Phytoplasma Detection Protocol

Buffers:

Hybridisation buffer

100ml hybridisation buffer
2.92g Sodium chloride
4g Blocking reagent (add slowly while stirring)
Mix at room temperature for 2 hours
Can be stored in aliquots at -20°C for up to 3 months

0.5M Sodium phosphate pH 7 (1 Litre) (for use in Primary Wash Buffer)

59.99g Sodium Dihydrogen Phosphate adjust to pH 7 with Sodium hydroxide

Primary Wash Buffer (1Litre)

2M Urea	120g
0.1% SDS	1g
0.5M Sodium phosphate pH 7	100ml

Store for up to 1 week at 4°C

20x Secondary Wash Buffer (1 Litre)

1M Tris Base	121g
2M NaCl	116.88g

pH to 10
Keep for up to 4 months at 4°C

1M $MgCl_2$ (100ml)

$MgCl_2$	9.52g

Secondary wash buffer-working dilution (200ml)

20x Secondary Wash Buffer	10ml
1M $MgCl_2$	0.4ml

Make up to 200ml with SDW
This buffer cannot be stored and must be made up fresh before washing a membrane.

Extraction Buffer AMES (200ml)

3% SDS	6g
20% Ethanol	40ml
0.5M Sodium Acetate	8.203g
10mM $MgCl_2$	2ml of 1M stock

10x TBE (1 Litre)

Tris Base	107.8g
Boric Acid	55g (harmful, wear mask)
EDTA	7.4g

DNA probe production:

From plasmid prep "nested hae III" a 1100bp fragment (approx.) will be amplified using universal primers, which anneal in the plasmid vector. The plasmid should be diluted 1 in 1000 in nuclease free water to provide a template for PCR, this working concentration of plasmid can be stored for up to 1 year at 4°C. PCR primer sequences are as follows pGEM T easy F 5′ CCG CGG GAA TTC GAT T, and pGEM T easy R 5′ GCG GCG AAT TCA CTA GTG ATT.

Example using Promega Taq other Taq DNA polymerases can be used, reagent concentrations should be altered according to manufacturer's instructions.

10x buffer	10µl
25mM $MgCl_2$	6µl
10mM each dNTP's	0.8µl
5µM pGEM T easy F	10µl
5µM pGEM T easy R	10µl
Taq polymerase	0.6µl
Nuclease free water	57.6µl

Diluted “nested hae III” plasmid	5μl
TOTAL	100μl

Cycling conditions are 94°C for 5 minutes followed by 30 cycles of 94°C for 30s, 52°C for 1min., and 72°C for 1min., with a final incubation step of 72°C for 5min.

Ensure the PCR has worked by gel electrophoresis as follows:

1. Make enough gel for the gel tank used the gel should be 3-5mm thick when poured, for the small gel tank 40ml of gel is enough.
2. Dilute the 10x TBE stock solution in water (100ml of 10x TBE plus 900ml water which makes a 1x TBE working solution).
3. Weigh out enough agarose to make a 1.2% solution (for 40ml of gel this will be 0.48g) and put into a conical flask, add 40ml 1x TBE working solution and swirl to mix.
4. Melt the agarose in the microwave mixing occasionally to insure all the agarose is melted (1 minute is enough for 40ml). Agarose heated in the microwave can boil over unexpectedly, wear heat protective gloves and be careful not to burn yourself.
5. Allow the agarose to cool for 5 minutes while setting up the casting tray, the small gel tray can be prepared in the tank, put in the tray, add the end stops and the comb which makes the wells. Otherwise the ends of the tray can be taped up.
6. Pour the agarose into the tray and pop any bubbles using a tip.
7. Allow to set for approximately 30 minutes.
8. Prepare your DNA by adding 8μl of PCR product to 2μl of blue loading buffer.
9. Remove the ends from the set gel and carefully remove the comb. Place the gel in the electrophoresis tank and cover with 1x TBE working solution.
10. Pipette the PCR samples into the wells in the gel and 5μl of Hyperladder I to a free well (markers/ standards).
11. Put the lid on the gel tank remembering that the DNA will move from positive to negative (towards the red electrode). Connect to the power pack and set the voltage to 5 volts for every cm between the electrodes (in the small tank this will be 80 volts).
12. Ensure the gel is running by checking for bubbles coming from the black electrode.
13. Leave for approximately 1 hour, when the blue dye is approximately 1cm from the end of the gel stop the power pack.
14. Move the gel to the staining solution (500ml water plus 50μl ethidium bromide solution, ethidium bromide is harmful wear gloves) and leave for 15 minutes to half an hour.
15. Visualise the DNA over a UV light, the PCR product should appear as a strong band slightly larger than the 100bp band in the standards lane.
16. Estimate The quantity of DNA produced by comparing to a mass ladder or by spectrophotometry. Hyperladder I shows quantities of DNA in each band in ng. Dilute the DNA to 10ng/μl with nuclease free water.

Labelling the probe:

17. Add 20μl of cross-linker solution to 80μl water (this working solution can be stored at 4°C for 1 week).
18. Pipette 10μl of DNA (10ng/μl) in a 0.5ml tube and denature the DNA by heating to 95°C for 5 minutes then place immediately on ice. It important to keep the DNA on ice at all times to maintain the DNA in single stranded form.
19. Centrifuge briefly to collect the DNA at the bottom of the tube.
20. Add 10μl of Reaction buffer.
21. Add 2μl of Labelling Reagent and mix by pipetting.
22. Add 10μl of Cross-Linker working solution (previously prepared), mix by pipetting and centrifuge briefly.
23. Incubate at 37°C for 30 minutes.

This reaction can be scaled up to produce more probe. Labeled probe can be kept on ice for up to 2 hours or for long-term storage aliquots may be stored in 50% glycerol at –20°C.

Dot-blot Hybridisation:

Preparing the membrane

24. Cut a suitable size piece of Hybond N+ nylon membrane; handle the membrane only with powder free gloves or with forceps at a corner. It is advisable to orientate the membrane by cutting off one corner. It may also be useful to mark squares gently onto the membrane with a pencil to provide clear testing areas.
25. For direct tissue blotting cut through a stem of Napier Grass to be tested with a sharp knife or scalpel. Immediately press the cut end onto the nylon membrane. Alternatively for dot blotting cut sections of stem with weight of approximately 0.2g and put into a 1.5ml tube with 10μl of extraction buffer (AMES) (this volume may need to be increased. Grind the stems well with a tube pestle, pipette 5μl of clarified homogenate onto the nylon membrane taking care not to damage the membrane.
26. 5μl of positive control should also be applied to the membrane, a 1 in 1000 dilution of the "nested hae III" plasmid prep can be used as a positive control. A previous positive and negative Napier Grass sample should be added to every membrane for comparison.
27. Fix the DNA to the membrane by baking at 80°C for 2 hours. This time may be reduced to 30 minutes by increasing the temperature to 120°C.

Hybridisation

28. Preheat of hybridisation buffer to 55°C (0.25ml/cm^2 of membrane will be required). Use a minimum of 15ml of hybridization buffer in the hybridization tube to ensure the membrane is well covered.
29. Place membrane in hybridisation buffer for 15 minutes at 55°C in the hybridization oven.
30. Add the labeled probe to the buffer, 5-10ng probe for every ml of buffer, leave in the hybridization buffer at 55°C overnight.

Washing

31. Preheat 200ml Primary Wash Buffer to 55°C.
32. Pour off hybridisation buffer and add 100ml Primary Wash Buffer leave in hybridization oven for 10 minutes at 55°C.
33. Repeat primary wash (step 32).
34. Transfer membrane to 100ml Secondary Wash Buffer in a fresh container.
35. Shake for 5 minutes at room temperature.
36. Repeat secondary wash (steps 34 and 35).

Signal generation and detection

37. Drain off excess wash buffer and place the membrane on a clean non-absorbent flat surface sample side up. Do not allow the membrane to dry out
38. Pipette 30μl of detection reagent for every cm^2 onto the membrane, leave for 2-5 minutes. Drain off excess detection reagent.
39. Wrap blots in cling film or between acetate sheets and smooth out any air pockets, place the membrane DNA side up in the film cassette (ensuring detection reagent is not leaking).
40. Film should not be exposed to light until after the development process is complete; films should be manipulated in a dark room with a safelight.
41. Remove a sheet of film from the packet and cut off one corner to orientate the film. Place on top of the membrane.
42. Close cassette and leave for 4 hours to overnight.
43. Remove film and develop.
44. Light will be produced by the membrane for up to 5 days so further films can be placed on the membrane.

Developing film

45. Prepare developer and fixer as specified by the manufacturer, pours into tanks for developing.
46. Place Film sheets in the developer tank for 5 minutes.
47. Remove film from the developer and rinse in water.
48. Place the film in the fixer tank for 5-10 minutes with moderate agitation.
49. Remove the film from the fixer and rinse in water (preferably running water) for 5-10 minutes.
50. Dry the film in a dust free area at room temperature.

Phytoplasma Detection Reagents and Equipment

Reagents required

- Taq DNA polymerase (eg. Promega 500units cat. No. M1865, other standard Taq would be fine)
- dNTP's
- DNA ladder with a range from approximately 0.2-10kb (e.g. Bioline's Hyperladder I or Promega 1kb DNA ladder)
- Amersham Biosciences AlkPhos Direct Labelling and Detection System with CDP-star RPN3690 £442.00 (for 2500cm^2)
- Hybond N+ Nylon membrane (Amersham)
- Film e.g. Kodak Biomax Light Film (Fisher catalogue number 306/7220/06 £103.02)
- Processing solutions: developer and replenisher (Fisher 43750 4R £13.10) and fixer and replenisher (Fisher 43751 4T £28.49)
- Nuclease free water
- Sodium chloride
- Urea
- Sodium Dodecyl Sulphate (SDS)
- Sodium Dihydrogen Phosphate
- Sodium Hydroxide
- Magnesium Chloride
- Tris base
- Glycerol
- Ethanol
- Sodium Acetate
- Agarose
- Boric Acid
- EDTA
- Ethidium Bromide

Equipment required

- PCR machine
- Gel electrophoresis equipment and agarose/ ethidium bromide
- UV cross-linker or oven capable of heating to 120°C
- Hybridisation oven plus hybridisation tubes
- Dark room
- Light-tight film cassette
- Three trays for developing films