many more commands that you could use to work more quickly and efficiently. You can find a mountain of useful information and practice exercises for using the command line on the internet.