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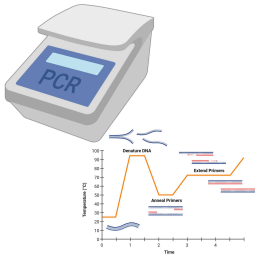
PCR reaction of marker regions (a.k.a metabarcodes)



Forked from PCR reaction of marker regions (a.k.a metabarcodes) for two kingdoms

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We use this protocol and it's working

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Abstract

This protocols is part of the ANU Biosecurity mini-research project #1 "Plant Pathogen Diagnostics: Visuals, subcultures, and genomics".

You will be provided four pots of 3-4 week old wheat plants that have been infected with different wheat pathogens. Each pot has been infected with one major pathogen. You will not know which pot has been infected with which pathogen. However, you will be provided a compendium of 10-15 wheat pathogens that will guide you to identify the infective agent for each treatment group. The fifth treatment group will be uninfected wheat plants which will be clearly identified. You can use treatment group #5 as negative control for your experiments.

In total, each group will obtain five pots each:

	A	B
	Treatment group 1	Unknown infective agent
	Treatment group 2	Unknown infective agent
	Treatment group 3	Unknown infective agent
	Treatment group 4	Unknown infective agent
	Treatment group 5	Uninfected control

This specific protocol is a step by step guide to perform PCR reactions on the extracted DNA samples of the five TG. The PCR reaction target conserved marker regions (a.k.a. metabarcodes) for fungi because our pathogens are by now known to be fungi. We use the ITS primers in this case.

The simplified idea of metabarcodes is that one can amplify a region that is conserved enough to generate primers that allow one to amplify this region from "all" members of the kindgom somewhat representatively. Within the amplicon though there are located hypervariable regions that are distinct between different species or genera. Matching these amplicons against a databases let's one identify the organism of this specific kingdom present in the sample.

At least this is the simple theory, however as you will see in the lectures there are many issues and biases to consider when applying these techniques in general and specifically to pathogen diagnostics for biosecurity purposes.

The final goal is to achieve the following:

- To measure and adjust DNA concentration for each sample to allow for equal amount of DNA being added to each PCR reaction.
- Perform PCR reaction for the fungal kingdom metabarcode using published ITS (Internal transcribed spacer) primers.
- Cross the fingers it all works well.

This protocol is applicable for week 4.

Protocols progress overview:

- Week 4 PCR reaction for 16S and ITS.

You will measure double stranded DNA concentration with a dye based method. Here the dye binds to double stranded DNA only and this will change the fluorescent of the dye in a way directly proportional to the DNA concentration. You will use the pre-mixed broad range dye kit https://www.thermofisher.com/order/catalog/product/Q32853.

The primers for the ITS PCR reactions are called ITSFor and ITSRev. These are based on White et. al., 1989 and Ohta, Nishi, Hirota and Matsuo, 2023. Their sequences are as follows.

ITSFor (a.k.a. ITS1-F_KYO2): 5'-TAGAGGAASTAAAAGTCGTAA-3'
ITSRev (a.k.a.LR6): 5'-CGCCAGTTCTGCTTACC-3'

The expected amplicon size is around 2 to 3 kb (kilobase pairs).

References:

- White, Bruns, Lee and Taylor, 1989, 'Amplification and direct sequencing of fungal ribosomal RNA Genes for phylogenetics', in PCR - Protocols and Applications - A Laboratory Manual, Academic Press

- Wuyts, Van de Peer, Winkelmans and De Wachter, 2002, 'The European database on small subunit ribosomal RNA', Nuc. Ac. Res., vol. 30 (1), pp. 183-185

- Ohta, Nishi, Hirota and Matsuo, 2023, 'DNA metabarcoding workflow utilising nanopore long-read sequencing and consensus generation for rapid identification of fungal taxa with high phylogenetic resolution, BioRxiv, doi: https://doi.org/10.1101/2023.04.14.536971

Attachments

PDF

MyTaqHSRedDNAPolymer...

252KB

Image Attribution

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Guidelines

You must have read, understood, and follow the health and safety instructions provided in the "Overview Mini-Research Project #1 BIOL3106/6106" provided on Wattle (ANU learning portal).

You must have signed and returned one copy of the "Student Safety Declaration Form For Practical Class Work" before starting any laboratory work.

You must have read and understood the Hazard Sheets (Risk assessment) of all chemicals listed bellow in the "Safety Warnings" section. These Hazard Sheets are provided on Wattle as part of the "Overview Mini-Research Project #1 BIOL3106/6106" document.

Materials

As always you need to bring a lab notebook, a printed version of this protocol, and a pen to record your adventures in the lab.

Consumables and culture material needed:

- 1.5 ml tubes. At least seven in total.
- strip tubes of 8.
- Primers @ 10uM. ITSFor, ITSRev; 20ul each.
- Nuclease Free water (ddH_2O)
- MyTaq HS Red DNA Polymerase Master Mix including MyTaq HS Red DNA Pol; 38.5ul total.
- Five tubes with 198ul broad range qubit.
- Ice bucket with ice.

Equipment needed:

- Benchtop centrifuge for 1.5ml/2ml tubes.
- Mini benchtop spinner for strip tubes of 8.
- Vortex.
- PCR machines. Up to two PCR machines with capacity for 96 PCR reactions each.

Troubleshooting

Safety warnings

This protocol does not require any hazardous substances.

You need to wear safety equipment at all times including lab coats, gloves, and safety goggles when handling chemicals and biological agents. While the major biological agents used for the wheat infection are pathogens commonly found in Australia, you must treat them as they were infective agents of general concern. Treat them with care. Do not remove them from the laboratory. Do not spread them via clothing. Use a dedicated notebook and pen to make notes during the mini-research project. Do not put anything into your mouth while in the laboratory. Wash your hands each time you leave the laboratory.

Before start

You must study the protocol carefully before you start. If anything is unclear post questions directly here on protocols.io.

Additional protocol specific notes:

- We recommend assembling all reaction components on ice and quickly transferring the reactions to a thermocycler preheated to the denaturation temperature (95°C). All components should be mixed prior to use.

Week 4: DNA quantification and dilution

37m

1 The first section describes the quantification of extracted DNA from week 3. Once you know the concentration for each TG you can generate a DNA stock solution at a final concentration of 10 ng/ul. This stock solution for each TG will be used for the following PCR reaction and DNA quality control measures in week 5.

2 You will receive your DNA extracts from the previous practical session in week 3. You should receive five tubes in total containing the DNA for each TG. 2m

3 Measure DNA concentration with Qubit broad range (BR).

3.1 You will receive five tubes containing 198uL BR Qubit reaction ready to use. Label the tubes with treatment group name. 2m

3.2 For each TG, add 2uL of DNA extracted to the corresponding BR Qubit tube. Make sure to add the correct DNA sample to the correctly labelled BR tube. 5m

3.3 Vortex your BR Qubit tube containing the DNA. 2m

3.4 Collect the liquid at the bottom of the tube with a quick "hand-centrifuge".

3.5 Incubate for 1-5 min. 5m

3.6 Wipe down the outside of your tube with a Kim wipe.

3.7 Measure your concentration using the Qubit machine. Demonstrators will facilitate the measurement. 5m

3.8 Note down the concentration of each TG DNA sample in ng/ul.

4 Label five 1.5 ml tubes with your research group name, treatment group name, date, and the DNA concentration 10 ng/ul. 5m

5 Now for each TG, dilute the DNA sample to a concentration of 10 ng/ul with nuclease free water. Make up a DNA total stock solution of 100ul.

The important formula here is:

n = c*V with n being the amount in ng, c the concentration in ng/ul, and V the volume in ul.

You want V = 100ul and c =10 ng/ul. This means you will have 1000ng in 100 ul.

The question is how much volume of the original DNA do you need to obtain 1000 ngs?

Here now n = 1000ng and c equals your measured concentration. Let's assume your measured c = 56 ng/ul for an imaginary sample. Than we can resolve the equation as follows.

n = c*V → V = n/c → 1000ng/56ng/ul = 17.85 ul

So you need to use 17.85 ul in 100 ul total volume to obtain 1000 ng.

This means you need to pipette 17.85 ul of extracted DNA (c = 56 ng/ul) and mix it with 82.15 ul nuclease free water to obtain your final 100 ul @ 10 ng/ul DNA stock solution.

5.1 For each TG, calculate the required volume of extracted DNA and nuclease free water to obtain your final 100ul @ 10 ng/ul DNA stock solution. 5m

5.2 Check with a demonstrator that your calculations are correct if you are unsure. 1m

5.3 For each TG, mix the the required volume of extracted DNA and nuclease free water to obtain your final 100 ul @ 10 ng/ul DNA stock solution. Mix the sample by flicking the tube with your finger several times, inverting it and spinning it down briefly to collect all liquid at the bottom of the tube. 5m

6 You now should have five DNA stock solution tubes (each 100 ul @ 10 ng/ul), one for each TG. These are the DNA solutions you will need for the PCR reactions below and the DNA quality control measures in week 5.

Week 4: ITS PCR reaction 33m

7 Now you will amplify the ITS regions in the DNA samples you extracted from wheat leaves.
You will use the DNA stock solutions and 50 ng per reaction.

You will use a regular polymerase to save some money.
Demonstrators will repeat PCR reaction with proof-reading polymerase to reduce errors on the selected three groups' DNA samples based on class discussion and selection in week 5.
You will use the following primer set for amplification.

ITSFor (a.k.a. ITS1-F_KYO2): 5'-TAGAGGAASTAAAAGTCGTAA-3'
ITSRev (a.k.a.LR6): 5'-CGCCAGTTCTGCTTACC-3'

7.1 Label strip tubes of 8 with your research group name, 1 to 6, and ITS. 5m
Note down the order 1 to 6 in your lab notebook and what they correspond to. E.g. 1 = TG1,
6= negControl.
You will use these strip tubes for the PCR now.

7.2 Make a master mix for the ITS PCR reaction for 7 samples. TG1 to TG5 and negative control. 10m
Keep master mix on ice at all times.

Mix the following amounts in the specified order to make a master mix for 7 reactions.

Cross off each reagents you added with a pen to make sure you added all reagents properly.

Once you added all the reagents (1-4), close the tube, vortex it, and spin it down to collect the liquid in the bottom of the tube.

	Reagents	uL per single reaction	uL for 7 reactions
	Water (ddH2O)	10.5	73.5
	ITSRev @ 10 uM	2	14
	ITSFor @ 10 uM	2	14
	5x MyTaq Red Reaction Buffer including MyTaq HS	5.5	38.5

	Reagents	uL per single reaction	uL for 7 reactions
	Red DNA Polymerase		
	Template DNA stock solution @ 10 ng/ul	5	NA DNA for each sample will be added once you aliquoted out the mastermix into individual tubes of the strip tubes.
	Total	25	140

PCR master mix table

7.3 Aliquot 20 ul of the master mix into each labelled tube of the strip tube of 8. Add the master mix only to the tubes labelled previously. Close all lids. 5m
Keep the PCR strip on ice.

7.4 For each TG, add 5 ul of the DNA stock solution (10 ng/ul) to the correct PCR tube. Mix by pipetting up and down. Close lid once you added the correct DNA stock solution. 8m
In the case of the negative control, add 5 ul of nuclease free water instead.
Keep reactions on ice.

7.5 Close the lids and hand over your strip tubes to the allocated demonstrator. 5m

8 The demonstrator will run the following PCR reaction with specific conditions for the ITS primers and amplicon length.

STEP 1: 95^{o}C for 30 sec; Initial denaturation
STEP 2: 95^{o}C for 20 sec; Denaturation
STEP 3: 58^{o}C for 20 sec; Primer annealing
STEP 4: 72^{o}C for 90 sec; Extension, 20-30 seconds/kb fragment size
GO TO STEP 2 for 34 additional cycles; Amplification
STEP 5: 72^{o}C for 2 min; Final extension
STEP 6: 20^{o}C for 1 hour Hold

9 The demonstrators will take out the PCR reactions for you and store them for week 5 in the fridge.