PCR Workshop – Bekasi, West Java, Indonesia.

Post activity report
31st May – 3rd June 2011

Lynne Jones

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Aim

Deliver PCR workshop, on behalf of OCPPO, to Indonesian Quarantine Scientists at Institute of Research Dissemination of Agricultural Quarantine Technology, Bekasi, West Java, 31st May – 3rd June 2011.

Presenters

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Background

After several emails between Ummu Rustiani and myself the tentative agenda for the workshop was that we would cover DNA and RNA (kit) extractions from plant material, nested PCR for phytoplasmas from DNA extracts and reverse-transcriptase PCR for Potyvirus from RNA extracts. Ummu expressed particular interest in presenting a PCR assay for lethal yellows (phytoplasma) of palms and RT-PCR for *Turnip Mosaic Virus* (potyvirus). I was not familiar with the specifics of these two assays and unfortunately did not receive the requested references in time to in include them in the workshop notes (Appendix 1), which instead contained generic protocol for phytoplasmas and potyvirus. I received an electronic copy of a journal article on protocol for lethal yellow by Harrison et al (2002) three days prior to leaving Australia for Indonesia and took the opportunity to read it during my transit to Indonesia.

On the Friday (27/5/2011) after arriving at the facility at Bekasi, I met primarily with Ummu, but also Dr Antario Dikon, the head of the facility, to establish how the workshop would run. We agreed that we would run the following phytoplasma PCR assays: first round PCR using generic phytoplasma primers P1 and P7 and two second round assays; one with generic primers R16f2n and m23sr, and the other with 16s Lethal Yellow primers (Harrison et al 2002). From what I could gather the latter 2nd round primers are routinely used by Indonesian Quarantine to screen for lethal yellowing in the embryos of imported oil palm nuts and from the spear leaf of oil palm seedlings germinated post entry. We also agreed that we would run the following potyvirus RT-PCR assays: two one-step RT-PCRs using generic potyvirus primers MJ1 and MJ2 and the other using specific Turnip Mosaic Virus primers (Kartiningtyas and Hidayat 2006). Both sets of assays were accompanied by 'house-keeping' PCR assays using primers supplied by Ummu (I did not receive the references). Kits and reagents used did not differ from the Draft Workshop Proposal put together by Ummu (Appendix 2). Important to note also was that the PCR protocol in the workshop booklet was based on the use of Qiagen reagents, which were available if required, and not those used during the workshop (GE Healthcare 'Ready-to-go beads').

Concerns prior to the workshop were that we would not have sufficient time to get through the proposed work load, and that there were no lethal yellow positive controls for the proposed phytoplasma assay of palms. Similar to Australian Quarantine, Indonesian Quarantine also find it very difficult to import verified positive controls for assays used to screen risk material. I took with me DNA extracts from unrelated plant hosts that had tested positive for endemic (non-lethal yellow) phytoplasmas here in Australia. The other concern was that the lethal yellow primers by Harrison et al (2002) had not been designed to amplify all phytoplasmas causing yellowing of palms, thus the inclusion of the universal second round assay in the workshop. To test the efficacy of the generic potyvirus assay, I also took RNA extracts from non-brassica hosts that I had found to amplify and sequence with the universal potyvirus primers, because although Grisoni et al (2006) with MJ1/MJ2 primers had successfully amplified *Turnip mosaic virus*, I had not had opportunity to validate their findings.

There were 14 participants (Appendix 3), with varying levels of PCR experience. Most were quarantine staff located at offices throughout Indonesia. Participants were separated into 5 groups to allow for a more

'hands on' experience. The criteria for the grouping, was to ensure that expertise in PCR techniques and English language skills were evenly distributed across the groups.

Workshop Delivery and Outcomes

Regardless of individual levels of experience, all participants proved to very competent technicians and performed all tasks with great enthusiasm and attention to detail. The combination of experience levels in groups worked well. We completed the workshop schedule within the allocated time.

Language barrier was flagged as notable hindrance to the smooth delivery of the workshop. But even without these communication difficulties, in my opinion the pace of the program would still have been too rushed and as it was did not allow enough opportunity for greater discussion among tutors and participants, particularly results interpretation. This was further confounded by some equipment malfunctions; most of the equipment, which is new, and supporting software had not been operated prior to this workshop. For example, the gel imaging system was very unstable and we were unable to obtain images from most of the nested PCR assays or any from the RT-PCR assays. Instead we had to rely on looking at the electrophoresis gels under UV illumination directly. Under different circumstances the fact that the PCR machines hadn't been run before the workshop with the selected assays would have provided a great opportunity to trouble shoot. There was what appeared to me to be non-specific annealing in some of the second round nested PCR reactions, perhaps due to the assay needing to be optimised for the reagents and/or PCR machine. This provided a great talking point, one that could have been extended substantially with more time. The interpretation of the RT-PCR results, or lack of, was the real victim unfortunately. I had about 15 minutes before I had to prepare to leave for the airport and there was much to talk about with this assay set that just couldn't be covered.

There were important outcomes from the workshop in terms of diagnostic standards used for the screening of palm tissues for Phytoplasmas in Indonesia. I believe participants gained a clearer understanding of the reasons behind using nested PCR for Phytoplasma detection and its application, and the need to source verified positive controls. Prior to the workshop the assumption among participants more familiar with the assay was that in the absence of a positive control, if the 'house-keeping' region was amplified, and the 1st round target band was not amplified, this was sufficient evidence to infer a negative result. The second round PCR was only to be used if first round P1/P7 product was amplified; i.e. it would be used to differentiate 'lethal yellow' of palms from other strains of phytoplasmas. This is actually in contrast to the reason behind the development of a nested PCR for phytoplasmas. Nested PCR can also be used as a method to amplify low copy targets: phytoplasmas are considered susceptible to being present in low titre across their host tissues, hence the development of nested PCR for the detection of these organisms. The importance of including a size ladder and positive control with assays was well illustrated by the presence of multiple bands/non-specific annealing in some of the universal 2nd round reactions; it is possible that inexperienced operators could mistake these bands for 'false' positives. And also by the presence of a band from one of the non-lethal yellow Phytoplasma positive controls in a couple of the 2nd round 'lethal yellow' specific PCRs; given the length the band (~1800bp) and the amount of template added from the 1st round PCR it was most likely product detection from the 1st round. Participants appeared to understand the significance of these points during a trouble shooting session for phytoplasma nested PCR assays.

Recommendations

After discussion with Ummu on how well we thought the aims of the workshop had been met we both came to the conclusion, that given each others' technical competencies, my limited Indonesian and Ummu's good English language skills that any future workshops conducted together might follow a 'train the trainer' model. For example, this might mean my travelling to Indonesia prior to the workshop to run through a diagnostic with Ummu. Ummu would then present the diagnostic at the workshop and I would perhaps stay on and act in a support or advisory role. It would also provide time to ensure that equipment and reagents are working. This of course would be a model applicable to any 'guest trainer' with little or no Indonesian language.

Potential Future Workshop Ideas

Ummu expressed much interest in running a Huanglongbing disease of Citrus PCR workshop in the future. It would obviously cover the extraction and screening of DNA from leaf tissue, but could also cover extraction and screening of DNA from the insect vector of the bacterium, *Diaphorina citri*. This would then have the added value of providing participants with exposure to insect DNA extraction techniques.

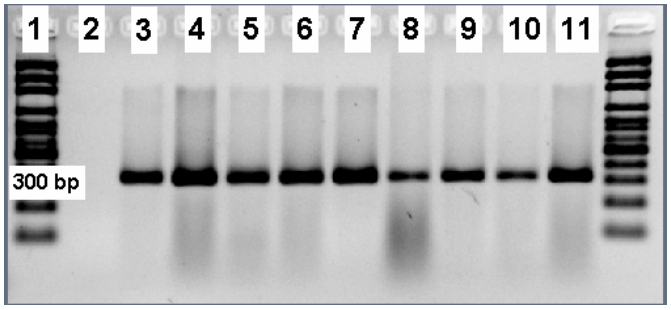
References

Harrison, N.A, Womack, M., Carpio M.L, 2002. Detection and Characterization of a Lethal Yellowing (16SrIV) Group Phytoplasma in Canary Island Date Palms affected by Lethal Decline in Texas. *Plant Disease* **86**, 676-681.

Kartiningtyas, Scri Hendrastuti Hidayat, 2006. Deteksi *Turnip Mosaic Virus* dalm jaringan benih dan daun. *Journal of HPT tropika* **6**, 32-40.

Appendix 1: Workshop Notes

Bekasi PCR Workshop



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Short Glossary of Terms

Aerosol – fine particle or liquid droplet (or both) suspended in air

Amplify – to increase

Buffer (solution) – maintains the pH of a solution when small amounts of acid or base are added

Degenerate primers – are just a mix of primers with similar sequences. That is, if you do not know exactly the sequence of the gene you are going to amplify, you insert "wobbles" in the PCR primers where there is more than one possibility. Example of a degenerate PCR primer:
`5'-TGG GAY ACN GCN GGN CAR GA-3'` (This gives a mix of 256 different oligonucleotides) where the Y = C or T, R = G or A, N = G, A, T or C. The more wobbles you introduce in the PCR primer the more degenerate it gets. (The degeneracy of the primer is produced during DNA synthesis, you do not need to order 256 different primers to get a 256 mix). Degenerate PCR is useful for identify new members of a gene family or orthologous genes from different organisms.

Deoxyribonucleic acid (DNA) - is a nucleic acid that contains the genetic instructions used in the development and functioning of all known living organisms (with the exception of RNA viruses). DNA consists of two long polymers of simple units called nucleotides, with backbones made of sugars and phosphate groups joined by ester bonds. These two strands run in opposite directions to each other and are therefore anti-parallel. Attached to each sugar is one of four types of molecules called bases. It is the sequence of these four bases along the backbone that encodes information. This information is read using the genetic code, which specifies the sequence of the amino acids within proteins. The code is read by copying stretches of DNA into the related nucleic acid RNA, in a process called transcription.

DNase – an enzyme capable of slicing through, and therefore destroying DNA bonds

dNTPs (deoxynucleoside triphosphates) – the building blocks from which the DNA polymerase synthesizes a new DNA strand, i.e. adenine (A), guanine (G), cytosine (C) and thymine (T)

Multiplex PCR - a variant of PCR that enables simultaneous amplification of many targets of interest in one reaction by using more than one pair of primers

Nested PCR – involves two sets of primers, used in two successive runs of polymerase chain reaction, the second set intended to amplify a secondary target within the first run product.

Nuclease – general term for DNase and RNase. It does not make a distinction between the two.

Nucleic acid – general term used to refer to deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). It does not make a distinction between the two.

PCR (Polymerase Chain Reaction) - is a scientific technique in molecular biology used to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence.

Polymerase - any of various enzymes, such as DNA polymerase, RNA polymerase, or reverse transcriptase, that catalyze the formation of strands of DNA or RNA using an existing strand of DNA or RNA as a template

Primer – a synthetically manufactured strand of nucleic acid that serves as a starting point for DNA replication during PCR

Reverse Transcriptase PCR (RT-PCR) – a variant of PCR where RNA strand is first reverse transcribed into its DNA complement (*complementary DNA*, or *cDNA*) using the enzyme reverse transcriptase, and the resulting cDNA is amplified using traditional PCR or real-time PCR

Ribonucleic acid (RNA) - Like DNA, RNA is made up of a long chain of components called nucleotides. Each nucleotide consists of a nucleobase (sometimes called a nitrogenous base), a ribose sugar, and a phosphate group. The sequence of nucleotides allows RNA to encode genetic information. For example, some viruses use RNA instead of DNA as their genetic material, and all organisms use messenger RNA (mRNA) to carry the genetic information that directs the synthesis of proteins.

RNase – an enzyme capable of slicing through and therefore destroying RNA bonds

RPM (revolutions per minute) – a measure of rotational speed for centrifuges

Template – a pattern used as a guide to making something. In the case of PCR, DNA or RNA extracted from a sample and used in a PCR reaction

Introduction

The polymerase chain reaction (PCR) is a technique widely used in molecular biology. It derives its name from one of its key components, a DNA polymerase used to amplify a piece of DNA by *in vitro* enzymatic replication. As PCR progresses, the DNA generated is used as a template for replication. This sets in motion a chain reaction in which the DNA template is exponentially amplified. With PCR it is possible to amplify a single or few copies of a piece of DNA across several orders of magnitude, generating millions or more copies of the DNA piece. PCR is used to amplify specific regions of a DNA strand (the DNA target). The original technology has since been extended upon to incorporate a range of PCR derivatives. Accessed 17:05 hrs, 30/3/2009 via internet at http://en.wikipedia.org/wiki/PCR#PCR_principles_and_procedure.

PCR provides a powerful diagnostic tool in plant pathogen detection, particularly when traditional methods of detection (e.g. cultural methods) are not possible. This manual describes PCR protocol that can be used to detect the presence of Phytoplasmas and Potyviruses.

Working Safely in a Laboratory: Operational Health and Safety (OHS)

Procedures should be developed to ensure that the health and safety of all laboratory users are maintained at all times. These procedures should be discussed with and approved by the Laboratory Manager. New users should be instructed on OHS procedures before they commence work in the laboratory. An up to date first aid kit, along with hazardous spill kits, should be readily accessible.

Some general laboratory safety rules

- Covered shoes should be worn and long hair tied back.
- Laboratory gowns should be worn in the laboratory.
- Gloves should be worn. Hands should be washed thoroughly with water and disinfectant after the removal of the gloves.
- No food or drink should be brought into the laboratory or consumed under any circumstances.
- No smoking in laboratories.
- Paperwork and books should be kept to a minimum in the laboratory.
- A file that includes material safety data sheets (MSDS) for all chemicals and reagents used in the laboratory should be kept in the laboratory. Material safety data sheets provide information on how to store, handle and dispose of chemicals and reagents used in the laboratory. They also provide information on what first aid measures should be taken should a staff member be exposed to a chemical or reagent used in the laboratory, and what measures should be taken if there is a chemical spill.
- Chemicals and other reagents should be stored, handled and disposed of in accordance with the information contained in their MSDS.
- Gloves, safety glasses and any other appropriate safety equipment should be worn when using hazardous chemicals as outlined in to their MSDS.
- Equipment should be used in accordance with the manufacturer's safe handling recommendations.
- Prior to exiting the laboratory all materials should be sanitised and put away. Waste should be sealed and work areas sanitised.
- Hands should be washed thoroughly with water and disinfectant before exiting the laboratory.
- All accidents and hazardous spills should be reported to the Laboratory Manager.

Specific OHS considerations

If the laboratory has equipment that emits ultraviolet (UV) light then a protocol for the operation of that equipment should be developed. Development of protocol should take into consideration the level, length and frequency of exposure, and importantly, the consequences associated with that exposure or the operator. Protocol should aim to identify and implement measures that reduce the risk of both short and long term exposure to the operator to a reasonable level. The level to aim for is zero (no risk).

If the laboratory employs the use of toxic chemicals, such as ethidium bromide, chloroform or phenol, the same considerations described above should be taken into account. The MSDS for any chemical or reagent used within a laboratory is an **extremely** important resource. The level of exposure to aim for is zero (no risk).

PCR Laboratory Set Up and Procedures: Contamination Prevention

PCR contamination remains an issue in laboratories. A primary source of contamination is the production of aerosols containing nucleic acids during some procedures. Another is the cross-contamination of equipment and reagents. PCR for diagnostic purposes requires that procedural limitations to avoid contamination be observed so that reactions yield valid results. Outlined below are the essential components of contamination control in a PCR laboratory.

Spatial separation of activities

Activities within a PCR laboratory typically include (1) sample preparation, (2) PCR reagent preparation, (3) PCR execution and (4) analysis. The most effective method in the control of contamination is to have separate rooms for each activity. But when this is not possible, different areas within the lab can be designated for each activity.

Key considerations when arranging your laboratory are:

- Aerosols containing nucleic acids can be produced during sample preparation.
- Aerosols containing nucleic acids can be produced during the operation of Gel equipment.
- PCR reagent preparation should be undertaken in a 'clean' part of the laboratory.
- In theory, the 'cleanest' part of the room will be where the air is coming into the laboratory.

A typical laboratory arrangement in which all activities are housed may look something like *Figure 1* below.

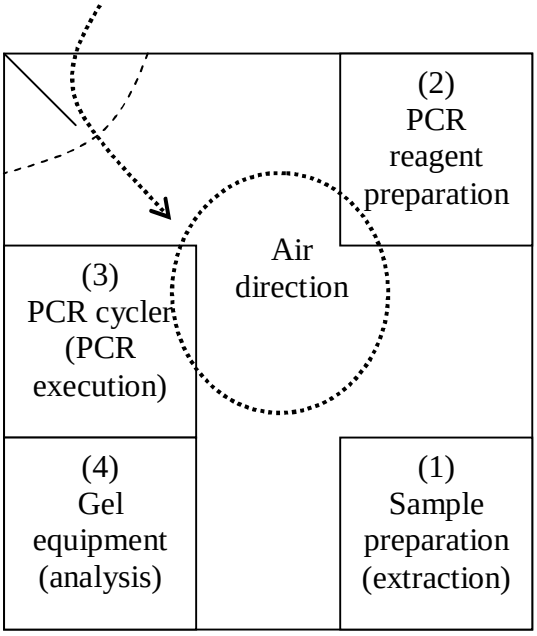

Figure 1. Spatial arrangement of typical activities undertaken in a PCR laboratory

Temporal separation of activities

A laboratory should arrange a schedule for the activities to be performed; one that avoids running different activities at the same time. Once an activity has been completed, place reagents and consumables away, and clean your work area before commencing the next activity.

A typical schedule would progress in the following order (*Table 1*).

Table 1. Timing of activities undertaken in a PCR laboratory

Time period	Activity
Day 1 Morning	(1) Sample preparation (extraction)
Afternoon	(2) PCR reagent preparation (3) PCR execution
Day 2 Morning	(4) Analysis

Dedicated pipettes', pipette tips and trays

Each activity should have its own set of set of pipettes, pipette tips and trays. Pipettes used for reagent preparation should never be used for pipetting nucleic acids or *vice versa*. When not in use, pipettes, pipette tips and trays used for sample preparation should be stored in sealed containers or plastic bags.

Physical aids – cabinets

The use of a designated cabinet with an ultraviolet (UV) light installed is **highly** recommended for PCR reagent preparation. As there is a risk of creating aerosols during sample preparation (extraction) you may also wish to perform this activity inside a designated hood or biosafety cabinet fitted with a UV light. These types of cabinets isolate the components within them from the surrounding environment and on completion of the activity, allow for that internal space to be sterilised through the application of UV light.

Physical aids – 'barrier' pipette tips

Use sterile, Nuclease (DNase and RNase) free 'barrier' (or filter) pipette tips for PCR activities. Unlike standard pipette tips (*Figure 2a*), barrier tips include a filter plug inside the hollow of the tip (*Figure 2b*). This plug prevents aerosols or liquids from coming in contact with the barrel of the pipette and contaminating subsequent samples or reactions. Occasionally though the plugs fail, and this is why it is important to have designated pipettes for each activity. The use of barrier tips is particularly important when pipetting nucleic acids or PCR products, and during the extraction of nucleic acids or proteins.

Figure 2a. Standard pipette tips

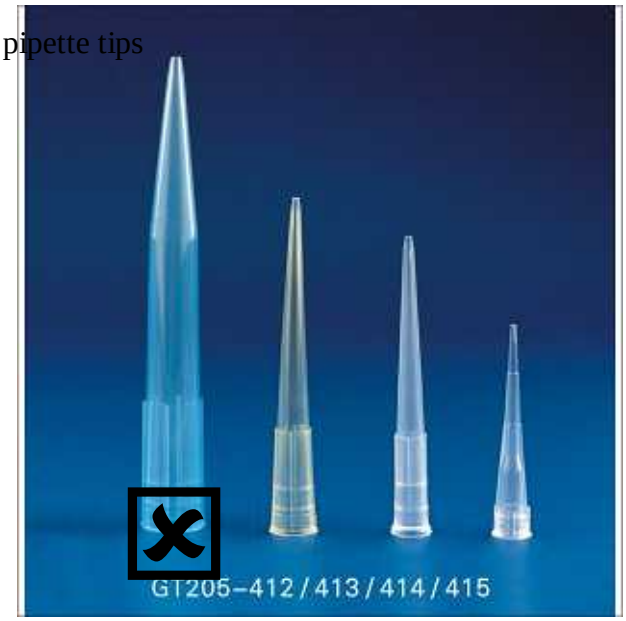

Figure 2b. Barrier (or filter) pipette tips.

Physical aids – gloves

Always wear gloves when performing each of the different activities. Always change gloves between activities. The wearing of gloves can do three things: (1) protect you against harmful chemicals, (2) protect the sample or reagent from contamination by you, and (3) with glove changes prevent cross contamination between activities.

The type of glove used is also important. The best all round gloves to use are chemical resistant, non-powered gloves. Chemical resistant gloves, for example nitrile gloves, provide the best protection against harmful chemicals. The powder in 'powdered' gloves has been known to inhibit PCR reactions. For this reason it is best practice to use the powder-free glove type in PCR laboratories.

PCR reagent and template storage

It is best practice to store PCR reagents and biological material (plant material and nucleic acids) in separate areas. Ideally this will mean storage in separate units (e.g. fridges or freezers) designated for either reagents or biological material. When this is not possible store your reagents in sealed containers at the top of the storage unit, and biological material in sealed containers at the bottom of the storage unit.

Always store PCR reagents at the temperatures recommended by the manufacturer. In most cases this will mean storage in a freezer or fridge.

PCR reagents

Pipette PCR reagents except for enzymes (polymerases) into smaller volumes before use. This reduces the potential of reagent degradation through repeated freezing and thawing. Also, and importantly, if one set of reagents becomes contaminated you will only need to discard a small amount of a reagent rather than the entire stock.

PCR reaction controls

In most cases when running PCR reactions there will be a number of samples to be assayed. It is better to make up a reagent master mix in which each of the reagent volumes per reaction are multiplied by the number of samples and added to the same tube. The reagent master mix is then mixed and pipetted at the calculated volume into each of the PCR reaction tubes.

The main purpose of the master mix is to minimise variation between the individual reaction tubes within an assay, since differences in the concentration of any can drastically affect results. Using master mixes also increases the volumes that are pipetted at any one time giving the added advantage of improved accuracy.

Always include (1) positive, (2) negative and (3) water controls with each PCR assay.

- (1) Positive controls demonstrate if the reaction has worked properly and indicate the size of the PCR product if the specific pathogen is present.
- (2) Negative or 'healthy' controls from uninfected plant material indicate if products generated in the reaction arise from plant-derived nucleic acids rather than the specific pathogen.
- (3) Water controls, where the reaction contains water instead of nucleic acid, can demonstrate if the reagent mix is free of nucleic acid contaminants that could give rise to false positives.

Additional components

- Wipe down non-electrical equipment (eg. pipettes) with 70-80% ethanol after use.
- Wipe down work areas with 70-80% or 1% sodium hypochlorite after completion of an activity.
- Use separate, sterile utensils for each extraction.
- Always use sterile DNase and RNase free tubes.
- Always use sterile DNase and RNase water for PCR reactions
- Don't resuse pipette tips or tubes, even if they have been cleaned and autoclaved.

References

Mifflin TE 2003. Setting up a PCR laboratory. In PCR Primer: A Laboratory Manual (2nd Ed). (ed. CW Dieffenbach and GS Dveksler), pp 5-14. CSHL Press, New York.

Sample Collection Protocol

<u>Important</u>: Label sample containers Clearly with a Unique Identifier

Materials and equipment

1. Safety blades, scalpel handle and blades, or sharp knife
2. 25 mL screw top tubes
3. Calcium chloride ($CaCl_2$) in lumps/Silicon gel
4. Facial tissues
5. Insulation tape/parafilm/cling wrap to seal tubes

Method

1. Collect up to 1-2 g fresh weight of plant material.
2. **Important**: keep plant material cool/refrigerated until ready to proceed with the next step. If using ice for field collections, do not store plant material directly against the ice.
3. Using a sharp blade, select and section up plant material into approximately 2-4 mm pieces.
4. Wrap in paper (facial) tissue and place over calcium chloride in 25ml plastic vials and place immediately in the refrigerator. Calcium chloride will dry the leaf material so it can be sent for testing. The vial should be ½ to $^1/_3$ full of calcium chloride.
5. Wrap parafilm (sealing tape)/insulation tape/cling wrap around join of lid and vial.
6. The following day replace the tissue wrap if necessary, check that the desiccant is not wet, and reseal vials. Material must be stored in the refrigerator or in a cool box with ice until fully desiccated.

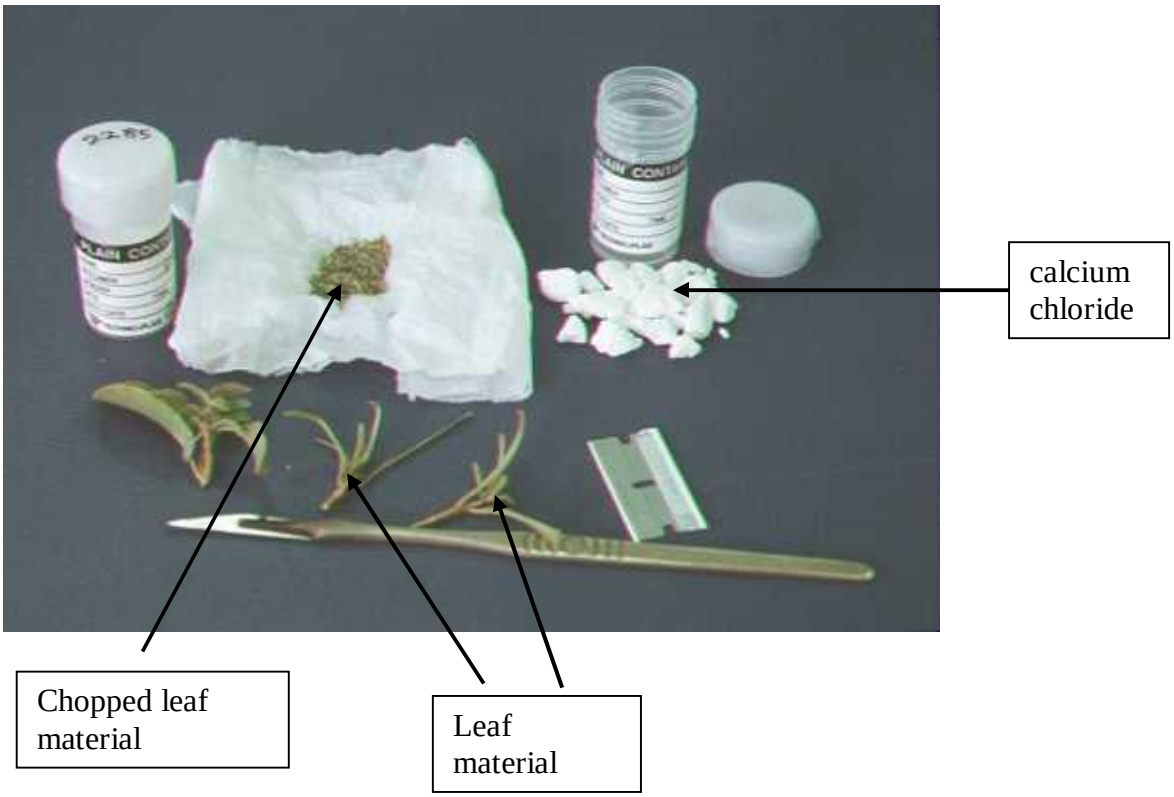

Figure 3. Equipment and reagents required for the collection of samples for testing.

DNA Extraction Protocol

(taken from Qiagen DNeasy® Plant Mini Kit protocol)

Materials and equipment

1. QIAGEN DNeasy® Plant mini kit
2. Sterile nuclease-free 1.5 ml centrifuge tubes
3. Pipettes
4. Sterile filter pipette tips
5. Sterile (acid washed & cleaned) sand (Silicon Dioxide - Sigma-Aldrich Cat. # 31623)/Liquid Nitrogen
6. Balance
7. Bench top centrifuge – to fit 1.5 – 2 mL microcentrifuge tubes; ≥14 000 rpm
8. Sterile, nuclease free, ultra pure water
9. Ice or cold blocks
10. Sterile mortars and pestles/sterile microcentrifuge tube and micropestle
11. Metal spatula
12. Vortex
13. Water bath or heating block at 65°C
14. Tube trays
    - *100% Ethanol*

NB. If using a new DNeasy® kit, add 100% ethanol to selected reagents before first use as directed by the protocol included with the kit.

Method

1. Pre-heat heating block or waterbath to 65°C
2. Label the tubes to be used during the extraction
3. If using sterile sand to disrupt tissues, weigh approximately 0.02g of dried leaf material and place in mortar; add a tiny amount of sand and grind leaf material to a powder with a pestle. If using liquid nitrogen to disrupt tissues, weigh either approximately 0.02g of dried leaf material or approximately 0.1g of fresh material and grind using a microcentrifuge tube and micropestle. **NB.** *If using liquid nitrogen to disrupt cellular/membrane walls do not allow tissue to become wet before adding the buffer AP1 and the RNase.*
4. With a metal spatula transfer the powdered plant material to a 1.5 ml centrifuge tube from the mortar and pestle. If tissue has been disrupted in a microcentrifuge tube, use this same tube for the next step.
5. Add 400 µl of QIAGEN buffer AP1 and 4 µl of RNase A (Supplied with the DNeasy® kit), cap tube, mix and incubate at 65°C for 10-30 minutes. Invert tubes several times during incubation.
6. Add 130 ul of QIAGEN buffer AP2 to extract. Invert 3 times to mix and place on ice for 5 minutes.
7. Place in centrifuge and spin at maximum speed for 5 minutes.
8. Pipette off as much of the aqueous layer as possible into a QIAshredder™ column (pink tube) and spin at maximum speed for 2 minutes.
9. Transfer up to 450 µl of flowthrough from QIAshredder™ column to a 1.5 ml centrifuge tube containing 675µl QIAGEN buffer AP3. Mix by pipetting.
10. Transfer 650 µl of extract onto a DNeasy® spin column and spin at 8000 rpm for 1 minute
11. Discard flow-through and add the rest of the sample to the column and spin at 8000 rpm for 1 minute

12. Place DNeasy® Mini spin column (white) in a new 2 ml collection tube and add 500 µl of QIAGEN buffer AW (wash buffer) and spin at 8000 rpm for one minute.
13. Discard flowthrough and add another 500 µl of QIAGEN buffer AW and spin at maximum speed for 2 minutes.
14. Discard flowthrough and collection tube. Ensure that the base of the column is dry (blot on tissue if it is not) and place in an appropriately labelled 1.5 ml centrifuge tube. Add 50 µl of AE buffer or nuclease free water directly to the filter (don't apply down the side of the tube), incubate at room temperature for 5 minutes before and centrifuging at 8000 rpm for 1 minute. Repeat this step into the same tube. Discard column and store DNA at -20°C.

RNA Extraction Protocol

(taken from Qiagen RNeasy® Plant Mini Kit protocol - 'Purification of Total RNA from Plant Cells and Tissues and Filamentous Fungi')

Materials and equipment

1. QIAGEN DNeasy® Plant mini kit
2. Sterile nuclease-free 1.5 ml centrifuge tubes
3. Pipettes
4. Sterile filter pipette tips
5. Sterile (acid washed & cleaned) sand (Silicon Dioxide - Sigma-Aldrich Cat. # 31623)/Liquid Nitrogen
6. Balance
7. Bench top centrifuge – to fit 1.5 – 2 mL microcentrifuge tubes; ≥14 000 rpm
8. Sterile, nuclease free, ultra pure water
9. Ice or cold blocks
10. Sterile mortars and pestles/sterile microcentrifuge tube and micropestle
11. Metal spatula
12. Vortex
13. Water bath or heating block at 56°C
14. Tube trays
    - *100% Ethanol*

NB. If using a new RNeasy® kit, add 100% ethanol to selected reagents before first use as directed by the protocol included with the kit. If using β-mercaptoethanol undertake steps 7 – 13 in a fume hood/fume extraction unit. 0.1% Sodium Sulfite can be added to the RLT Buffer to improve RNA recovery from difficult hosts.

Mortar and pestle preparation

- It may be necessary to cook mortar and pestles in an oven set at 240ºC for 4 hours prior to use. **NB.** DO <u>NOT</u> place the mortar and pestles in the oven enclosed in bags. Allow to cool before use.

Method

1. Set heating block to 56ºC
2. Label the tubes to be used during the extraction
3. Cut tips of 1 mL pipette tips (1/sample + a couple extra) with sterile scissors/scalpel blade for use in step 9 of the procedure.
4. In a fume hood/fume extraction unit add 10 µL β-mercaptoethanol (β-ME) per mL of Buffer RLT from the RNeasy® Kit. Calculate volume of Buffer RLT needed by

multiplying 450 µL of buffer by number of samples. **NB.** *If using liquid nitrogen to disrupt cellular/membrane walls do not allow tissue to become wet before adding the buffer RLT.*

5. If using sterile sand to disrupt tissues, weigh approximately 0.02g of dried leaf material and place in mortar; add a tiny amount of sand and grind leaf material to a powder with a pestle. If using liquid nitrogen to disrupt tissues, weight either approximately 0.02g of dried leaf material or approximately 0.1g of fresh material and grind using a microcentrifuge tube and micropestle.
6. With a metal spatula transfer the powdered plant material to a 1.5 ml centrifuge tube from the mortar and pestle. If tissue has been disrupted in a microcentrifuge tube, use this same tube for the next step.
7. Add 450 µL Buffer RLT to tissue powder.
8. Incubate at 56ºC for 1-3 minutes. **NB.** *Avoid incubation step if plant tissue is high in starch.*
9. Transfer sample to QIAshredder spin column (lilac) using the modified pipette tips (with cut tips) and spin for 2 minutes at maximum speed.
10. Carefully transfer supernatant of the flow through to a new tube without disturbing the cell debris pellet in the collection tube.
11. Add 0.5 volume of ethanol (95-100%) to this supernatant.
12. Immediately transfer the sample to an RNeasy® spin column (pink) and spin for 15 seconds at 10 000 rpm. Discard the flow through.
13. Add 700 µL Buffer RW1 and centrifuge for 15 seconds at 10 000 rpm. Discard flow through.
14. Add 500 µL Buffer RPE to the RNeasy® spin column and centrifuge at 15 seconds at 10 000 rpm. Discard flow through.
15. Add another 500 µL Buffer RPE to the RNeasy® spin column and centrifuge at 2 minutes at maximum speed. Discard flow through.
16. Place RNeasy spin column into a new 2 mL collection tube (supplied with kit) and centrifuge at maximum speed for 1 mnute.
17. Place RNeasy spin column into a new 1.5 mL collection tube (supplied with kit). Add 30 µL RNase-free water directly to the spin column membrane and spin for 1 minute at 10 000 rpm to elute RNA.

Additional notes

- The optional on-column DNase digestion is not used in this protocol
- It is very important to use glassware and consumables that are RNase free. Refer to the RNeasy Mini Handbook for further details on how to inactivate RNase on laboratory consumables.

On Completion of Extractions

1. UV irradiate/sterilise the work ares along with the mortar and pestles, tube racks and other associated equipment used in the maceration step.
2. Then place mortar & pestles and racks into cleaning washes: 1% Sodium hypochlorite for 12 hours followed by 0.2M HCL for 12 hours. Remove and clean with detergent, rinse with tap water and then reverse osmosis/distilled water x 3 times.
3. Leave mortar & pestles and racks to drain.
4. Autoclave mortar & pestles and return racks to shelves.

PCR Protocol

Materials and equipment

1. Pipettes
2. Sterile filter pipette tips
3. Sterile nuclease-free water
4. PCR Reagents – buffer, dNTPs, $MgCl^2$, enzymes, etc
5. 10 µm primers
6. 1.5 mL centrifuge tubes
7. 0.2 mL PCR tubes
8. Freezer
9. Ice or cold blocks
10. Centrifuge or lab fuge
11. PCR Machine

Method

Two negative and two positive controls are to be used along with two water controls (DNA grade H_2O only). Set up as per the following example.

1 = water control	5 = test sample 3	9 = -ve control
2 = -ve control	6 = test sample 4	10 = water control
3 = test sample 1	7 = +ve control	
4 = test sample 2	8 = +ve control	

NB. Steps 2 to 6 should be undertaken in a designated enclosed PCR room (free of potential contaminates, eg. DNA/PCR derived aerosols) or a PCR cabinet. Steps 1, 7-10 should be undertaken outside the designated enclosed PCR room or PCR cabinet. Physical separation of the steps reduces the risk of reagent contamination.

1. Bring DNA template aliquots to room temperature. Gently mix (DNA can sheer if it is mixed too violently) and softly spin down (using a labfuge or centrifuge on low rpm).
2. Defrost and mix the PCR reagents before softly spinning them down (using a labfuge or centrifuge on low rpm). Keep reagents cool on ice while out on the bench.
3. Make-up the PCR mastermix into a 1.5 mL microcentrifuge tube. Mix well and softly spin down (using a labfuge or centrifuge on low rpm). Keep mastermix cool on ice while out on the bench.
4. Place individual PCR reagents back into freezer.
5. Aliquot PCR mastermix into labelled (with PCR #) 0.2 mL thin-walled PCR tubes.
6. Add sterile nuclease-free water to 'water control' PCR tubes.
7. Close all lids except for the second (or last) 'water control' tube and transfer the tubes to the area outside the designated PCR room or PCR cabinet where the DNA/PCR template is to be added. **NB.** *Leaving the second (or last) of the two water control PCR tubes open will give some indication of any possible background contamination. By closing the first of the two water controls will indicate whether contamination, if present, is from the reagents.*
8. Open lids individually to add DNA/PCR template. Close lids immediately after adding DNA/PCR template.
9. Gently mix and spin down tubes using a labfuge or centrifuge on low rpm.
10. Place tubes in PCR machine, select program and start cycle.
11. Return DNA/PCR templates to freezer/fridge.

Additional notes

- Handle all reagents carefully, but be particularly careful with enzymes since they are highly sensitive to temperature and mechanical damage. Remove enzymes from freezer at the last possible moment and add immediately to master mix, return immediately to freezer. Avoid too much pipetting and vortexing once enzymes are added (mix by flicking and inversion).
- Overlay samples with mineral oil if the thermal cycler does not have a heated lid.
- Ensure all volumes to be added are sensible, this may mean diluting to give realistic volumes (eg add 2 μl of a 1:10 dilution, rather than 0.2 μl).

Phytoplasma assay

Quality control PCR

PCR was used by Weisberg *et al* 1991 for bacterial phylogenetic studies. This test will non-specifically amplify the 16S rDNA of any bacteria. This generic bacterial PCR can be used to check the quality of DNA extracts from plants or most bacteria. The PCR ensures that DNA is present or that there are no inhibitors in the DNA extracts that retard the activity of the DNA polymerase.

Primers: Published Primers (Weisberg *et al* 1991)

*f*D2	5'-AGA GTT TGA TCA TGG CTC AG-3'
*r*P1	5'-ACG GTT ACC TTG TTA CGA CTT-3'

Weisberg, WG, Barns, S, Pelletier, DA and Lane DJ. 1991. 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology* **173**, 697-703.

Phytoplasma nested PCR

Nested PCR involves two sets of primers, used in two successive runs of polymerase chain reaction, the second set intended to amplify a secondary target within the first run product. In the case of nested PCR for phytoplasma the first primer set amplifies a region of the 16S and 23S ribosomal DNA of the phytoplasma. The second primer amplifies a region within the first amplification. So, if you ran the first and the second primer amplifications next to each other on a gel, the second amplification would be slightly smaller than the first, because it is within the first primer set. The reason a nested PCR is performed is that it is more sensitive to picking up low copy Nucleic Acids. Phytoplasmas have been known to not only be unevenly distributed across host tissues, but also be present at very low titre in host tissues. Nested PCR increases the chance of their detection if present. *Table 2* and *Figure 4* below outline primers commonly used for the detection of phytoplasmas and their location along the 16S and 23S region.

Table 2. PCR primers used to detect phytoplasmas

Primer name (direction)	Primer sequence	Position	Reference
P1 (forward)	AAG AGT TTG ATC CTG GCT CAG GAT T	6-30, 16S	Deng and Hiruki (1991)
P7 (reverse)	CGT CCT TCA TCG GCT CTT	51-68, 23S	Schneider et al (1995)
P3	GGA TGG ATC ACC TCC TT	1518-1534, 16S	Schneider et al (1995)
rP3	AAG GCG GTG ATC CAT CC	1518-1534, 16S	
R16F2n (forward)	GAA ACG ACT GCT AAG ACT GG	149-168, 16S	Lee et al. (1993).
R16R2	TGA CGG GCG GTG TGT AGA AAC CCC G	1373-1397, 16S	Lee et al. (1993).
m23SR (reverse)	TAG TGC CAA GGC ATC CAC TGT G	20-51, 23S	Padovan et al. (1995)
fU5	CGG CAA TGG AGG AAA CT	369-386, 16S	Lorenz et al. (1995)
rU3	TTC AGC TAC TCT TTG TAA CA	1229-1249, 16S	Lorenz et al. (1995)

Deng SJ and Hiruki C 1991 Amplification of 16S rRNA genes from culturable and non-culturable Mollicutes. J. Microbiol. Methods 14:53-61 Deng, S.J. and Hiruki, C. (1991b) Genetic relatedness between two nonculturable mycoplasmalike organisms revealed by nucleic acid hybridization and polymerase chain reaction. *Phytopathology*. 81(12): 1475-1479.

Lee,I.-M., Hammond, R.W., Davis R.E. and Gundersen, D.E. (1993) Universal amplification and analysis of pathogen 16S rDNA for classification and identification of mycoplasma-like organisms. *Phytopathology*, 83:834-842

Lorenz, K-H, Schneider, B., Ahrens, U. and Seemuller, E. 1995. Detection of the apple proliferation and pear decline phytoplasmas of ribosomal and nonribosomal DNA. *Phytopathology* 85:771-776.

Padovan, A.C., Gibb, K.S., Bertaccini, A., Vibio, M., Bonfiglioli, R.E., Magarey, P.A. and Sears, B.B. (1995) Molecular detection of the Australian grapevine yellows phytoplasma and comparison with Grapevine yellows phytoplasmas from Italy. *Australian Journal of Grape and Wine Research*, **1**, 25-31.

Schneider, B., Cousin, M.T., Klinkong, S. and Seemüller, E. (1995). Taxonomic relatedness and phylogenetic positions of phytoplasmas associated with diseases of faba bean, sunnhemp, sesame, soybean, and eggplant. *Journal of Plant Diseases and Protection* **102**, 225-32.

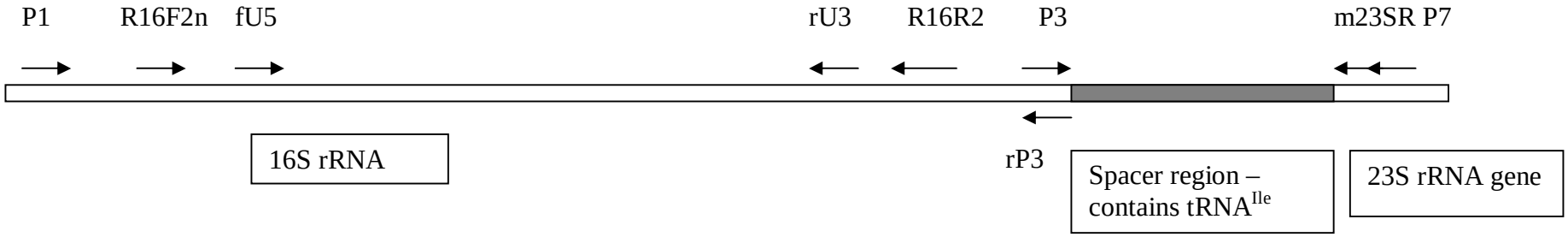

Figure 4. Schematic for location of priming regions along the 16S rRNA – 23SrRNA genes used to detect phytoplasmas

PCR Diagnostic: 16S rDNA Quality Control PCR (Weisberg *et al.* 1991) using Qiagen Taq DNA Polymerase (Cat. # 201205).

Date: **Operator:**

PCR #	Accession #	Results	Notes	**PCR #**	Accession #	Results	Notes
1				21			
2				22			
3				23			
4				24			
5				25			
6				26			
7				27			
8				28			
9				29			
10				30			
11				31			
12				32			
13				33			
14				34			
15				35			
16				36			
17				37			
18				38			
19				39			
20				40			

PCR Master Mix	**x 1**	**x**	**Final Concentration**
Sterile distilled water	18.30		
10 x Buffer (plus 15 mM $MgCl_2$)	2.5		1 x Buffer (1.5mM $MgCl_2$*)
$MgCl_2$ (25 mM)	0.5		0.5 mM*
dNTPs (10mM mix)	0.5		0.2 mM
5' primer end FD1 (10µM)	1.0		0.4 µM
3' primer end rP2(10µM)	1.0		0.4 µM
Taq (5 units/uL)	0.2		1 unit

- 1 µL of DNA template * 2.0mM $MgCl_2$ in total
- **Total final reaction volume = 25 µL**

PCR cycling conditions			
Temp	*Time*		
95°C	1 minutes		Initial denaturation
95°C	45 seconds	30 cycles	Denaturation
55°C	30 seconds		Annealing
72°C	30 seconds		Extension
72°C	10 minutes		Final Extension
25°C	1 minute		

References: The expected PCR product size is approximately 1400-1500 bp; load 5 µL of PCR reaction into a 1% agarose gel, run for 20 minutes at 100 Volts

PCR Diagnostic: Nested Phytoplasma – 1st Round P1-P7 PCR using Qiagen Taq DNA Polymerase (Cat. # 201205).

Date: **Operator:**

PCR #	Accession #	Results	Notes	**PCR #**	Accession #	Results	Notes
1				21			
2				22			
3				23			
4				24			
5				25			
6				26			
7				27			
8				28			
9				29			
10				30			
11				31			
12				32			
13				33			
14				34			
15				35			
16				36			
17				37			
18				38			
19				39			
20				40			

PCR Master Mix	**x 1**	**x**	**Final Concentration**
Sterile distilled water	18.8		
10 x Buffer (plus 15 mM $MgCl_2$)	2.5		1 x Buffer (1.5mM $MgCl_2$
dNTPs (10mM mix)	0.5		0.2 mM
5' primer end P1 (10µM)	1.0		0.4 µM & fD1
3' primer end P7 (10µM)	1.0		0.4 µM & rP2
Taq (5 units/uL)	0.2		1 unit

- 1 µL of DNA template
- **Total final reaction volume = 25 µL**

PCR cycling conditions			
Temp	*Time*		
95°C	1 minute		Initial denaturation
95°C	1 minute	35 cycles	Denaturation
55°C	1 minute		Annealing
72°C	1.5 minutes		Extension
72°C	10 minutes		Final Extension
25°C	1 minute		

References: The expected PCR product size is approximately 1800 bp; load 5 µL of PCR reaction into a 1% agarose gel, run for 20 minutes at 100 Volts

PCR Diagnostic: Nested Phytoplasma – 2nd Round R16F2n/m23SR PCR using Qiagen Taq DNA Polymerase (Cat. # 201205).

Date: **Operator:**

PCR #	Accession #	Results	Notes	**PCR #**	Accession #	Results	Notes
1				21			
2				22			
3				23			
4				24			
5				25			
6				26			
7				27			
8				28			
9				29			
10				30			
11				31			
12				32			
13				33			
14				34			
15				35			
16				36			
17				37			
18				38			
19				39			
20				40			

PCR Master Mix	**x 1**	**x**	**Final Concentration**
Sterile distilled water	19.3		
10 x Buffer (plus 15 mM $MgCl_2$)	2.5		1 x Buffer (1.5mM $MgCl_2$
dNTPs (10mM mix)	0.5		0.2 mM
5' primer end R16F2n (10µM)	1.0		0.4 µM
3' primer end m23SR (10µM)	1.0		0.4 µM
Taq (5 units/uL)	0.2		1 unit

- 0.5 µL of DNA template
- **Total final reaction volume = 25 µL**

PCR cycling conditions			
Temp	*Time*		
95°C	1 minute		Initial denaturation
95°C	1 minute	35 cycles	Denaturation
55°C	1 minute		Annealing
72°C	1.5 minutes		Extension
72°C	10 minutes		Final Extension
25°C	1 minute		

References: The expected PCR product size is approximately 1500 bp; load 5 µL of PCR reaction into a 1% agarose gel, run for 20 minutes at 100 Volts

Potyvirus assay

Quality control RT-PCR

An RT-PCR was developed from sequence data from the NADH dehydrogenase ND2 subunit (*ndhB* gene) by Thompson *et al* 2003 as a generic quality control for RNA studies (Figure 5). This test will non-specifically amplify across the splice junction between 'exon 1' and 'exon 2' mRNA region of the *ndhB gene* (Figure 5) and can be used to check the quality of extracts from a range of RNA plant viruses. The PCR ensures that RNA is present or that there are no inhibitors in the RNA extracts that retard the activity of the RNA enzymes. If you use total nucleic acid extracts directly without any DNAse treatment you will most likely get two bands. The band of about 900 bp is from DNA and the one of about 200 bp is from RNA. It appears to work with most host types. Mango might be an exception.

Primers: Published Primers (Thompson *et al* 2003)

AtropaNad2.1a	5'-GGACTCCTGACGTATACGAAGGATC-3'
AtrophaNad2.2b	5'-AGCAATGAGATTCCCCAATATCAT-3'

Thompson JR, Wetzel S, Klerks MM, Vaskova D, Schoen CD, Spak J, Jelkmann W. 2003. Multiplex RT-PCR detection of four aphid-borne strawberry viruses in Fragaria spp. in combination with a plant mRNA specific internal control. *Journal of Virological Methods* **111**, 85-93.

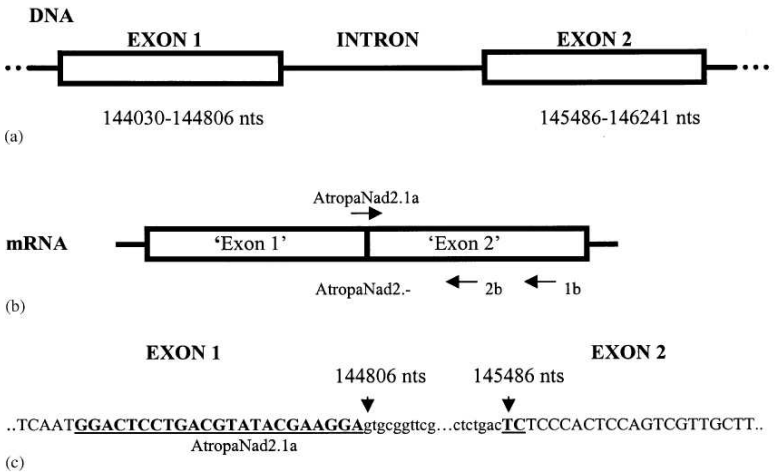

Fig. 1. Schematic of internal control primers in *ndhB* gene. (a) The *ndhB* gene in chloroplast DNA, consisting of two exons (1 and 2) separated by an intron. Nucleotide positions are marked below. (b) The *ndhB* gene in chloroplast mRNA showing the location of the AtropaNad2 primers. (c) Location of AtropaNad2a primer (bold type, underlined) in exons 1 and 2 (large case letters). Part of intron sequence shown in small case letters.

Figure 5. Schematic of *ndhb gene* and priming sites taken from Thompson *et al* 2003.

Potyvirus RT-PCR

RT-PCR used by Grisoni *et al* 2006 to identify potyviruses infecting vanilla through direct sequencing of the RT-PCR amplicon. The generic degenerative potyvirus primers used where first developed by Marie-Jeanne *et al*. (2000) to amplify a 327 bp fragment spanning conserved motifs MVWCIEN to QMKAAA in the core of the Coat Protein of potyviruses.

Primers: Published Primers (Marie-Jeanne *et al*. 2000; Grisoni *et al* 2006)

MJ1 5'- ATGGTHTGGTGYATHGARAAYGG-3'
MJ2 5'- TGCTGCKGCYTTCATYTG-3'

Grisoni M, Moles M, Farreyrol K, Rassaby L, Davis R, Pearson M. 2006. Identification of potyviruses infecting vanilla by direct sequencing of a short RT-PCR amplicon. *Plant Pathology* **55**, 523–529.

Marie-Jeanne V, Ioos R, Peyre J, Alliot B, Signoret P, 2000. Differentiation of Poaceae potyviruses by reverse transcription-polymerase chain reaction and restriction analysis. *Journal of Phytopathology* **148**, 141–51.

PCR Diagnostic: *ndhB gene* (AropaNad2.1a/ArtopaNad2.2b) Quality Control RT-PCR using Qiagen One-step PCR Reagents (Cat. # 210212).

Date: **Operator:**

PCR #	Accession #	Results	Notes	**PCR #**	Accession #	Results	Notes
1				21			
2				22			
3				23			
4				24			
5				25			
6				26			
7				27			
8				28			
9				29			
10				30			
11				31			
12				32			
13				33			
14				34			
15				35			
16				36			
17				37			
18				38			
19				39			
20				40			

PCR Master Mix	**x 1**	**x**	**Final Concentration**
Sterile RNase-free water	11.75		
5 x RT-PCR Buffer	5.0		1 x buffer
dNTPs (10mM)	1.0		0.4 mM each dNTP
3’ primer end – 2.1a (10 uM)	0.625		0.25 uM
5’ primer end – 2.2b (10 uM)	0.625		0.25 uM
RT-PCR Enzyme Mix	1.0		

- 5.0 µL of DNA template
- **Total final reaction volume = 25 µL**

PCR cycling conditions			
Temp	*Time*		
50°C	Manual hot start		
50°C	30 minute		Reverse Transcript.
95°C	15 minute		Initial activation step
94°C	1 minute	Repeat steps x 34	Denaturation
55°C	40 seconds	Cycles	Annealing
72°C	1 minute		Extension
72°C	10 minutes		Final Extension

References: The expected PCR product size is approximately 200bp (if no DNAse treatment there will be a second band @ 900bp); load 10 µL of PCR reaction into a 2% agarose gel, run for 50 minutes at 70 Volts

PCR Diagnostic: Potyvirus (MJ1/MJ2) RT-PCR using Qiagen One-step PCR Reagents (Cat. # 210212).

Date: **Operator:**

PCR #	Accession #	Results	Notes	**PCR #**	Accession #	Results	Notes
1				21			
2				22			
3				23			
4				24			
5				25			
6				26			
7				27			
8				28			
9				29			
10				30			
11				31			
12				32			
13				33			
14				34			
15				35			
16				36			
17				37			
18				38			
19				39			
20				40			

PCR Master Mix	**x 1**	**x**	**Final Concentration**
Sterile RNase-free water	14.75		
5 x RT-PCR Buffer	5.0		1 x buffer
dNTPs (10mM)	1.0		0.4 mM each dNTP
3’ primer end – MJ1 (10 uM)	0.625		0.25 uM
5’ primer end – MJ2 (10 uM)	0.625		0.25 uM
RT-PCR Enzyme Mix	1.0		

- 2.0 µL of DNA template
- **Total final reaction volume = 25 µL**

PCR cycling conditions			
Temp	*Time*		
50°C	Manual hot start		
50°C	30 minute		Reverse Transcript.
95°C	15 minute		Initial activation step
94°C	30 seconds	Repeat steps x 34	Denaturation
50°C	1 minute	Cycles	Annealing
72°C	1 minute		Extension
72°C	10 minutes		Final Extension

References: The expected PCR product size is approximately 327bp; load 10 µL of PCR reaction into a 2% agarose gel, run for 50 minutes at 70 Volts

Gel Electrophoresis

Gel electrophoresis is a technique used for the separation of PCR products using an electric current applied to a gel matrix.

Figure 6. Gel electrophoresis unit (or gel rig): power supply and buffer tray containing an agarose gel. (Accessed at 10:45 hrs, 2/4/09 by internet at http://en.wikipedia.org/wiki/Gel_electrophoresis)

Important – Wear gloves and protective clothing when handling agarose gels and associated materials/equipment as toxic chemicals may be used in this procedure. Develop appropriate protocol for the disposal of contaminated materials.

PCR Electrophoresis

Electrophoresis conditions

- 1 - 2 % Agarose Gel made up with 1 x TBE (or TAE) buffer
- 5 µL – 10 µL of PCR product
- Loading dye
- 100 base pair ladder (5 µL aliquots)
- Run gel for 20-30 minutes at 80-100 Volts (1% agarose gel)/ 40-60 minutes at 70-80 Volts (2% agarose gel)

Materials and equipment

1. Balance
2. Microwave or hotplate
3. Power pack, gel rig and gel trays with removable combs
4. 100 mL microwave/heat safe wide-necked bottle
5. Agarose powder
6. 1 x TBE buffer solution
7. SYBR® Safe DNA gel stain *10,000X concentrate in DMSO (store in fridge when not in use, defrost before use)
8. UV illumination system

Method

1. Make up your gel solution in a 50 – 250 mL Schott Bottle. Leave the lid LOOSE.

2. Weight out the agarose. First tare the bottle weight and then add the required amount of agarose powder, before adding the required amount of 1.0 TBE.
3. Dissolve the agarose by applying heat – takes up to 3 minutes in the microwave with the setting on high. Keep an eye on the gel so that it doesn't boil over and swirl occasionally. Use heat resistant gloves when handling the Schott Bottles containing the heated agarose solution. The solution can become superheated and extreme caution should be taken.
4. Place the gel solution to cool a little while you prepare the electrophoresis tray and cell.
5. Add 1 µL of SYBR® Safe DNA gel stain per 10 mL of agarose gel solution. Mix well and pour into the gel tray to set (remember to place the gel combs into the tray).
6. Remove the gel comb once the gel is set. Remove any tape or wedges. Fill the buffer tray to the recommended level with 1 x TBE buffer. Transfer the gel to the gel rig. Make sure the buffer is just covering the gel.
7. Aliquot each PCR sample reaction/loading dye mixture into an individual well and at least 1 DNA ladder per row.
8. Place the electrophoresis cell lid on (make sure the gel wells are located nearest to the black [+ve] electrodes in the tray and that the colour of the plugs/leads on the lid is matched in colour to the electrodes in the tray). Connect the cell power leads to the power pack and turn on the mains power.
9. Set the voltage and time appropriate for your gel before pressing start.
10. Transfer the gel to the UV illumination system for visualisation.
11. Once you have finished taking images, dispose of the gel in a safe manner.

Additional note
If the PCR reaction buffer or DNA ladder does not include a dye, then the sample can be mixed with loading dye.

Gel Image Analysis

The dye added to the gel forms linkages to the PCR products. If the dye molecules fluoresce under ultraviolet light, a photograph can be taken of the gel under ultraviolet lighting conditions. The image below (*figure 7*) is of a photograph of PCR products from a Huanglongbing assay run on a 2% agarose gel

By including a DNA 'ladder' (molecular weight marker) beside the PCR products during gel electrophoresis the size or lengths of the products can be determined. A DNA ladder is essentially a solution of DNA molecules of different, but known lengths. It is very important to include ladders with your PCR products in diagnostic gels, because it is the length of the product that provides the strongest argument for or against the presence of the target under investigation.

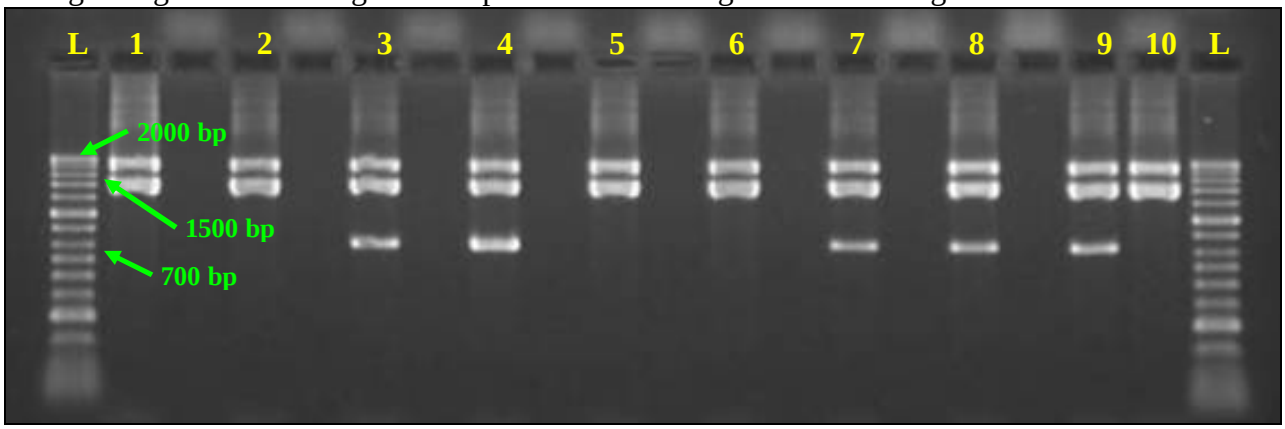

Figure 7. Huanglongbing PCR products: 'L' = DNA ladder; 1 and 10 = negative controls; 8 and 9 = positive controls; 2 to 7 = samples screened during assay.

Appendices

Appendix 1: General background on DNA extraction and PCR principles

General DNA Extraction Steps

1. Break open the cells: cell disruption. For example, by grinding. Cell disruption is a particularly important step when working with plants. Unlike animal cells, plant cells have an additional cell wall composed of cellulose.
2. Disrupt or remove cell membrane so that cellular contents (including DNA) are released into solution. As the cell membrane is composed of lipids, this is commonly achieved through the addition of a detergent.
3. Remove tissue and cellular debris from the solution.
4. Isolate and clean DNA free of salts, complex carbohydrates (eg. starch) and other organic compounds (eg. polyphenols) that may inhibit PCR.
5. Suspend DNA in nuclease free water or an elution buffer.

Reagent components of the polymerase chain reaction (PCR)

1. Nucleic acid (DNA or RNA) template
2. A set of two primers, which are complimentary to the DNA reagents at the 5' (five prime) or 3' (three prime) ends of the DNA region of interest. In cases were PCR reactions are multiplexed, i.e. where two or more regions are being targeted, there will be two or more sets of primers.
3. *Taq polymerase* or another DNA polymerase with a temperature optimum at around 70°C.
4. dNTPs (Deoxynucleoside triphosphates)
5. Buffer solution, providing a suitable chemical environment for optimum activity and stability of the DNA polymerase.
6. Magnesium ($Mg2^{+}$) or manganese ($Mn2^{+}$) ions. The exact role of these ions in the reaction is not clear. However, if they are not in sufficient quantity the reaction will not proceed.

NB. Reagent components can be purchased as separate items or as part of a kit. In other instances concentrated master mixes comprising of several or all the reagents can be purchased.

How PCR works (information accessed at 09:20 hours 6/4/09 via internet at http://en.wikipedia.org/wiki/Polymerase_chain_reaction)

The PCR usually consists of a series of 20 to 40 repeated temperature changes called cycles; each cycle typically consists of 2-3 discrete temperature steps. Most commonly PCR is carried out with cycles that have three temperature steps. The temperatures used and the length of time they are applied in each cycle depend on a variety of parameters. These include the enzyme used for DNA synthesis, the concentration of divalent ions and dNTPs in the reaction, and the melting temperature (Tm) of the primers.

1. *Denaturation step*: this step is the first regular cycling event and consists of heating the reaction to 94-98°C for 20-30 seconds. It causes melting of DNA template and primers by disrupting the hydrogen bonds between complementary bases of the DNA strands, yielding single strands of DNA.
2. *Anealing step*: the reaction temperature is lowered to 50-65°C for 20-40 seconds allowing annealing of the primers to the single-stranded DNA template. Typically the annealing temperature is about 3-5 degrees Celsius below the Tm of the primers used. Stable DNA-

DNA hydrogen bonds are only formed when the primer sequence very closely matches the template sequence. The polymerase binds to the primer-template hybrid and begins DNA synthesis.

3. *Extension/elongation step*: the temperature at this step depends on the DNA polymerase used; commonly a temperature of 72°C is used with this enzyme. At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction. The extension time depends both on the DNA polymerase used and on the length of the DNA fragment to be amplified. As a rule-of-thumb, at its optimum temperature, the DNA polymerase will polymerize a thousand bases per minute. Under optimum conditions, i.e., if there are no limitations due to limiting substrates or reagents, at each extension step, the amount of DNA target is doubled, leading to exponential (geometric) amplification of the specific DNA fragment.

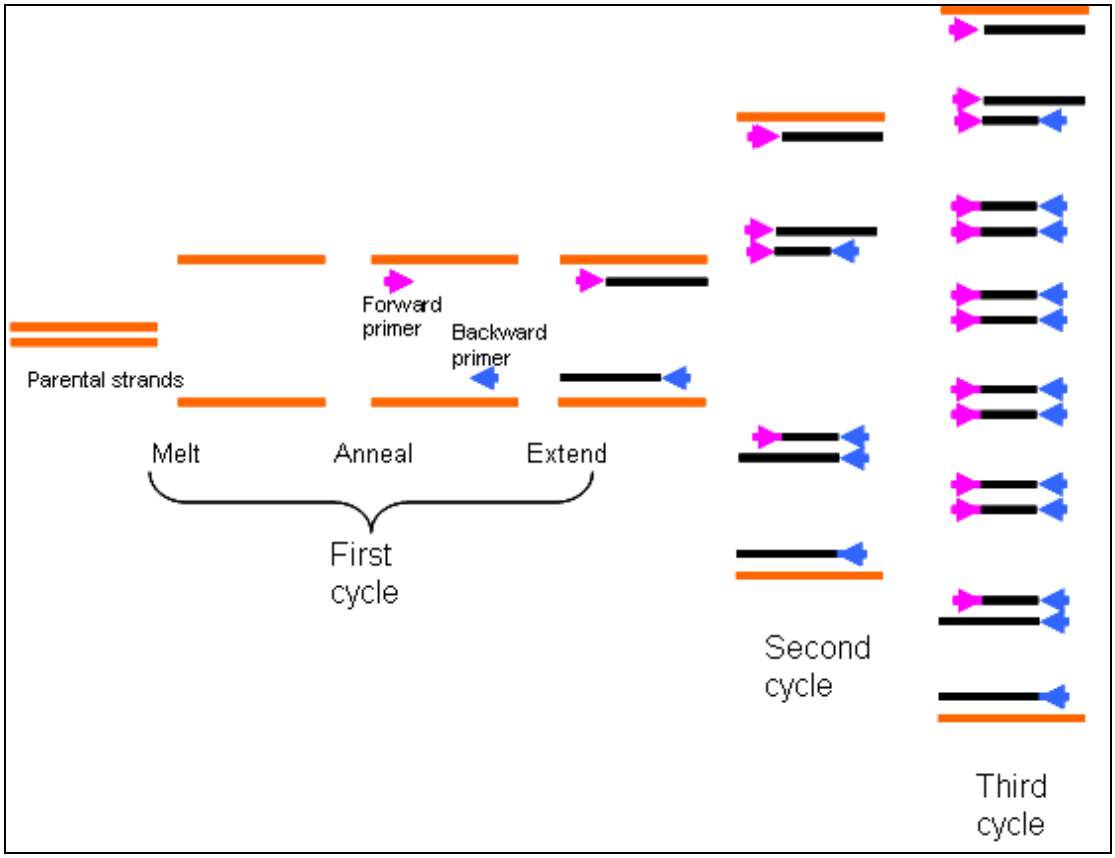

Figure 8. The different steps in PCR. (Image accessed 09:30 hrs 6/4/09 via internet at http://www.obgynacademy.com/basicsciences/fetology/genetics/).

Appendix 2: common chemical/reagent definitions, calculations and conversions

Mass-volume percentage

Sometimes referred to as weight-volume percentage or percent weight per volume and often abbreviated as % m/v or % w/v. It describes the mass of solute in grams (g) per 100 mL of the resulting solution. Mass-volume percentage is often used for solutions made from a solid solute dissolved in a liquid. For example, a 40% w/v sugar solution contains 40 g of sugar per 100 mL of resulting solution.

Volume-volume percentage

Sometimes referred to as percent volume per volume and abbreviated as % v/v) describes the volume of the solute in mL per 100 mL of the resulting solution. This is most useful when a liquid - liquid solution is being prepared, although it is used for mixtures of gases as well. For example, a 40% v/v ethanol solution contains 40 mL ethanol per 100 mL total volume.

Molar Solutions

A 1 molar solution is a solution in which 1 mole of a compound is dissolved in a total volume of 1 litre. For example, the molecular weight of sodium chloride (NaCl) is 58.44 therefore 1 mole is 58.44g. If you dissolve 58.44g of NaCl in a final volume of 1 litre, you have made a 1M NaCl solution. To make a 0.1M NaCl solution, you could weigh 5.844g of NaCl and dissolve it in 1 litre of water or 0.5844g of NaCl in 100mL of water or make a 1:10 dilution of a 1M sample. You might also see some solution concentrations written as 1 N (N='normal'), these are equivalent to 1 M for univalent chemicals such as NaOH, HCl. The molecular weight (MW) of a chemical is nearly always printed on the container it was purchased in. If not, you can look it up on the internet. There are many WWW resources listing chemicals, just make sure you are looking up the correct one.

Molarity conversions

- 1 M solution is = 1 mole per litre
- 1 mM solution is = 1 milli-mole per litre=0.001; M =10^{-3} M
- 1 µM solution is = 1 micro-mole per litre=0.001; mM=0.000001 M =10^{-6} M
- 1 nM solution is = 1 nano-mole per litre=0.001; µM=0.000001; mM=0.000000001 M =10^{-9} M
- 1 pM solution is = 1 pico-mole per litre=0.001; nM=0.000001; µM=0.000000001 mM=0.000000000001; M =10^{-12} M

Working with primer (custom oligonucleotide) concentrations

The paperwork accompanying primers will generally include information such as 'nmoles' of primer and 'µg' of primer, etc.

- To make a 100 µM stock solution = add (number of nmoles of primer x 10) µL nuclease-free water and dissolve
- To make a 10 µM working solution = dilute the 100 µM stock by 10 (1:10); for example, 1 µL of 100 µM stock, plus 9 µL nuclease-free water
- To convert µg to µmoles: µmoles of primer = µg of primer per MW of primer
- To calculate concentration from µmoles (nmoles): M = µmoles per µL (µM = µmoles per mL)

To reconstitute

- Spin primer tube to bring pellet to bottom
- Add recommended volume of water to tube

- Allow to stand for at least 5 minutes at room temperature
- Vortex for 15 seconds to mix

Appendix 2: Draft Workshop Program

DRAFT
SHORT TRAINING ON IDENTIFICATION OF BIO MOLUCULAR PLANT PATHOLOGIC APPROACH

I. Introduction

Background

Plant pests recognized as major problem in several agricultural commodities in Indonesia caused yield losses. Particularly, some horticultural products contributes high economic added value to rural areas. For instance, economic losses for *Ralstonia solanacearum* in some of horticultural product reported which may able to reach more than 80%. Other impact was also on social and enviroment impact as well.

Capacity building on accurate detection and identification plant pests is very important to decide the right decision in the risk mitigation and pests control in the frame integrated pest management system. Collaboration training on detection and identification of pests between Indonesia and Australian under frame of WGAFFC may be more reality benefit if the application of technology could be able to touch to the farmers.

Task of Indonesian Agricultural Quarantine is not the only to protect the country itself from the incursion of quarantine pest but also as dynamic facilitator as lead agency of NPPO in the market access which refer to the SPS requirement

There are listed technical diagnosis of plant diseases pathogens as international standards. Polymerase Chain Reaction (PCR) is the most effective techniques because of highest sensitivity . Sensitivity of PCR techniques could be resulted only from 1 DNA molecule. This technique also develop for plant diseases diagnosis and more over for the pathogen which low density population in the existing plant tissues and other scientific report may cover for latent symptoms.

Objectives

1. Improve capacity plant pathologist of quarantine analyst and member of NPPO for identification of plant pathogen using bio-molecular approach.

2. Able to work individual using PCR techniques and its substantial concept particularly on such pathogen lethal yellowing on palm oil and other.

Participant :

Participants should be a person who work in the quarantine laboratory and some laboratory from Directorate General of Horticulture with the total participant 15 persons. Background of the participants should be a person who familiar with PCR techniques or have main duties in Biomoleculer Laboratory.

Instructur :

This training is collaboration between Australia-Indonesia Government as continuation activity in the training on the application Remote Microscope Program for Indonesia. Tutor or expertise of training from AQIS (Australian Quarantine Inspection Service) or other research institute/univerties of Australia namely Dr. Lynne Jones as Plant Pathologist.

II. Materials and Methods :

Method:

Training will be carried out in the class for general overview as introduction of the training, objective and the implementation target after training. Practical laboratory will be conducted in Biotechnology Laboratory, Applied Research Institute of Agricultural Quarantine (ARIAQ), Bekasi, Jakarta, Indonesia.

During the training ARIAQ provided translation of bahasa Indonesia language for optimizing in the achievement of knowledge.

Syllabus of the training containing:

1. Introduction of biomolecular approach for identication of plant disease.
2. Application of PCR techniques and its concept for diagnosis of bacteria, virus and fungi as causal pathogen of some horticultural commodities including ornamental plants.
3. Recognition of Lethal Yellowing Disease and likes in bio-molecular laboratory practical.

Materials
List of chemicals as below will be used in the training may be prepared by AQIS while the laboratory apparatuses will be provided by ARIAQ.

No	Item	Number per Unit
1	Ready to go RT PCR bead GE	96 reactions/box
2	RNeasy RNA plant mini kit Qiagen	50 reactions/box
3	Ready to go PCR bead	96 rx/box
4	DNeasy plant mini kit Qiagen	50 rx/box
5	Microtube 1.5 ml	500 pcs/box
6	Micropippette tips: 1-10 µl 2-20 µl 20-200 µl 100 – 1000 µl	 1000 pcs. 1000 pcs. 1000 pcs. 1000 pcs.
7	Agarose	100 g
8	TAE 50X	500 ml
9	TE 1X	250 ml
10	Blue loading dye 6x	1 unit
11	DNA ladder 100 bp	100 rxn
12	Ethidium bromide/ Cybergreen	100 ml
13	Rubber glove size S,M,L	Each 1 box
14	PCR Primer for *Peronosclerospora sorghi*	1 unit
15	PCR primer for Turnip Mosaic Virus	1 unit
16	PCR Primer for *Lethal Yellowing Phytoplasma*	1 unit
17	2-Mercaptoethanol	solution
18	Ethanol 70%	solution
19	Ethanol 96%	solution
20	Liquid Nitrogen	-
21	Internal Control Primer (for DNA)	1 unit
22	Internal Control Primer (for RNA)	1 unit
23	Liquid Nitrogen	20 Liters

24	Disposable masker	1 box
25	Dnaway and Rnaway	solution

Tentative Schedule of Training.

Date	Time	Activity	Lecture and Assistant
30th May	09.00-10.00	Introduction	ARIAQ Team
	10.00-10.15	Tea Break	
	10.15-12.00	Introduction of Biomoleculer technique & Intoduction of PCR concept and PCR steps	Lyne
	12.00-13.30	Lunch	
	13.30-15.00	Intoduction of PCR concept and PCR steps (continued) & Application of PCR Concept for Diagnosing plant pathogenic organisms	Lyne
	15.00-15.15	Tea Break	
	15.15-17.00	Outline of details for remaining activity	Lyne
~~31th May~~	~~08.00-17.00~~	~~Field visit in Infection area of Turnip Mosaic Virus and Peronosclerospora including handling of samples from the field.~~	
01st June	08.00-12.00	DNA / RNA Extraction (Including tea break time) for Cabbage, Maize, and Oil Palm Seeds	Lyne & Assistant
	12.00-13.30	Lunch	
	13.30-17.00	Electroforesis	Lyne & Assistant
		PCR & RT-PCR (Overnight)	
02nd June	08.00-12.00	Electroforesis	Lyne & Assistant
		DNA Visualization	
	12.00-13.30	Lunch	
	13.30-17.00	Discuss	
03th June	08.00-17.00	Remote Microscope Program	Michael Thompson

Accommodation and Daily Allowance

During the training the participants will be stay in the dormitory, providing the meals with the budget under subsided price of IAQA amount Rp. 175.000,- per day per person. Daily allowance for one person to cover their personnel requirements of Rp. 100.000,- Transportation of participants will be covered by Aus Aid.

~~Field Visit~~

~~Field visit will be conduct in the cabbage and maize plantation in Cinangneng and Dramaga, Bogor - West Java. This place reported as Turnip Mosaic Virus (TuMV) and Peronosclerospora infection area. TuMV is one of problem in cabbage plant more over as seedborne pathogen. Peronosclerospora is a latent pathogen that difficult to identify.~~

Appendix 3: Participants

List Of Participants

Advance Short Training on Identification
of Biomolecular Plant Pathologic Approach

No.	Nama dan NIP	UPT asal
1.	Leny Hartati H., SP, M.Si NIP. 19751209 200312 2 001	Balai Besar Karantina Pertanian Belawan
2.	Damraeni Juhari, SP NIP. 19760528 200501 2 001	Balai Besar Karantina Pertanian Makassar
3.	Iman Suryaman, SP NIP. 19641103 198903 1 001	Balai Besar Karantina Pertanian Soekarno-Hatta
4.	Ir. Agus Suparto NIP. 19611210 198903 1 001	Balai Besar Karantina Pertanian Surabaya
5.	Ir. Suyatman NIP. 19660311 199203 1 002	Balai Besar Karantina Pertanian Tanjung Priok
6.	Dra. Derhani Lumban Gaol NIP. 19610901 198903 2 002	Balai Besar Uji Standar Karantina Pertanian
7.	Ir. Suwardi NIP. 19660307 199403 1 001	Balai Karantina Pertanian Kelas II Medan
8.	Ir. Septa Indah, M.Si NIP. 19640907 199003 2 001	Balai Karantina Pertanian Kelas I Palembang
9.	Ir. Slamet Riyadi, MP NIP. 19680628 200112 1 001	Direktorat Perlindungan Hortikultura
10.	Ir. Desmawati NIP. 19631218 199103 2 001	Direktorat Perlindungan Hortikultura
11.	Ir. Sabirin NIP. 19621120 198903 1 002	Direktorat Perlindungan Perkebunan
12.	Slamet Budiawan, SE, SP NIP. 19650223 198603 1 003	Balai Uji Terap Teknik dan Metode Karantina Pertanian
13.	Ade Syahputra, SP NIP. 19790110 200801 1 005	Balai Uji Terap Teknik dan Metode Karantina Pertanian
14.	Ina Retnowati	SEAMEO BIOTROP Bogor