Soil microbiology workshop

4TH- 14TH SEPTEMBER 2023

Charles Sturt University

Gulbali Institute
Agriculture Water Environment

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Background

The study of soil microbiology allows researchers to investigate the function of soil microbes and the interactions they have with soil properties. Studying microbial functions focuses on processes that soil microbes perform. These processes are tightly linked with carbon and nitrogen cycling and can include mineralisation, nitrification, and nitrogen fixation. These processes can be measured by quantifying the functional gene associated with a specific process. For example, the process of nitrogen mineralisation is associated with the functional genes neutral and alkaline metallopeptidase (*npr* & *apr*), bacterial nitrification is associated with the functional gene of ammonium monooxygenase (*amoA*), and the process of nitrogen fixation is associated with the dinitrogenase reductase (*nifH*) gene. Soil DNA needs to be extracted in order to quantify the functional gene of a soil microbial process through quantitative polymerase chain reaction (qPCR). This allows researchers to determine the effects of treatments on a particular soil microbial process.

What is qPCR?

Quantitative polymerase chain reaction is a DNA measurement technique that aims to turn a small amount of DNA into a larger amount. As it does this, qPCR detects a fluorescence in real time that gives readings through the amplification process that can be used to calculate relative and absolute amounts of DNA.

The steps to PCR

Initiation requires the DNA template that has been extracted from the soil sample of interest.

Denaturation is the first step in PCR where heat is applied to break the hydrogen bonds and separate the double-stranded DNA into two single strands. This step is usually performed at 94-98 ℃ and can last up to 5 minutes.

Annealing is the second step of PCR and is performed at a lower temperature to allow for the primers to bind to the target DNA strands. The primers are designed to identify the target of interest (reference gene) and the region of sequence between the primers is called the amplicon. If this temperature is too low, non-sepecific binding may occur.

The final step of PCR is **extension** and is the period of elongation where the polymerase extends the primer to form a new DNA strand. This process occurs multiple times (normally 25-35 cycles) so that the region of interest (reference gene) is amplified exponentially.

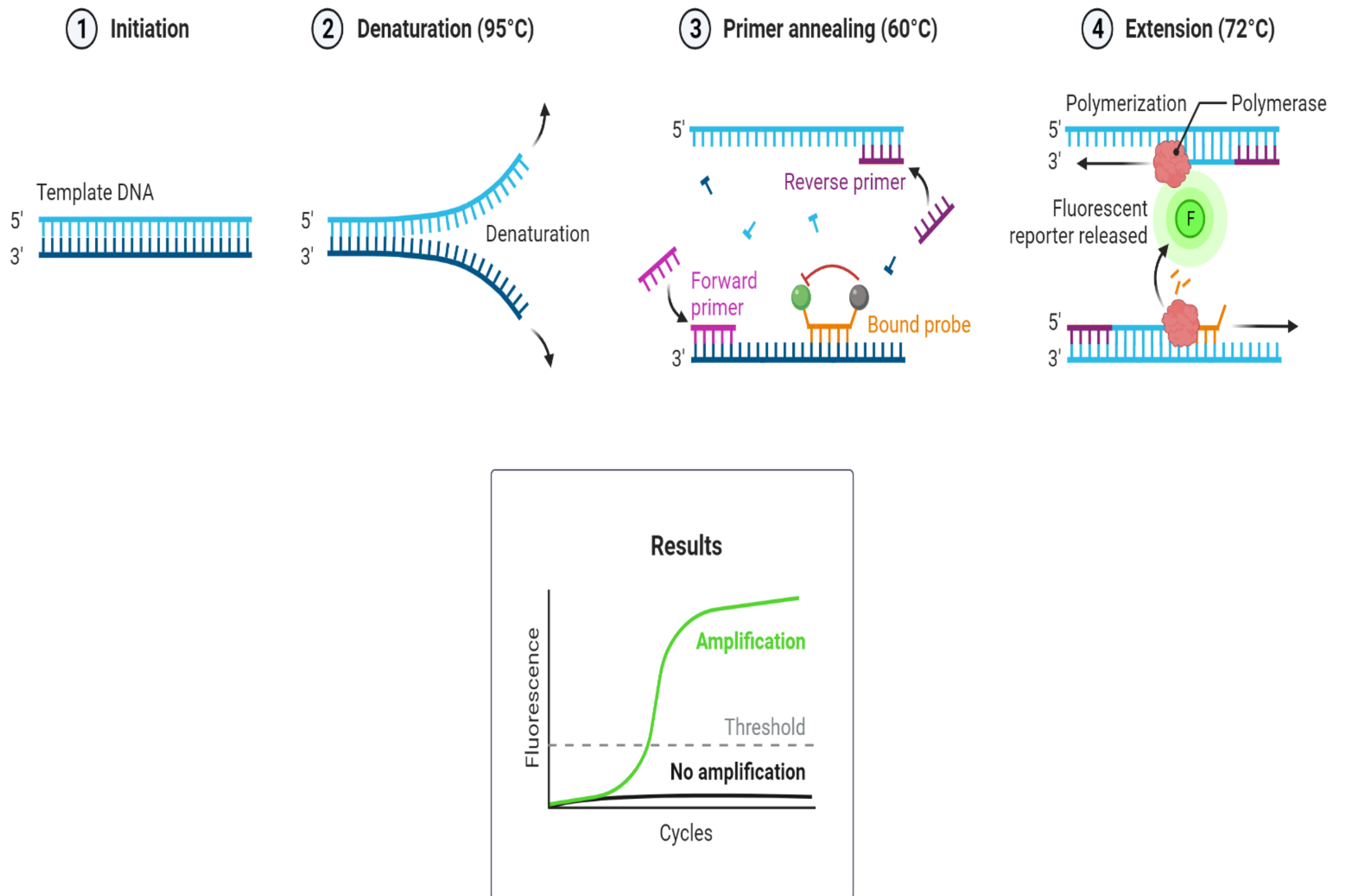
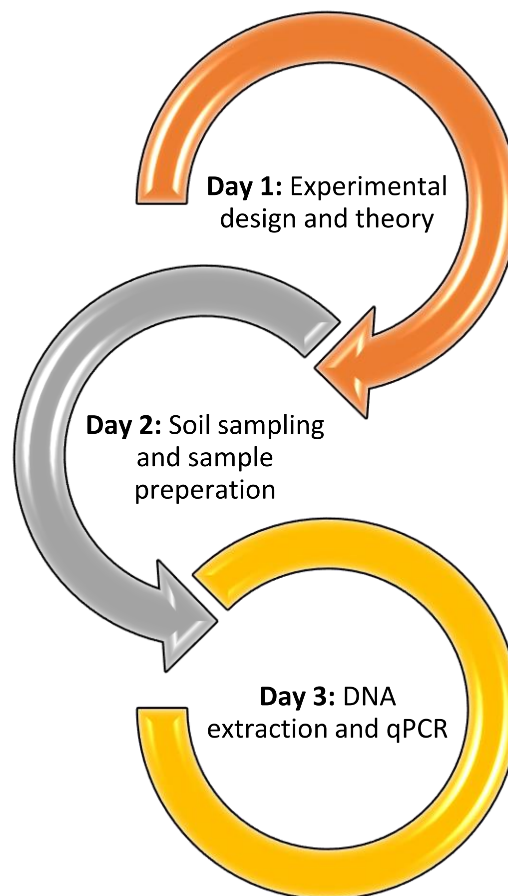


Figure 1: *illustration showing the main steps of PCR (initiation, denaturation; annealing and extension) and the results of fluorescence vs cycle number (Biorender.com).*

This workshop

This workshop will focus on microbial function and soil processes with analysis performed using qPCR. The three days will be broken down into a theoretical component (day 1) where DNA extraction, primer selection, reagent preparation, qPCR run cycles, results, and analysis are all discussed in preparation of co-designing a short experiment using an established field site that will be conducted on day 2 & 3. A practical soil sampling component at a local field site (day 2) will be followed by a laboratory day (day 3) where DNA is extracted and analysed via qPCR.



Field site, treatments, and design

The field site we will be using is located at Mangoplah NSW and is funded by Meat and Livestock Australia (MLA). It is investigating the effect of acidic soils on different pasture species and legume mixes (Figure 2). We will use this established field trial in the design and implementation of our microbiology experiment.

Field design

- ☐ Mixes of multiple different pasture species with legumes (72 plots)
  - Chicory
  - Plantain
  - Phalaris
  - Lucerne
  - Vetch
  - Sulla
- ☐ No lime vs Lime at 5 t/ha
- ☐ Lime has been incorporated prior to sowing (2022)
- ☐ Plots are 10 x 12 m for each pasture species

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***Figure 2**: Mangoplah field trail including limed (green) and unlimed (orange) plots with different pasture species mixes with 6 replicates.*

Functional gene selection

To effectively design an experimental plan with a soil microbial component, you need to identify and select the microbial processes relevant to your objectives and aims. Identifying your target process (e.g. mineralisation, nitrification, nitrogen fixation, etc.) will enable the selection of functional genes that can be quantified from the extracted DNA. Many different functional genes can be found in peer-reviewed literature and Table 1 lists some examples focusing on N cycling processes.

Table 1: *Examples of functional genes and their processes in soil.*

Process	Gene	Function in soil
Mineralisation	*Npr* Neutral metallopeptidases	Bacterial decomposition of organic matter to mineral N via *Bacillus* spp. *NPR* encodes the main extracellular proteases in soil.
	Apr Alkaline metallopeptidases	Bacterial decomposition of organic matter to mineral N via *P. fluorescens*. *APR* encodes the main extracellular proteases in soil.
Nitrification	*amoA* Ammonium monooxygenase bacteria	Oxidation of ammonium to nitrate by bacteria. Common in neutral soils with adequate moisture.
	Arch-amoA Ammonium monooxygenase bacteria	Oxidation of ammonium to nitrate by archaea. Common in acidic soils and hostile soil conditions.

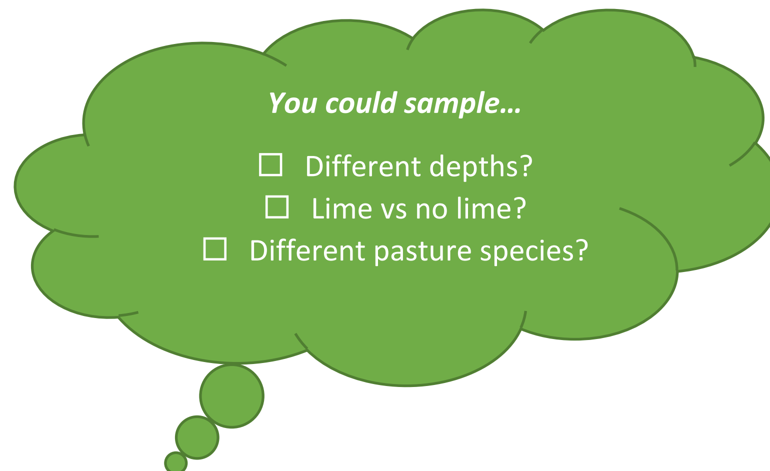
MIC qPCR

Functional genes will be quantified using a Magnetic Induction Cycler (MIC) qPCR. The MIC (Figure 3) is a small and compact PCR machine that only weighs 2 kg, is a 48 well instrument and has relatively fast run times. Bluetooth connection enables multiple MIC instruments to be connected to one computer, enabling bulk samples to be completed at once.

Figure 3: *MIC qPCR*

Creating your objectives and hypothesis

Based off the field trial information, you can now design a short experiment targeting a microbial process with a functional gene of interest. Think of ways that soil microbial process is affected and how you can best use the field treatments to test your hypothesis.



Soil sampling and preparation

Sampling needs to be done in a methodical and repeatable way to ensure the soil sampled is representative of the treatment plots. We commonly use 20 cores per treatment plot which can be divided into various depths and sample in a 'W' or 'Z' shape when sampling.

If you are also taking soil samples for chemical or physical analysis to be linked with microbial data, ensure those samples are subsampled from the same microbial sample.

Ethanol is used between treatments to prevent cross contamination. Once soil is collected and labelled, it should be stored in a cold place until transported back to the laboratory where samples are thoroughly mixed, labelled and stored at -20℃.

Soil microbiology in experiments

The following steps are broken down into multiple sections:

These steps are all performed to quantify your target gene of interest. The storage of DNA and certain reagents at -80°C means you do not have to perform all the steps at once.

4.1 DNA extraction

After soil sampling your treatments, the next step is to extract the DNA of each sample using a DNA extraction kit. The quality of the kit will determine the ease and effectiveness of DNA extraction. Things like high clay content and organic matter can require additional cleaning steps to ensure the extracted DNA is of good quality. These types of soils may require extra disruption steps. We will be using DNeasy PowerSoil Pro Kits (qiagen.com) (Figure 4) in this workshop.

Once you have extracted your DNA, you will need to check the purity of the sample using a NanoDrop. This is done by measuring the ratio of absorbance at 260 nm divided by the reading at 280 nm. Good quality DNA will have a A_{260}/A_{280} ratio between 1.7-2.0. A low ratio may indicate contaminants and require the use of spin columns or re-precipitation.

Figure 4: QIAGEN DNeasy PowerSoil DNA extraction kit.

DNA soil extraction protocol- adapted from QIAGEN

Important points before starting

- Perform all centrifugation steps at room temperature (15–25°C).
- If Solution C1 has precipitated, heat at 60°C until precipitate dissolves.

Procedure (*tick as you go*)

☐ 1. Add 0.25 g of soil sample to the PowerBead Tube provided. Gently vortex 10 sec to mix.

☐ 2. Add 60 µl of Solution C1 and vortex briefly 5 secs.

☐ 3. Secure PowerBead Tubes horizontally using a Vortex Adapter for 24 (1.5–2.0 ml) tubes

☐ 4. Vortex at maximum speed for 5 min. If many samples use 10 mins.

☐ 5. Centrifuge tubes at 10,000 x g for 30 s.

☐ 6. Transfer the supernatant to (400-500 ul) a clean 2 ml Collection Tube.

☐ 7. Add 250 µl of Solution C2 and vortex for 5 s. Incubate at 2–8°C for 5 min.

☐ 8. Centrifuge the tubes for 1 min at 10,000 x g.

☐ 9. Avoiding the pellet, transfer up to 600 µl (550 ul) of supernatant to a clean 2 ml Collection Tube.

☐ 10. Add 200 µl of Solution C3 and vortex briefly. Incubate at 2–8°C for 5 min.

☐ 11. Centrifuge the tubes for 1 min at 10,000 x g.

☐ 12.Avoiding the pellet, transfer up to 750 µl (700 ul) of supernatant to a clean 2 ml Collection Tube.

☐ 13. Shake to mix Solution C4 and add 1200 µl to the supernatant. Vortex for 5 s.

☐ 14. Load 675 µl onto an MB Spin Column and centrifuge at 10,000 x g for 1 min. Discard flow-through.

☐ 15. Repeat step 14 twice, until all the sample has been processed.

☐ 16. Add 500 µl of Solution C5. Centrifuge for 30 s at 10,000 x g.

☐ 17. Discard the flow-through. Centrifuge again for 1 min at 10,000 x g.

☐ 18. Carefully place the MB Spin Column into a clean 2 ml Collection Tube. Avoid splashing any Solution C5 onto the column.

☐ 19. Add 100 µl of Solution C6 to the center of the white filter membrane. Alternatively, you can use sterile DNA-free PCR-grade water for this step (cat. no. 17000-10).

☐ 20. Centrifuge at room temperature for 30 s at 10,000 x g. Discard the MB Spin Column. The DNA is now ready for downstream applications (store at –20°C to –80°C).

DNA extraction- tips and tricks

Cleaning

- Ensure that all spoons, spatulas and tubes are cleaned with 70% ethanol between samples.

Sample integrity

- Take precautions and consider contamination between samples.

Two useful approaches...

- Randomise samples to be extracted (you don't want all samples from the same treatment in order).
- (for Next generation amplicon sequencing) Include an extraction blank. A tube that goes through all steps of a DNA extraction but does no have any sample added.

Tips for efficiency

- label all tubes before you commence DNA extractions. Time between steps is short and quality of the DNA extraction can be compromised by delays in some places.
- Think about where the DNA is at all times. E.g. is it bound to the column, is it in solution.

4.2 Primer selection

To detect the functional gene that you are targeting from the DNA extracted from your soil samples, you must use a primer that is specific to that gene. Each primer is commercially prepared through the primer code and comes in pairs (forward and reverse). The primer code (Table 2) is often be found in the methodology section of published peer-reviewed literature.

Table 2: *Specific primers and codes for a range of functional genes.*

Gene	Primer	Primer code	Reference
amoA	*amoA* F	GGGGTTTCTACTGGTGGT	Rotthauwe et al. 1997
	amoA R	CCCCTCKGSAAAGCCTTCTTC	
Arch-amoA	*Arch-amoA* F	STAATGGTCTGGCTTAGACG	
	Arch-amoA R	GCGGCCATCCATCTGTATGT	
apr	*apr* I F	5'-TAY GGB TTC AAY TCC AAY AC-3'	Bach et al 2001
	apr II R	5'-VGC GAT SGA MAC RTT RCC-3'	
npr	*npr* I F	5'-GTD GAY GCH CAY TAY TAY GC-3'	
	npr II R	5'- ACM GCA TGB GTY ADY TCA TG-3'	

4.3 qPCR cycle settings

The cycle settings determine the duration and temperature that the qPCR will perform denaturing, annealing and extension steps for. Cycle settings for qPCR will vary between each functional gene and also within the literature. The settings you decide on will be the input settings for the qPCR run (section 4.6) and will determine the overall run time. Table 3 has some examples of different run times for functional genes. Note the slightly different run times between the literature for the same functional gene.

Table 3*: Examples of different qPCR cycle settings for functional genes that will be used to run the MIC.*

Gene	Denaturation	Cycle number	Cycle	Extension	Reference
amoA* & Arch-*amoA	95°C for 5 min	40	95°C for 5 secs, 60°C or 30 secs, 72°C for 45 secs	95°C for 5 mins	Kaveney et al., 2020
apr* & *npr	95°C for 5 min	30	94°C for 30 secs, 53°C or 49 °C for 30 secs, 72°C for 20 secs	72°C for 10 mins	Bach et al., 2001
apr	95 °C for 15 mins	40	94°C for 30 secs, 60 °C for 30 secs, 72°C for 30 secs	72°C for 5 mins	Sakurai et al., 2007
npr	95 °C for 15 mins	40	94°C for 60 secs, 55 °C for 60 secs, 72°C for 60 secs	72°C for 5 mins	Sakurai et al., 2007

4.4 qPCR reagent mastermix

Once you have selected your functional genes, primers and run cycle settings, you can now prepare the qPCR reagents and mastermix (Figure 5). This is a multi-step process performed in a preparation room (a different area to where you handle/ extract DNA).

The qPCR reagent mix is specific to the functional gene and the qPCR instrument you are using and includes all the reagents necessary to perform qPCR on your extracted DNA. We are using a MIC PCR and thus will use a 'Sensifast Perfecta SYBR green FastMix mastermix' reagent.

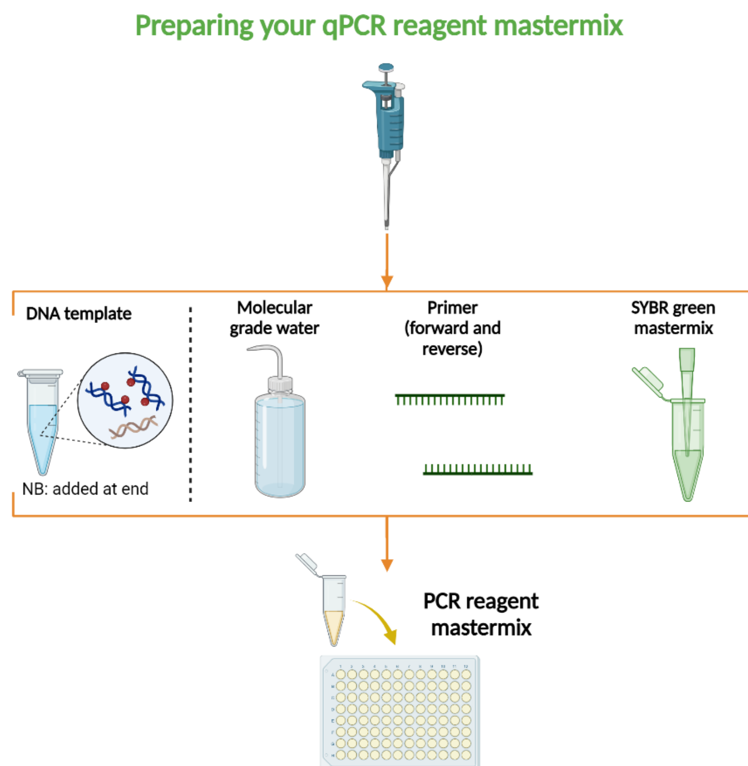


Figure 5: The components required to make the PCR reagent mastermix. Note the reagent mastermix is prepared in a DNA free area and the DNA template is added at the end.

Preparing the qPCR reagent mix

1. **Primers**- Perform a 1/10 dilution for each of the primers

   (20 ul of primer/ 180 ul of MG water)

   This can be done 4 times (4 sets of 1/10 dilution for working standards) for large batch quantities. Keep these frozen.

2. **PCR mix**- create a PCR mix (Figure 4) using the parameters set by the paper and MIC.

You will need to consult the literature to establish the parameters (volumes) for the qPCR mix for the relevant functional gene. *Ensure that units are in volume, not concentration! Papers will report in both and use it interchangeably.*

Primer preparation example:

We are investigating nitrification functional genes (*AOA and AOB)* and the paper specified concentrations of primers at 0.6 uM of AOB *amoA* primer and 0.5 uM of primer for AOA *Arch-amoA* primer. These concentrations need to be converted to volumes to work out how much primer should be added to the qPCR reagent mix.

Calculate the primer volume required when the total reagent volume is 20 ul and primer concentration is 10 uM.

C1V1=C2V2

C1	10 uM
V1	?
C2	0.6 uM
V2	20 ul

V1=C2V2/C1
=0.6 X 20/10
=1.2 ul AOB

V1=C2V2/C1
=0.5 X 20/10
=1 ul AOB

Once you have determined the volumes required, you can create the qPCR reagent mix (Table 4). It can be useful to make large batches of reagent mix if you are analysing many DNA samples. Table 4 shows an example of the volumes require for a single reagent mix or for 50 reagent mixes.

NB: the DNA extract is not added to the qPCR reagent mix in this step. All PCR prep will occur before any DNA contact i.e. clean room at the start of the day before any DNA contact.

Table 4: *an example of the volumes required to make individual or multiple (x50) reagents for AOB amoA and Arch-amoA primer sets.*

Product	AOB- ***amoA***		Arch-*amoA*	
	Single	**x 50**	**Single**	**x 50**
Base pairs	*491bp*	*491bp*	*635bp*	*635bp*
MG water	5.6 ul	280 ul	6 ul	300 ul
Primer FWD	1.2 ul	60 ul	1 ul	50 ul
Primer REV	1.2 ul	60 ul	1 ul	50 ul
Sensifast	10 ul	500 ul	10 ul	500 ul
DNA	2 ul	100 ul	2 ul	100 ul
Total	**20** ul	**1000** ul	**20** ul	**1000** ul

3. **PCR mix-** Aliquot 18 ul (20 ul total once you add 2 ul DNA) of qPCR mix into MIC tubes. An automatic dispenser is ideal as it will draw up 180 ul and dispense automatically to 10 tubes etc. These can be prepared in bulk before adding your extracted DNA.

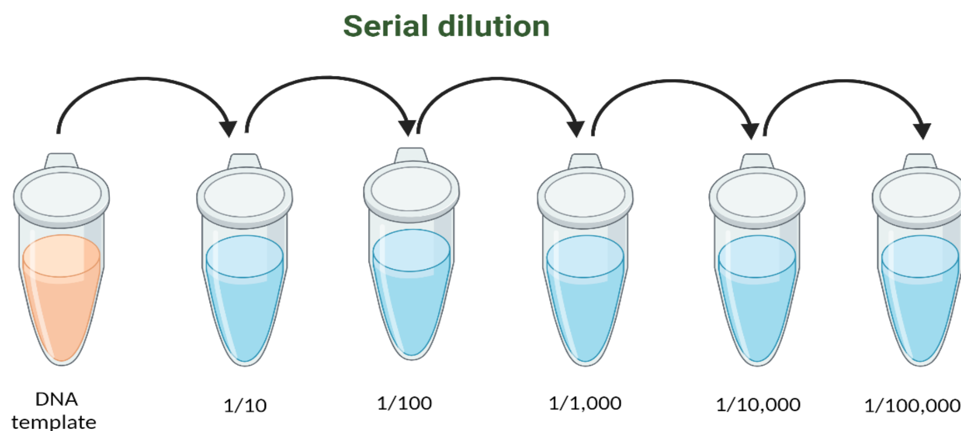
4. **DNA-** Move to a different 'clean' area and add 2 ul of your DNA extract to the MIC tube. Each DNA extract is replicated three times (3 tubes). Also run no-template controls (NTC) which contain only master mix and no DNA, as well as blanks.

4.5 Preparation of a standard curve

There are two ways you can quantify your gene copy numbers: absolute or relative quantification. Absolute quantification relates the PCR signal to a standard curve determined from known concentrations. Relative quantification will use the C_t value (threshold value) where treatment samples are compared against each other. You can also use the serial dilution standard curve method presented below which is semi-quantitative and relative to the starting template.

Pick the method based on your research question and hypothesis. Consider if relative quantification is sufficient to answer these. In most cases, relative quantification of functional genes is sufficient to address experimental questions.

1. 1/10 serial dilution of a DNA extract (Figure 6)
   i. Pick a DNA extract that had a good PCR run with a lower CT ~20
   ii. This sample will be diluted for calibration
2. Label 6 1.5 ml Eppendorf tubes
3. Add 18 ul of TE buffer (Tris-EDTA buffer) to numbers 2-6
4. The first tube will have chosen DNA template
5. In tube 2, add 2 ul of DNA to the 18 ul of TE buffer. Briefly shake by hand.
6. Draw up 2 ul from tube 2 and add to tube 3. Repeat this for each tube (shown in pic).
7. Add 2 ul of each serial dilution to PCR mastermix tube for AOA and AOB primer series.



4.6 Running the MIC

MIC- turn on power at back. Blue is idle and green in action. Use the worksheet to develop a program for amplification. The amplification protocol is sourced from papers (Table 3). Using an

Figure 6: *Example of semi-quantitative serial dilution using DNA template.*

example from Table 3, the settings would be as follows:

- Initial denaturation- 95°C for 5 minutes- product dependent on Sensifast etc
- Cycling- 40 cycles of 5 secs @ 95°C, 30 secs @ 60 °C and 45 secs @ 72 °C
- Melting- 72- 95 °C @ 0.3 °C/sec. This is a standard run.

Should take approx. 1.5 hr to complete cycle

Consumables List

- ☐ General equipment for DNA extraction
  - Microcentrifuge (1.5mL capacity) 12 or 24 space, this will determine how many samples you can do at once when used to the protocol.
  - Vortex
  - Pipettes (range of sizes 1-10uL/ 20uL, 200uL, 1000uL)
  - Sample homogenization: if available a bead beater or equivalent would be useful.
- ☐ Molecular Grade Water (eluting DNA, making up primers etc).
- ☐ DNA Extraction from Soil
- ☐ PCR Primers
- ☐ PCR Mastermix/ cocktail
- ☐ qPCR Thermocycler
- ☐ MIC PCR tubes

Results and interpretation

When using a standard curve, the MIC qPCR software (Figure 7) can automatically calculate the gene copy numbers. Gene copies per gram of dry soil can be determined when calculated against the gravimetric soil moisture content.

Things to check before proceeding:

- Positive control = application
- Negative control = no or low amplification (fluorescence kicks up late in cycling > 25-30 cycles)
- NTC= no or low amplification (fluorescence kicks up late in cycling > 25-30 cycles)
- Perform a melt analysis- gives a good indication of primer dimer issues

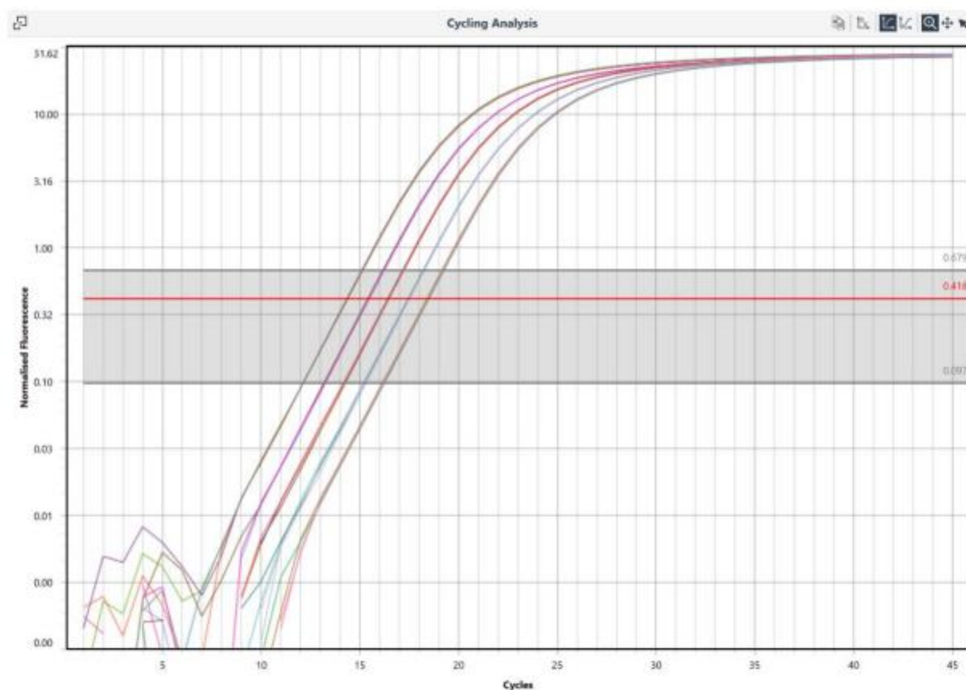
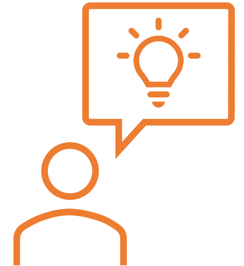


Figure 7: *qPCR curves of example samples with the fluorescence (y-axis) plotted against the number of cycles (x-axis). Sourced from Biosystems MIC user manual V2.12.*

Troubleshooting- what to do when something goes wrong

DNA extraction



- Did my extraction obtain DNA? Check nanodrop/ qubit or run DNA out on an agarose gel (not preferred).
- Is the quality of my DNA ok? Look at A_{260}/A_{280} for DNA. Good quality DNA will have a A_{260}/A_{280} ratio between 1.7-2.0. A low ratio may indicate contaminants and require the use of spin columns or re-precipitation.

PCR run

- Check if positive control amplified? If yes, the problem is likely to be the samples. If no, the problem could be the PCR mix or cycling conditions
- If you suspect the issue is your samples and you are sure you have DNA step 1, consider spiking a sample (adding DNA you know amplifies i.e. positive control to an aliquot of one of your samples) and test if you get amplification. Still no amplification, could indicate the presence of PCR inhibitors (common with soil and plant samples).

Further links

- https://www.thermofisher.com/au/en/home/life-science/cloning/cloning-learning-center/invitrogen-school-of-molecular-biology/pcr-education/pcr-reagents-enzymes.html
- https://www.thermofisher.com/au/en/home/life-science/cloning/cloning-learning-center/invitrogen-school-of-molecular-biology/pcr-education/pcr-reagents-enzymes/pcr-troubleshooting.html

Field trips and tours

Murrumbidgee Irrigation Area tour

The Murrumbidgee Irrigation Area (MIA) is located in the Riverina, NSW. Two main rivers service the MIA: the Murrumbidgee and Tumut rivers. The MIA region is home to SunRice, the Rice Research Institute and is Australia's predominant rice growing region. Other irrigation crops including cotton, macadamias, oranges, grapes and corn are grown in the MIA using irrigation licenses to access water. Broadacre agriculture crops including wheat, canola and barley are also grown throughout the winter and spring months using irrigation water to supplement rainfall. Average yearly rainfall is lower in the western region of the MIA averaging 450 mm annually, whilst eastern regions of the MIA can reach 700 mm rainfall annually.

We will be visiting the Yanco Agricultural Institute Research Station in Leeton. This is where research regarding irrigated crops, particularly rice and cotton, is conducted via the Department of Primary Industries. We will also visit the Southern Cotton Gin in Whitton NSW. This independently owned ginning facility services the MIA and aims to gin cotton of a high quality in an efficient manner.

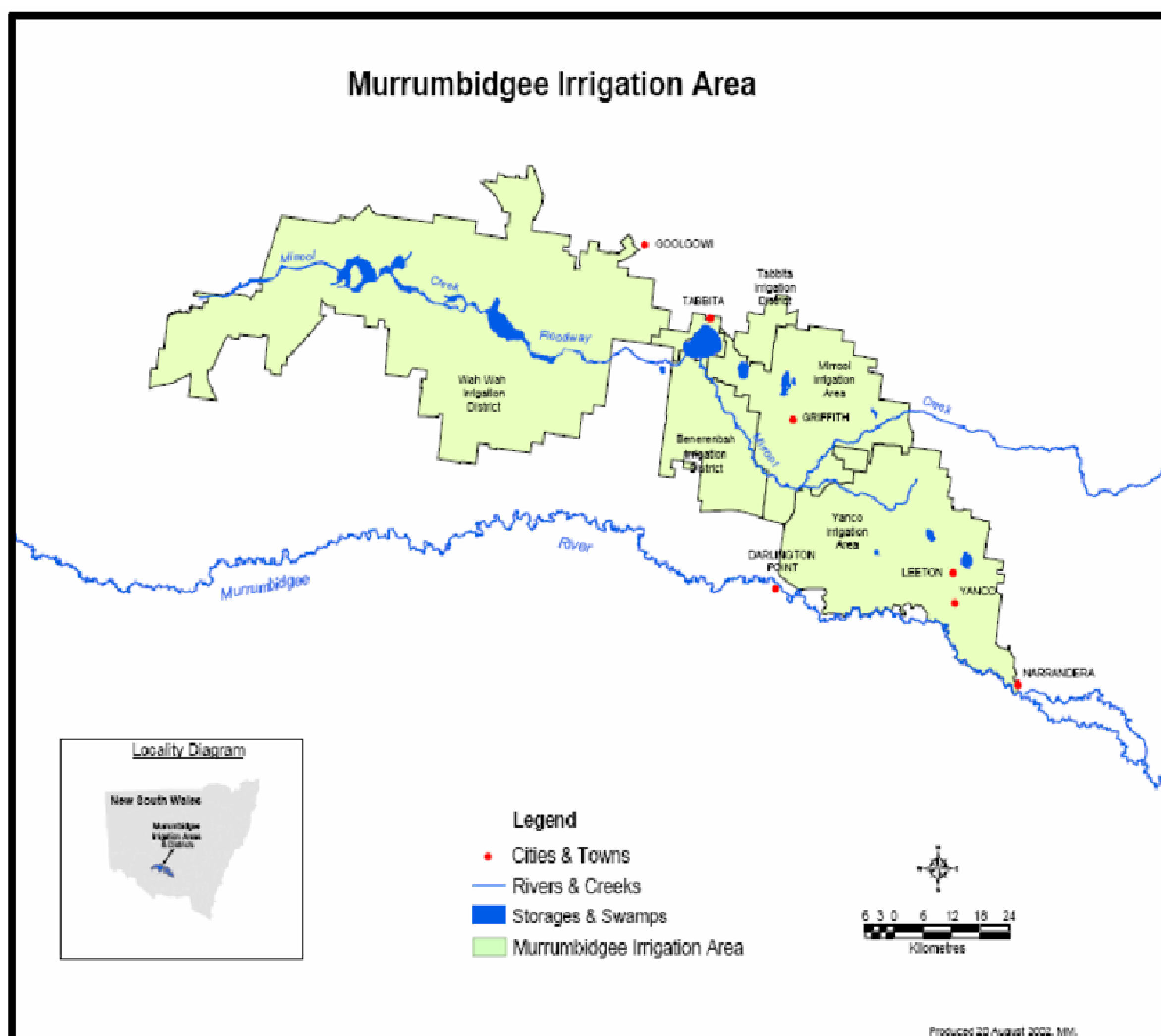


Figure 8: *The Murrumbidgee Irrigation Area*

Dryland broadacre agriculture tour

The dryland broadacre farm we will be visiting is located near Young, NSW and is owned by the Kaveney family. The business currently farms over 1500 ha across multiple farms where the primary enterprise is mixed broadacre crops grown during winter and spring including canola, wheat and barley. These crops rely on rainfall and are not supplemented by irrigation water. The cropped paddocks are rotated every 4-5 years with a perennial pasture of lucerne and sub-clover mix. The pasture is cut during spring and summer for animal hay (fodder). The use of a pasture phase enables soil organic matter to build up after declining during the cropping phase. The farm also runs a sheep enterprise where merino ewes (sheep grown for wool) are crossed over Poll Dorset rams (meat producing sheep). Their lambs are raised, fattened, and sold at market as prime lamb for human consumption on the domestic market. Lambs are sold when they are approximately 50 kg liveweight and the price received is often $/kg or $/lamb.

Harvest for broadacre crops occurs annually during November and December and grain is transported to local receival depots where it is stored before being sold. The price is determined by the buyer but the farmer can decide when and how grain much to sell.

The biggest limitation to growth in this cropping area the risk of frost (temperature drops below 0 ℃ during the night) at flowering. If a late frost occurs, sterilization of the flowers and significant yield reductions can occur. Farmers will delay the sowing of crops by 1-2 weeks during dry years in an attempt to mitigate frost risk but frost events are occurring later in the season. If a farmer delays sowing too late, there is the risk that soil moisture will get too low during critical reproductive phases throughout the spring and the crop will not produce grain. Variable rainfall during spring and soil constraints (acidic and dispersive soils) also limit production.

Figure 9: *The Kaveney family at the end of harvest time.*

Figure 11: *Prime lambs getting ready to be taken to the saleyards*

Figure 10: *Harvest time at the farm with the header harvesting wheat and emptying grain into the tractor and chaser-bin.*