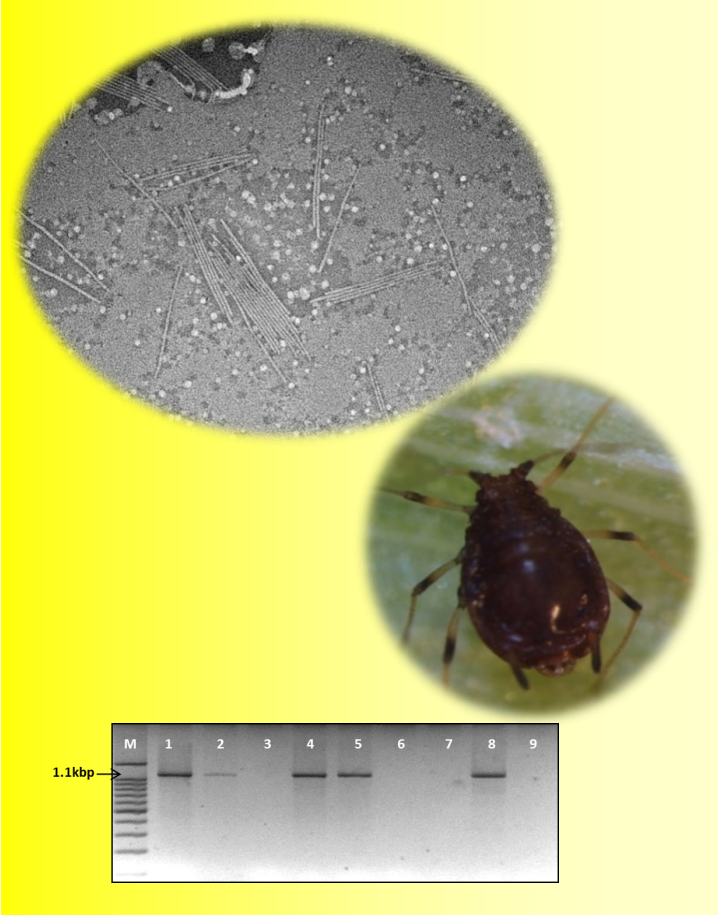
DIAGNOSTICS OF PLANT VIRUSES

TRAINING MANUAL



Training Workshop on Diagnostics of Plant Viruses

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DIAGNOSTICS OF PLANT VIRUSES

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SAMPLE COLLECTION

Materials:

- Disposable gloves
- Scissors
- Collection plastics
- Newspaper/Paper towels
- Marker
- Masking tape/Rubber bands
- Cotton
- Tissue paper
- 70% alcohol

Procedure:

1. Cut sample tissue from the plant with clean scissors.

   Note: Wipe clean scissors with 70% alcohol in every collection of sample.

2. Put in collection plastic with wet cotton. Tie a knot to close or use a rubber band. Another way is to wrap the sample in newspaper or paper towel then secure using a masking tape.

   Note: Properly label each sample. Don't forget to include the place and date of collection.

3. Fill-up sampling details in the collection form.

4. Extract the DNA or RNA immediately after collection or store samples in a refrigerator then process it the next day.

   Note: RNA can be easily degraded, that is why it is important to immediately process the samples. Also some plant tissues oxidized quickly like banana leaves. However, some plant samples can be stored in silica gel like tomato, squash and pepper leaves.

LIST OF PLANT SAMPLES

Sample No.	Crop	Place of Collection	Farmer's or Farm's Name	Variety	Age of Plant (days)	Farm Size	Symptoms	Estimated %Infection	Date of Collection
1	Squash	Tayug, Pangasinan	DA Paraiso	Suprema	120	500 sq.m.	Leaf curling, moscaic	30	Feb 10, 2015

INDIRECT ELISA

Materials:

- Blade or scissors
- Collection plastic (4x6in)
- Paper towels
- Wash Bottle
- Pipettes and Tips
- Disposable gloves

Equipment:

- ELISA Reader
- Weighing balance
- Incubator (Set at 37 °C)

Buffers and Solutions:

- PBS Buffer (pH7.4)
- Coating Buffer (pH9.6)
- Blocking Buffer
- Antibody Buffer
- Washing Buffer (PBS-T)
- Substrate Buffer (pH 9.8)
- Virus-specific antibody
- Goat anti-rabbit enzyme conjugate (GARAP)
- p-ntrophenylphosphatae (pNPP)
- 3M NaOH (Stop solution)

Procedure:

1. Grind leaf samples in coating buffer in a dilution of 1:10.
2. Load 200 µl plant sap per well of the ELISA plate.
3. Incubate overnight at 4 °C.
4. After incubation, remove the plant sap from the wells. Fill the wells with washing buffer; let it stand for 5 min then empty the plates. Repeat 3 times.
5. Add 300 µl blocking solution to each well and incubate at room temperature for 1 hr.
6. Repeat step 4 (Washing).
7. Add 100 µl virus-specific antibody with a dilution of 1:200 in antibody buffer to each well.
8. Incubate at 37 °C for 2-3 hrs.
9. Repeat washing as in step 4.
10. Add 100 µl goat anti-rabbit enzyme conjugate (GARAP) with a dilution of 1:1000 in antibody buffer.
11. Incubate at 37 °C for 2-3 hrs.
12. Repeat washing step.
13. Add 100 µl of p-nitrophenylphosphatase (pNPP) in substrate buffer to each well.
14. Incubate at room temperature for 30-60 min. Observe color reaction.
15. Stop reaction by adding 50 µl 3M NaOH.
16. Assess results by:
    - Visual observation
    - Absorbance Reading at 405 nm using an ELISA Reader.

DOT-BLOT ELISA

Materials:

- Blade or scissors
- Collection plastic (4x6in)
- Paper towels
- Wash Bottle
- Pipettes and Tips
- Disposable gloves

Equipment:

- Weighing balance

Buffers and Solutions:

- Nitrocellulose membrane (45 µm)
- TBS Buffer (pH8.0)
- Blocking Buffer
- Washing Buffer (TBS-Tween 20)
- Substrate Buffer (pH 8.0)
- Virus-specific antibody
- Goat anti-rabbit enzyme conjugate (GARAP)
- Nitro Blue Tetrazolium (NBT)
- 5-bromo-4cholro-3-indolyl phosphate (BCIP)
- 1.5% Sodium hypochlorite (Stop solution)

Procedure:

1. Gently draw a grid pattern on the nitrocellulose membrane (NCM) with a pencil and ruler. Dip membrane onto PBS buffer then air-dry before using.
2. Grind samples at a ratio of 1:20 (w/v) in TBS buffer.
3. Drop 2-3 µl plant sap onto the membrane. Incubate overnight at room temperature.
4. Evenly cover the membrane with blocking buffer in a plastic container with cover then incubate at room temperature for 1 hr.
5. Remove blocking buffer. Dry container with clean paper towel.
6. Add 1:200 (v/v) of virus specific antibody in TBS buffer.
7. Incubate at room temperature for 1-2 hr.
8. After incubation, remove antibody and buffer then rinse 3 times with TBS-T with 10 min interval.
9. Add 1:1000 (v/v) goat anti-rabbit enzyme conjugate (GARAP) in blocking buffer.
10. Incubate at room temperature for 1-2 hr.
11. Remove conjugate buffer. Incubate in TBS buffer at room temperature for 30 min.
12. Remove buffer. Soak membrane in 5 ml substrate buffer with 33 µl NBT and 16.5 µl BCIP.
13. Observe color reaction. After color reaction (purple or blue) developed in the positive check, pour off the substrate buffer.
14. Add 1.5 % sodium hypochlorite and incubate at room temperature for 5 min to eliminate color background and stop the reaction.

COMPOUND ELISA
(BBTV Detection)

Materials:

- Blade or scissors
- Collection plastic (4x6in)
- Paper towels
- Wash Bottle
- Pipettes and Tips
- Disposable gloves

Buffers and Solutions:

- General Extract Buffer (GEB 1X)
- Carbonate Coating Buffer (1X)
- PBST Buffer (Wash Buffer) (1X)
- ECI Buffer (1X)
- PNP Buffer (1X)

Equipment:

- ELISA Reader
- Weighing balance

Procedure:

1. Prepare capture antibody.

   Note: All antibodies and enzyme conjugates should be prepared in a container made of a material such as polyethylene or glass that does not readily bind antibodies. Do not use polysterene, polypropylene or polycarbonate

   Prepare the volume of carbonate coating buffer needed for the test. *Example: If the dilution given on the bottle of concentrated capture antibody is 1:200, and you are preparing 10 ml of the capture antibody solution, you should mix 10 ml of carbonate coating buffer with 50 µl of concentrated capture antibody. Mix the prepared antibody solution thoroughly and use immediately.*

2. Coat plate. Pipette 100 µl of the prepared capture antibody solution into each well.

3. Incubate plate. Cover the plate with cling plastic wrap and incubate in a humid box for 4 hrs at room temperature or overnight in the refrigerator (4°C). *Do not store coated plates longer than 24 hrs.*

4. Wash plate. Empty the wells into a sink or container. Fill the test wells completely with 1X PBST, and then quickly empty them again. Repeat 2 more times.

   Hold the plate upside down and tap firmly on folded paper towel to remove excess liquid or if you have an automated plate washer, calibrate the washer and wash the plate 3x.

5. Grind and dilute the samples. Select samples showing symptoms. Young leaf tissue is recommended. Seed, stem and other tissue can also be tested depending on the crop.

   Grind plant tissue in General Extract Buffer (GEB) at 1:10 ratio (*tissue weight in g: buffer volume in ml*). You can use mortar and pestle or any grinding devices. Be sure to wash and rinse the grinding device thoroughly between samples.

6. Dispense the sample. Following the loading diagram (see sample diagram), dispense 100 µl of prepared sample into sample wells. Dispense 100 µl of positive control into positive control wells, and dispense 100 µl of sample extraction buffer into buffer wells.

7. Incubate plate. Set the plate, cover it with cling wrap and incubate in humid box for 2 hrs at room temperature or overnight in the refrigerator (4°C).

8. Prepare enzyme conjugate.

   Note: always prepare enzyme conjugate within 10 min before use. Bottles of alkaline phosphatase enzyme conjugate and detection antibody are supplied as a concentrate and must be diluted with ECI buffer before use. Please follow the recommended dilution stated in the product.

   Example: If the dilution given on the bottles of concentrated detection antibody (A) and alkaline phosphatase enzyme (B) conjugate is 1:200, and you are preparing 10 ml of enzyme conjugate solution, you should first dispense 10 ml of ECI buffer. Then, add 50 µl of A and 50 µl of B to the ECI buffer.

   After adding the reagents from A and B, mix thoroughly the enzyme conjugate solution well.

9. Wash plate. When sample incubate is complete, wash the plate. Use the quick flipping motion to dump the wells into a sink without mixing the contents. Fill all the test wells completely with 1X PBST, and then quickly empty them. Repeat for 2 more times. After washing tap the plate firmly on folded paper towel to remove all droplets of wash buffer.

   Note: It is important to inspect the test wells. It should be free from any plant tissue and dirt. If plant tissue is present repeat the wash step and tap firmly. Avoid touching the bottom of the plate.

10. Add enzyme conjugate. Dispense 100 µl of prepared enzyme conjugate solution per well.

11. Incubate the plate. Cover the plate with cling wrap and incubate in humid box for 2 hrs at room temperature.

12. Prepare PNP solution. Each PNP tablet will make 5 ml of PNP solution, at a concentration of 1 mg/ml about enough for five 8-well strips. About 15 min before the end of incubation step, measure 5 ml of room temperature 1X PNP buffer for each tablet you will be using. Then without touching the tablets, add the PNP tablets to the buffer.

    Note: Do not touch the PNP tablets or expose the PNP solution to strong light. Light or contamination could cause background color in negative wells.

13. Wash plate. Follow the same wash procedure previously described.

14. Add PNP substrate. Dispense 100 µl of PNP substrate into each testwell.

15. Incubate plate. Cover the plate with cling wrap and incubate in humid box for 60 min at room temperature. Plates should be protected from direct or intense light.

16. Evaluate results.

    Qualitative interpretation. Examine each well by eye, or measure on a plate reader at 405 nm. Air bubbles should be removed; it can alter results at the time of reading.

    Wells in which color develops indicate positive results. Wells in which there is no significant color development indicate negative result. Test results are valid only if positive control wells give a positive result and buffer wells remain colorless.

    Results may be interpreted after more than 60 min of incubation as long as negative wells remain virtually clear.

    Quantitative interpretation. Appropriate controls should be included for reference, since ELISA values may differ in different microtiter plates due to possible plate-to-plate variation in sensitivity. Overlapping range of specific and non-specific reaction values causes difficulty in interpretation. In this case, it is necessary to include large number of known healthy control samples and determine statistically a threshold level for infection. *To establish thresholds, several authors have used the mean value for healthy controls plus three times their standard deviation ($\bar{x}$ + 3SD).*

    Alternatively, values more than twice those healthy controls have been considered infected.

TOTAL PLANT DNA EXTRACTION

(Dellaporta Miniprep)

Materials:

- Blade or scissors
- Mortar and pestle (sterilized)
- 1.5 mL Eppendorf tubes
- Pipettes and Tips
- Face mask
- Disposable gloves
- Paper towels

Equipment:

- Fume hood
- Water bath or Dri-bath incubator
- Centrifuge
- Vortex
- Weighing balance

Buffers and Solutions:

- Nitrocellulose membrane (45 µm)
- Dellaporta extraction buffer
- 20% Sodium Dodecyl Sulfate (SDS)
- 5M Potassium acetate (KAC)
- Isopropanol
- 80% ethanol
- Sterile distilled water or TE buffer

Procedure:

1. Collect 2 leaf discs about 0.9cm in diameter.
2. Homogenize in 500 µl Dellaporta extraction buffer using a mortar and pestle.
3. Transfer in a 1.5 ml microfuge tube then add 33 µl 20% SDS. Gently invert tube to mix the solution.
4. Incubate at 65°C for 10min.
5. Add 160 µl 5M KAC and mix by gently inverting the tube.
6. Centrifuge at 13,000 rpm for 10 min.
7. Transfer supernatant into a new 1.5 ml microfuge tube, avoiding the plant tissue debris.
8. Repeat centrifugation. Transfer 500µl supernatant into a new tube.
9. Add 0.5 volume (250 µl) isopropanol and invert tube gently.
10. Centrifuge at 13,000 rpm for 10min.
11. Carefully discard supernatant
    Note: Make sure that the pellet does not become aspirated. It may be necessary to leave some (about 15 µl) supernatant behind. The following step will delete any problems it may cause.
12. Add 500 µl 80% ethanol and centrifuge at 13,000 rpm for 5min.
13. Carefully discard as much supernatant as possible.
14. Air-dry for 1hr or speed-vacuum for 5min.
15. Resuspend pellet in 500 µl sterile distilled water or DEPC-treated water.

CTAB DNA EXTRACTION

(For Banana Samples)

Materials:

- Blade or scissors
- Mortar and pestle (sterilized)
- 1.5 mL Eppendorf tubes
- Pipettes and Tips
- Face mask
- Disposable gloves
- Paper towels

Buffers and Solutions:

- CTAB extraction buffer
- 2-mercaptoethanol
- Phenol:Chloroform:Isoamyl (PCI) 25:24:1
- 95% ethanol
- 70% ethanol
- Sterile distilled water or TE buffer

Equipment:

- Fume hood
- Water bath or Dri-bath incubator
- Centrifuge
- Vortex
- Weighing balance

Procedure:

1. Clean working station and pipettors with 70% alcohol.
2. Cut 0.5 g leaf sample using sterile blade or clean scissors.
3. Homogenize leaf sample using a sterile mortar and pestle with 4 mL CTAB extraction buffer and 8 µl 2-mercaptoethanol.
4. Transfer 500 µL leaf extract in a sterile 1.5 mL sterile microfuge tube.
5. Incubate in a water bath at 60 °C for 1 hr. Gently swirl tubes every 15 min.
6. After incubation, add 400 µL Phenol:Chloroform:isoamyl (PCI 25:24:1). Mix by vortexing for 15 sec.
7. Centrifuge at 10,000 rpm for 5 min.
8. Collect 500 µL of the upper phase using a wide-bore tip and transfer it to a new 1.5 mL microfuge tube.
9. Precipitate the DNA by adding 2 volumes of ice-cold 95% ethanol. Mix well by gently inverting the tube. Incubate in the freezer for 1 hr or overnight.
10. Centrifuge at 10,000 rpm for 15 min.
11. Discard the supernatant. Wash the pellet with 500 µL ice-cold 70% ethanol and gently invert the tube for 3-5 min.
12. Short spin then discard the ethanol. Air-dry the pellet for 2-3 min at room temperature in the laminar flow or until the wall of the tube is already dry.
13. Resuspend the pellet in 100 uL TE buffer or DNAse-free water.
14. Store at -20°C.

RNA EXTRACTION

Materials:

- Blade or scissors
- Mortar and pestle (sterilized)
- 1.5 mL Eppendorf tubes
- Pipettes and Tips
- Face mask
- Disposable gloves
- Paper towels

Buffers and Solutions:

- Trizol® Reagent (Invitrogen)
- Chloroform
- Isopropanol
- 75% ethanol
- RNAse-free water or TE buffer
- RNAse Away
- 70% alcohol

Equipment:

- Fume hood
- Water bath or Dri-bath incubator
- Refrigerated centrifuge
- Vortex

Procedure:

1. Clean working station and pipettors with 70% alcohol or RNAse Away.
2. Homogenize about 100 mg leaf sample in 1 ml Trizol® Reagent using an ice-cold mortar and pestle.
3. Incubate at room temperature for 5 min.
4. Centrifuge at 12,000xg for 10 min at 8°C.
5. Transfer the supernatant using a wide-bore tip into a new 1.5 ml microfuge tube then add 200 µl chloroform.
6. Shake vigorously by hand for 15 sec.
7. Incubate at room temperature for 3 min then centrifuge at 12,000xg for 10 min at 8°C.
8. Collect the aqueous phase into a new 1.5 ml microfuge tube then add 500 µl ice-cold isopropanol.
9. Incubate at -20 °C for 2 hrs or overnight if precipitate fails to form.
10. Discard supernatant and wash pellet with ice-cold 75 % ethanol.
11. Centrifuge at 7,500xg for 5 min at 8°C.
12. Discard ethanol then air-dry pellet for 10 min in a laminar flowhood.
13. Resuspend pellet in 50 µl RNAse-free water or TE buffer.
14. Incubate at 55 °C for 10 min before storing at -20 °C or in a biofreezer (-80 °C).

GEL ELECTROPHORESIS

Materials:

- Casting tray
- Gel comb
- Pipettes and Tips
- Disposable gloves

Buffers and Solutions

- 0.5X TBE
- Agarose
- GelRed
- 1 KB plus DNA Ladder

Equipment:

- Gel electrophoresis system
- Weighing balance
- Microwave oven

Procedure:

1. Prepare 1.2% agarose gel in 0.5X TBE buffer.
2. Completely dissolve the agarose in the buffer using a microwave.
3. Let the solution to slightly cool down (~5 min).
4. Pour the solution slowly into the casting tray with the gel comb in place. Avoid forming any bubbles.
5. Let the agarose gel to solidify (~30 min) then carefully remove the gel comb.
6. Place the solidified agarose gel into the electrophoresis unit. Fill in with 0.5X TBE buffer until the gel is fully submerged.
7. Pipette several 2 µl loading dye (depending on the number of samples) in a piece of parafilm.
8. Mix 5 µl PCR product into the dye by carefully pipetting the solution in and out of the tip. Avoid forming of bubbles.
9. Load the sample mixture into each well of the gel. Avoid spilling over the sides of the wells to prevent contamination.
   Note: Start loading samples on the second well.
10. Lastly, load 2 µl DNA ladder on the first well.
11. Run samples for 30-45 min or until the dye has migrated about 75-80% of the gel.
12. After the run, stain the gel by submerging it in the GelRed solution for about 15-30 min.
13. Visualize the amplified DNA bands using a gel documentation system.

GEL DOCUMENTATON AND ANALYSIS OF THE RESULTS

Materials:

- Disposable gloves
- Kimwipes
- Paper towel or tissue

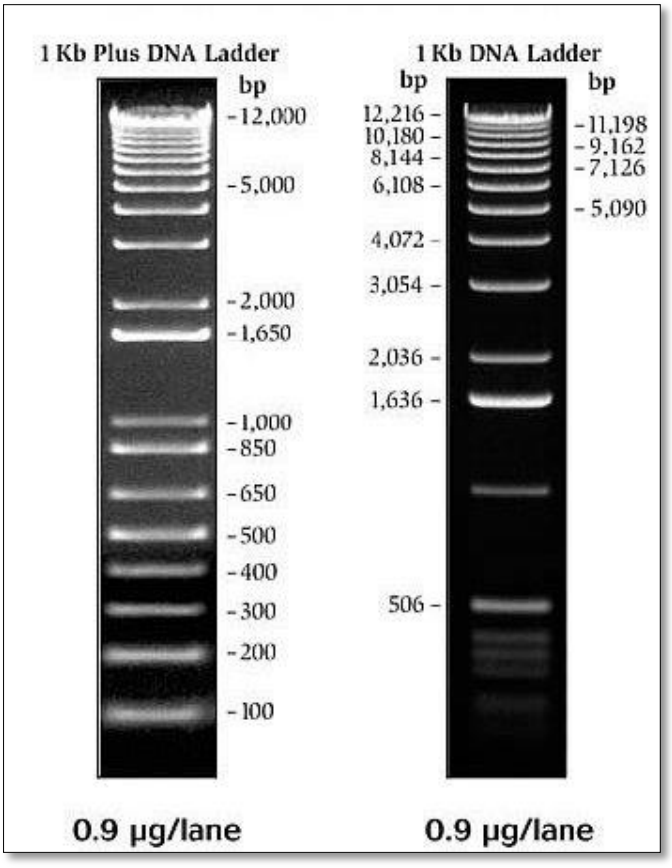
Equipment:

- GelDoc
- Computer

Procedure:

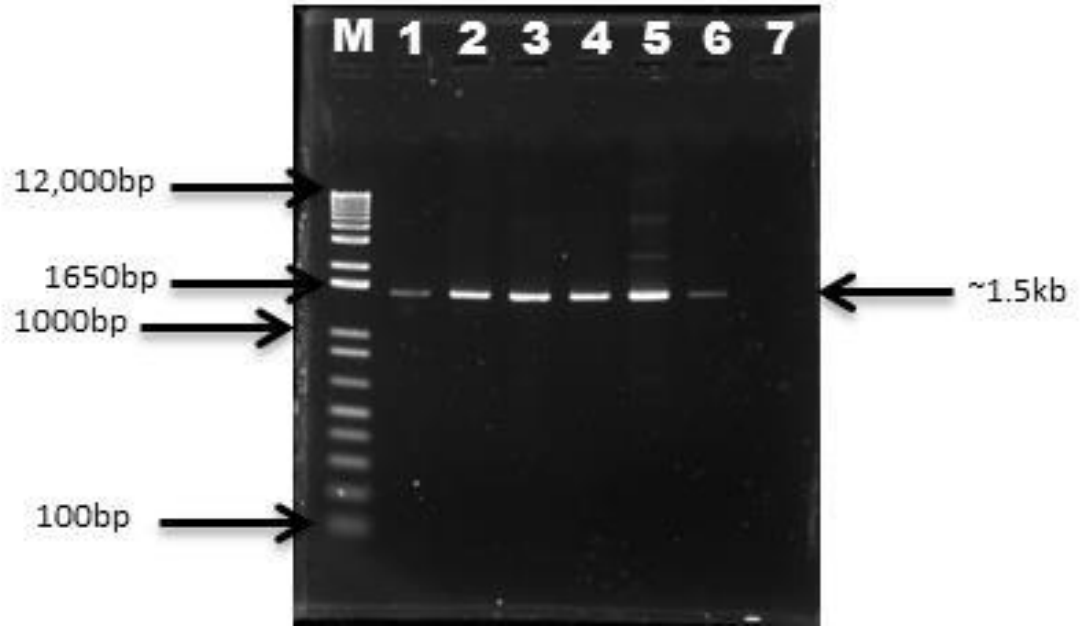
1. After staining the gel, the PCR result can be viewed using a gel documentation system or GelDoc.

2. Analyze the result using the DNA ladder.



Example of DNA ladders (Invitrogen)

3. If the PCR run is successful, the PCR product is within the expected DNA sequence size.



Agarose gel showing the expected band size of around 1.5kb after PCR amplification.

4. The presence of the expected band, confirms the presence of the virus.

   Note: It is important that in every gel electrophoresis for PCR analysis, ***DO NOT FORGET TO INCLUDE THE DNA LADDER.***

POLYMERASE CHAIN REACTION (PCR)

Materials:

- 0.2ml PCR tubes
- 1.5 ml microfuge tubes
- 70% ethanol
- Pipettes and Tips
- Disposable gloves
- Paper towel or tissue

Equipment:

- PCR machine
- Centrifuge

Buffers and Solutions

- 10X PCR buffer
- 50 mM $MgCl_2$
- 10 mM dNTPs
- 10 µM forward and reverse primers
- RNAse-free water
- Taq Polymerase
- Template DNA

Procedure:

1. Before starting:
    a. Calculate the amount of the PCR reagents needed according to the number of samples to be tested. Give allowance for pipetting error.
    b. Clean working bench, pipettors and microfuge rack with 70% ethanol.
    c. Thaw reagents (PCR buffer, $MgCl_2$, dNTPs, forward and reverse primers, RNAse-free water and DNA samples) on ice.
    d. Flick tubes to mix its content then short spin.
    e. Prepare and label 0.2ml PCR tubes.
2. Prepare a cocktail mix. Add all the PCR reagents in appropriate amounts in a 1.5 ml microfuge tube.
3. Lastly add the Taq polymerase to the cocktail.
4. Gently flick the tube to mix the reagents then short spin the tube using a centrifuge.
5. Dispense the PCR mix on each labeled 0.2 ml PCR tube. Add the DNA template then gently mix the contents.
6. Place tubes in the thermal cycler then begin run with the appropriate PCR program.

PCR Troubleshooting

CAUSE	SOLUTION
Problem: Faint bands or no PCR product	
a. Too little DNA template in the reaction	Increase the amount of the DNA template.
b. Damaged or degraded DNA template	Assure the purity and integrity of the DNA template. Be careful in handling DNA. Prevent freeze-thawing of the DNA samples by preparing aliquots.
c. Insufficient *Taq* Polymerase	Increase the DNA polymerase concentration in increments of 0.5 units per 100 µL of reaction.
d. Insufficient number of cycles	Increase cycle number by 5 to 10 cycles.
e. Presence of PCR inhibitors	Re-purify DNA samples.
f. To low $MgCl_2$ concentration	Increase magnesium concentration in increments of 0.1mM.
g. Too long or too short denaturation time.	Adjust denaturation time in increments of 5sec.
h. Too high annealing temperature	Lower annealing temperature in increments of 2°C. Compute the melting temperature (T_m) of the primers. The annealing temperature should be 5°C less to the primer T_m.
i. The primer extension period is too short.	Increase extension time in increments of 1 minute.
Multiple bands or smearing	
a. Too much DNA template	Decrease the amount of template DNA
b. Too low annealing temperature	Increase annealing temperature in increments of 2°C.
c. Too high concentration of *Taq* Pol	Decrease enzyme concentration in increments of 0.5 units per 100-µL reaction.
d. Magnesium concentration is too high.	Decrease the magnesium concentration in increments of 0.1mM.
e. Denaturation time is too short or too low	Increase the denaturation time in increments of 5sec. and temperature by 1°C.
f. Too many cycles	Reduce the cycle number by 5 to 10 cycle.
g. Extension time is too long	Reduce the extension time in increments of 1min.
h. Review primer design and composition.	Design new primers.

PCR Cocktail & Thermal Profile Using Degenerate Primers for *Begomoviruses*

1. PCR Cocktail

PCR Cocktail Preparation			
Components	**Stock Concentration**	**Final Concentration**	**1x**
DEPC-treated Water			17.65
PCR Buffer	10x	1x	2.5
$MgCl_2$	50mM	2.5 mM	1.25
dNTPS	10mM	0.2 mM	0.5
Primer F	10µM	0.5 µM	0.5
Primer R	10µM	0.5 µM	0.5
Taq	5U/µl	0.06	0.1
DNA			2
Total Reaction Volume			25 µl
Aliquot 23µl to each DNA tube			

2. PCR Profile

Steps	Temperature (°C)	Time	Number of Cycles
Denaturation	94	1 min	
Annealing	57	2 min	30x
Elongation	72	2 min	
Final Extension	72	10 min	1
Hold	16	∞	-

3. Primer Sequence (amplifies the fragment of DNA-A including the 5' end of CI, IR, V2 and the 5' end of the coat protein (CP), Tsai etal., 2011)

Primer Name	Sequence	Amplicon Size (bp)
PAL1v1978B	GCATCTGCAGGCCCACATBGTYTTHCCNGT	~1.5 Kb
PAR1c715H	GATTTCTGCAGTTDATRTTHTCRTCCATCCA	

PCR Cocktail & Thermal Profile for *Banana bunchy top virus* (BBTV)

1. PCR Cocktail

PCR Cocktail Preparation			
Components	**Stock Concentration**	**Final Concentration**	**1x**
DEPC-treated Water			17.65
PCR Buffer	10x	1x	2.5
$MgCl_2$	50mM	2.5 mM	1.25
dNTPs	10mM	0.2 mM	0.5
Primer F	10µM	0.5 µM	0.5
Primer R	10µM	0.5 µM	0.5
Taq	5U/µl	0.06	0.1
DNA			2
Total Reaction Volume			25 µl
Aliquot 23µl to each DNA tube			

2. PCR Profile

Steps	Temperature (°C)	Time	Number of Cycles
Initial Denaturation	94	4min	1
Denaturation	94	1 min	29x
Annealing	61	1 min	
Elongation	72	2 min	
Final Extension	72	10 min	1
Hold	16	∞	-

3. Primer Sequence (amplifies the BBTV-R genome)

Primer Name	Sequence	Amplicon Size (bp)
D11	GGAAGAAGCCTCTCATCTGCTTCAGACARC	~1.1 Kb
D12	TTCCCAGGCGCACACCTTGAGAAACGAAAG	

RT-PCR/PCR Cocktail & Thermal Profile Using Degenerate Primers for *Potyviruses*

1. RT-PCR Cocktail

PCR Cocktail Preparation			
Components	**Stock Concentration**	**Final Concentration**	**1X**
DEPC-treated Water			2.2
Reaction Mix	2X	1X	5
Primer F	10µM	0.5 µM	0.2
Primer R	10µM	0.5 µM	0.2
SuperScript III RT			0.4
RNA			2
Total Reaction Volume			10 µl
Aliquot 8 µl to each DNA tube			

2. PCR Profile

Steps	Temperature (°C)	Time	Number of Cycles
cDNA Synthesis	55	30 min	1
Initial Denaturation	94	2 min	1
Denaturation	94	15 sec	
Annealing	55	30 sec	40x
Elongation	68	1 min	
Final Extension	68	5 min	1
Hold	16	∞	-

3. Primer Sequence (amplifies the 3' terminal portion of the genomes of various *potyviruses*, Gibbs and Mackenzie, 1997)

Primer Name	Sequence	Amplicon Size (bp)
Potyvirid 1	CACGGATCCCGGG(T)17VGC	~1.6 Kb
Potyvirid 2	ACCACAGGATCCGGBAAYAAYAGYGGDCARCC	

RT-PCR/PCR Cocktail and Thermal Profile for *Papaya ringspot virus* (PRSV)

1. RT-PCR Cocktail

PCR Cocktail Preparation			
Components	**Stock Concentration**	**Final Concentration**	**1X**
DEPC-treated Water			2.2
Reaction Mix	2X	1X	5
Primer F	10µM	0.5 µM	0.2
Primer R	10µM	0.5 µM	0.2
SuperScript III RT			0.4
RNA			2
Total Reaction Volume			10 µl
Aliquot 8 µl to each DNA tube			

2. PCR Profile

Steps	Temperature (°C)	Time	Number of Cycles
cDNA Synthesis	55	30 min	1
Initial Denaturation	94	2 min	1
Denaturation	94	15 sec	
Annealing	58	30 sec	40x
Elongation	68	1 min	
Final Extension	68	5 min	1
Hold	16	∞	-

3. Primer Sequence (amplifies the PRSV-CP gene, Bateson etal., 1994)

Primer Name	Sequence	Amplicon Size (bp)
MB11	GGATCCATGTCCAAAAATGAAGCTGTGGATGCT	~900 bp
MB12	TCAATTGGCGCATACCCAGGAGAGT	

MECHANICAL INOCULATION

Materials:

- Mortar and pestle
- Wash bottle
- Carborundum (500mesh)/Celite
- Disposable gloves
- Labels and water resistant marker
- Detergent soap

Buffers and Solutions

- 0.01M Phosphate Buffer pH 7.2
- Sodium sulphite (2%)

Procedure:

1. Arrange plants to be inoculated.
2. Homogenize infected leaf sample in 0.01M Phosphate buffer at 1:10 dilution using a mortar and pestle. Add 2% Sodium sulphite.
3. Add a small amount of celite (0.5-1% w/v) onto the inoculum or spread small amount of carborundum into the leaf to be inoculated.
4. Moisten two fingers with the inoculum then gently rub onto the first and second fully expanded leaves while supporting it with the other hand.
5. Rinse inoculated plants with tap water within 2-5 min.
6. Observe test plants for symptoms at least twice a week for a month.
7. Label with date of inoculation and name of virus inoculation.

Note: To prevent cross contamination, change or wash thoroughly gloves between samples. Also separate test plants of different samples.

Optimum stage of most common test plant species.

Test plant species	Number of leaves	Remarks
Abelmoschus esculentus	1-2	
Capsicum annuum	2-3	
Chenopodium amaranticolor	3-4	for local symptoms only
Chenopodium quinoa	3-4	for local symptoms only
Cucumis sativus	2 cotyledons	remove leaves, except top leaf
Cucurbita maxima	2 cotyledons	
Datura metel	2-3	
Gomphrena globosa	about 6	
Gossypium herbaceum	1-2	
Nicotiana benthamiana	3-4	
Nicotaina glutinosa	3-4	
Nicotiana tabacum 'Xanthii'	1-2	
Solanum lycopersicum 'Money-maker'	1-2	
Solanum melongena	1-2	
Vigna unguiculata	2 cotyledon	

Source: www.q-bank.eu

INSECT TRANSMISSION OF VIRUS

I. Non - Persistent Mode of Transmission

Materials:

- Insect vector, *Aphis gossypii*
- Test plants
- Symptomatic plants/Inoculum
- Camel hair brush
- Close container with screen window

Procedure:

1. Starve the non-viruliferous insect vectors for 30 minutes before virus acquisition.

2. Place the starved insect vectors to symptomatic host plant for 15-20 minutes to acquire virus.

3. Transfer the viruliferous insect vectors (15-20 aphids) to the uninfected/clean test plant and allow the insects to feed and transmit the virus for 1-2 hours.

4. Manually remove or eliminate the insects by spraying insecticide.

5. Transfer the test plants to insect-free/insect-proof cages.

6. After 1-2 months, check the test plants for virus infection symptom/s and collect samples for ELISA and/or PCR tests.

INSECT TRANSMISSION OF VIRUS

IIa. Persistent Mode of Transmission (*Banana bunchy top virus*)

Materials:

- Insect vector, *Pentalonia nigronervosa*
- Test plant, 1-mo old banana seedling
- Symptomatic plants/Inoculum
- Camel hair brush
- Close container with screen window

Procedure:

1. Starve the non-viruliferous insect vectors for 30 minutes before virus acquisition.

2. Place the starved insect vectors to symptomatic host plant for 24 hours to acquire virus.

3. Transfer the viruliferous insect vectors (15-20 aphids) to the uninfected/clean test plant and allow the insects to feed and transmit the virus for 24 hours.

4. Manually remove or eliminate the insects by spraying insecticide.

5. Transfer the test plants to insect-free/insect-proof cages.

6. After 1-2 months, check the test plants for virus infection symptom/s and collect samples for ELISA and/or PCR tests.

Note: In the case of Banana bunchy top virus, symptom expression may take (up to 3 months) depending on the susceptibility of the host plant.

INSECT TRANSMISSION OF VIRUS

IIb. Persistent Circulative Mode of Transmission (*Tomato leaf curl virus*)

Materials:

- Aviruliferous whiteflies, *Bemisia tabaci*
- ToLCV infected tomato (source of inoculum)
- Tomato seedlings (healthy)
- Aspirator
- Screen cages

Procedure:

1. Collect/aspirate whiteflies and allow having access on ToLCV infected tomato plant for 24-48 hours.

2. Remove whiteflies from the source and transfer 10-20 whiteflies to healthy tomato seedling inside insect proof screen cages.

3. Allow the viruliferous whiteflies to feed on test plants for 48 hours then remove or spray insects with insecticide.

4. Observe the inoculated plants for virus symptoms (leaf curl) after 3-4 weeks until 2 months and collect samples for PCR.

BUFFERS AND SOLUTIONS

Indirect ELISA

PBS Buffer (pH7.4)

Sodium Chloride (NaCl)	8 g
Monobasic Potassium Phosphate (KH_2PO_4)	0.2 g
di-Sodium hydrogen Orthophosphate ($Na_2HPO_4x12H_2O$)	1.44 g
Potassium Chloride (KCl)	0.2 g
Distilled water	1000 ml

Coating Buffer (pH9.6)

Sodium Carbonate (Na_2CO_3)	1.5 g
Sodium Hydrogen Carbonate ($NaHCO_3$)	2.93 g
Distilled water	1000 ml

Washing Buffer (PBS-T)

PBS Buffer	1000 ml
Tween 20	0.05%

Blocking Buffer (must be freshly prepared)

PBS Buffer	500 ml
BSA or skim milk	1%

Antibody Buffer (must be freshly prepared)

PBS Buffer	100 ml
Egg albumin	0.2%

Substrate Buffer (pH 9.8)

Diethanolamine	97 ml
Distilled water	800 ml

Adjust pH to 9.8 then volume to 1000 ml.

Dot Blot ELISA

TBS Buffer (pH8.0)

Tris	6.057 g
Distilled water	800 ml
Adjust pH to 8.0.	
NaCl	8.766 g
Volume to 1000 ml.	

Washing Buffer (TBS-Tween 20)

TBS Buffer	1000 ml
Tween 20	0.5 ml

Blocking Buffer

Skim Milk	3 g
Glycine	2 g
TBS-Tween 20	100 ml

Substrate Buffer (pH 8.0)

Tris	1.214 g
Dissolve in 80 ml distilled water then adjust pH to 8.0.	
NaCl	0.5844 g
$MgCl_2x6H_2O$	0.102 g
Volume up to 100 ml.	

Nitro Blue Tetrazolium (NBT) Solution

Nitro Blue tetrazolium chloride	0.5 g
Dimethylformamide (70%)	10 ml
Store at 4°C.	

5-bromo-4-chloro-3-indolyl phosphate (BCIP)

Disodium salt BCIP	0.5 g
Dimethylformamide (100%)	10 ml

Compound ELISA

General Extract Buffer (GEB 1X)

Dissolve in 1000 ml of 1X PBST:

Sodium sulfite (anhydrous)	1.3g
Polyvinylpyrrolidone (PVP) MW 24-40,000	20g
Sodium azide	0.2g
Powdered egg (chicken) albumin, Grade II	2g
Tween-20	20g

Adjust pH to 7.4. Store at 4°C

Carbonate Coating Buffer (1X)

Dissolve in distilled water to 1000 ml

Sodium carbonate (anhydrous)	1.59g
Sodium bicarbonate	2.93g
Sodium azide	0.2g

Adjust pH to 9.6. Store at 4°C

PBST Buffer (Wash Buffer) (1X)

Dissolve in distilled water to 1000 ml:

Sodium chloride	8g
Sodium phosphate, dibasic (anhydrous)	1.15g
Potassium phosphate, monobasic (anhydrous)	0.2g
Potassium chloride	0.2g
Tween-20	0.5g

Adjust pH to 7.4

ECI Buffer (1X)

Add to 1000 ml 1X PBST:

Bovine serum albumin (BSA)	2g
Polyvinylpyrrolidone (PVP) MW 24-40,000	20g
Sodium azide	0.2g

Adjust pH to 7.4 Store at 4°C

PNP Buffer (1X)

Dissolve in 800 ml distilled water:

Magnesium chloride hexahydrate	0.1g
Sodium azide	0.2g
Diethanolamine	97g

Adjust pH to 9.8 with hydrochloric acid.

Adjust final volume to 1000 ml with distilled water. Store at 4°C

Total DNA Extraction

STOCK SOLUTIONS

1 M Tris-Base

Tris-Base	60.55 g
Distilled water	500 ml

0.5 M EDTA

EDTA	18.61 g
Distilled water	100 ml

5 M NaCl

Sodium chloride (NaCl)	29.22 g
Distilled water	100 ml

Dellaporta Extraction Buffer

Stock Concentration	Final Concentration	100 ml	500 ml
1M Tris-Base pH 8.0	100 mM	10 ml	50 ml
0.5M EDTA	8.5 mM	1.7 ml	8.5 ml
5 M NaCl	500 mM	10 ml	50 ml
Distilled Water	-	78.3 ml	391.5 ml
Sterilize			
2-mercaptoethanol	10 mM	78 µl	391 µl

5M Potassium Acetate (KAC)

KAC	49.07 g
Distilled water	100 ml

Sterilize

20 % SDS

Sodium dodecyl sulphate (SDS)	10 g
Distilled water	100 ml

Sterilize

CTAB Extraction Buffer

Stock Concentration	Final Concentration	100 ml	500 ml
1M Tris-HCl pH 8.0	0.1 M	10 ml	50 ml
0.5M EDTA	0.02 M	4 ml	20 ml
5 M NaCl	1.4 M	28 ml	140 ml
CTAB	2% (w/v)	2 g	10 g
Distilled Water	-	58 ml	290 ml

Sterilize

TE Buffer

Stock Concentration	Final Concentration	100 ml	1 L
1M Tris-HCl pH 7.5	10 mM	1 ml	10 ml
0.5M EDTA pH 8.0	1 mM	200 µl	2 ml
Distilled Water	-	98.8 ml	988 ml

Sterilize.

Gel Electrophoresis

10x TBE Buffer

Tris Base	54.5 g
Boric Acid	27.2 g
0.5 M EDTA	20 ml
Distilled water	480 ml

Note: TAE can also be used.

0.5x TBE Buffer

10x TBE buffer	50 ml
Distilled water	950 ml

10X Loading Dye

Sucrose	6.5 g
1M Tris-HCl pH 7.5	100 µl
0.5M EDTA	200 µl
Bromophenol Blue	0.03 g
Distilled water	9.7 ml

6X Loading Dye

10X Loading dye	600 µl
Distilled water	400 µl

1Kb plus DNA Ladder (0.1 µg/ml)

DNA Marker (1 µg/ml)	50 µl
6X Loading Dye	85 µl
Sterile distilled water	365 µl

1.2% Gel

Agarose	0.48 g
0.5X TBE buffer	40 ml

Melt agarose using a microwave.

GelRed Solution

5M NaCl*	2 ml
GelRed	30 µl
Distilled water	98 ml

*optional

**or add 4 µl GelRed directly to the melted agarose gel (40 ml)

Note: Avoid exposing the solution in light as GelRed is light sensitive.

PCR

10mM dNTPs

100mM ATP	10 µl
100mM TTP	10 µl
100 mM GTP	10 µl
100 mM CTP	10 µl
RNAse-free water	60 µl

Reconstitution of primers

1. Centrifuge tubes for a few seconds then add the appropriate volume of TE buffer.
2. Rehydrate for 2 min then vortex for 15 sec.
3. Short spin to collect contents in the bottom. Store at -20 °C.

Mechanical Inoculation

Phosphate Buffer

Solution A KH_2PO_4	1.36 g
Distilled water	1000 ml
Solution B $Na_2HPO_4 \times 2H_2O$	1.78 g
Distilled water	1000 ml

0.01M Phosphate Buffer, pH 7.2

Solution A	51 ml
Solution B	49 ml

Phospahte Buffer (10X)

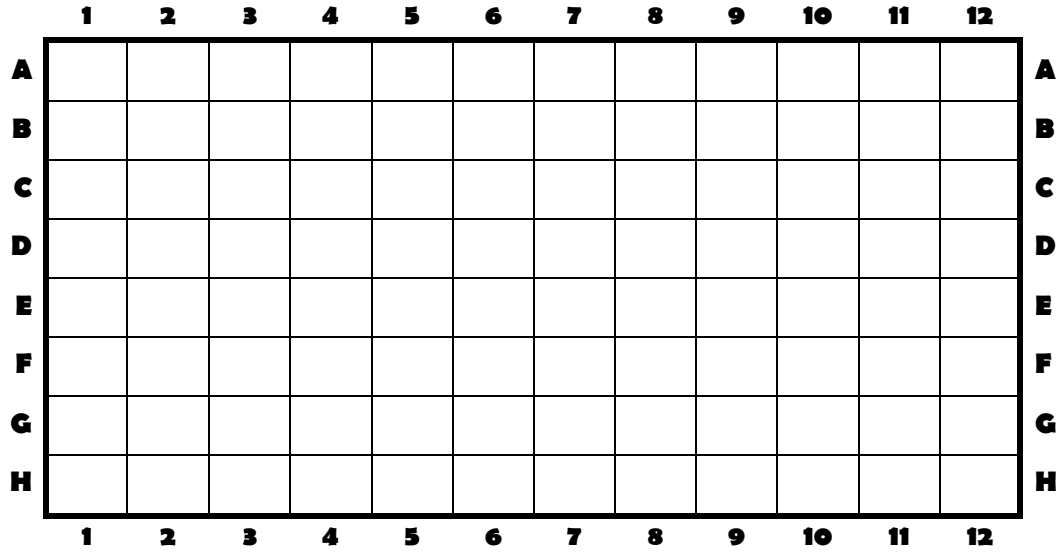
KH_2PO_4	2.72 g
$Na_2HPO_4 \times 2H_2O$	14.2 g
Distilled water	800 ml
Adjust pH to 7.2 then volume to 1000 ml	

0.01M Phosphate Buffer, pH 7.2

Phosphate buffer (10X)	100 ml
Distilled water	900 ml

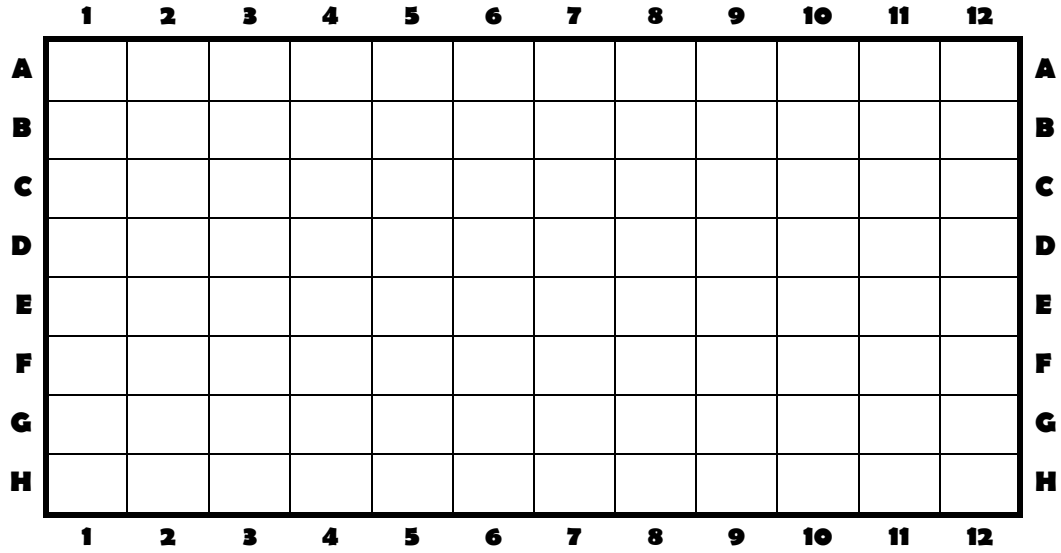
ELISA Form

Type of ELISA: _____ Date: _____
Antibody: _____ Plate Number: _____
Crop: _____ Performed by: _____



REMARKS: _____

Type of ELISA: _____ Date: _____
Antibody: _____ Plate Number: _____
Crop: _____ Performed by: _____



REMARKS: _____

PCR Form for Begomoviruses

PCR Experimental Set-up for Begomoviruses				
Date:	Ref. Expt. #:		Ref. Gel #:	
Title/Description:			Thermalcycler:	
Time Start:	Time End:		Total Run Time:	
Test Parameters				
PCR Profile:			**DNA Samples:**	
Denaturation 94°C 1 min Annealing 57°C 2 min Elongation 72°C 2 min Final Extension 72°C 10 min		30x		
Primer Pair: PAL1v1978B/PAR1c715H				
Expected Product Size: ~1.5kb				
Target Gene(s): partial DNA-A				

PCR Cocktail Preparation				
Components	**[Stock]**	**[Final]**	**1x=25µl**	
DEPC Water			17.65	
PCR Buffer	10x	1x	2.5	
MgCl2	50mM	2.5mM	1.25	
dNTPS	10mM	0.2mM	0.5	
Primer F	10µM	0.2µM	0.5	
Primer R	10µM	0.2µM	0.5	
Taq			0.1	
DNA			2	
Total Reaction Volume			25	
Aliquot **23µl** to each DNA tube				

Gel Electrophoresis		**Notes/Remarks:**
%Gel:	Voltage:	
Run Time:	Staining Time:	

Documentation
Set-up by:

PCR Form for BBTV

PCR Experimental Set-up for BBTV				
Date:	Ref. Expt. #:		Ref. Gel #:	
Title/Description:			Thermalcycler:	
Time Start:	Time End:		Total Run Time:	
Test Parameters				
PCR Profile:		**DNA Samples:**		
Initial Denaturation 94°C 4 min				
Denaturation 94°C 1 min ┐				
Annealing 61°C 1 min ├ 29x				
Elongation 72°C 2 min ┘				
Final Extension 72°C 10 min				
Primer Pair: D11/D12				
Expected Product Size: ~1.1kb				
Target Gene(s): DNA-R				
PCR Cocktail Preparation				
Components	**[Stock]**	**[Final]**	**1x=25µl**	
DEPC Water			17.65	
PCR Buffer	10x	1x	2.5	
MgCl2	50mM	2.5mM	1.25	
dNTPS	10mM	0.2mM	0.5	
Primer F	10µM	0.2µM	0.5	
Primer R	10µM	0.2µM	0.5	
Taq			0.1	
DNA			2	
Total Reaction Volume			25	
Aliquot **23µl** to each DNA tube				
Gel Electrophoresis		**Notes/Remarks:**		
%Gel:	Voltage:			
Run Time:	Staining Time:			
Documentation				
Set-up by:				

RT-PCR Form for Potyviruses

PCR Experimental Set-up for Potyviruses				
Date:	Ref. Expt. #:		Ref. Gel #:	
Title/Description:			Thermalcycler:	
Time Start:	Time End:		Total Run Time:	
Test Parameters				
PCR Profile:		**DNA Samples:**		
cDNA Synthesis	55°C 30min			
Initial Denaturation	94°C 2 min			
Denaturation	94°C 15 sec			
Annealing	55°C 30sec			
Elongation	68°C 1 min			
	40x			
Final Extension	68°C 5 min			
Primer Pair: Potyvirid1/Potyvirid2				
Expected Product Size: ~1.6kb				
Target Gene(s): 3' terminal portion of the genome				
PCR Cocktail Preparation				
Components	**[Stock]**	**[Final]**	**1x=10µl**	
DEPC Water			2.2	
Reaction mix	2x	1x	5	
Primer F	10µM	0.5µM	0.2	
Primer R	10µM	0.5µM	0.2	
Superscript III RT			0.4	
RNA			2	
Total Reaction Volume			10	
Aliquot **8µl** to each DNA tube				
Gel Electrophoresis		**Notes/Remarks:**		
%Gel:	Voltage:			
Run Time:	Staining Time:			
Documentation				
Set-up by:				

RT-PCR Form for PRSV

PCR Experimental Set-up for PRSV				
Date:	Ref. Expt. #:		Ref. Gel #:	
Title/Description:			Thermalcycler:	
Time Start:	Time End:		Total Run Time:	
Test Parameters				
PCR Profile:		**DNA Samples:**		
cDNA Synthesis	55°C 30min			
Initial Denaturation	94°C 2 min			
Denaturation	94°C 15 sec			
Annealing	58°C 30sec			
Elongation	68°C 1 min			
Final Extension	68°C 5 min			
Primer Pair: MB11/MB12				
Expected Product Size: ~900 bp				
Target Gene(s): CP gene				

(40x)

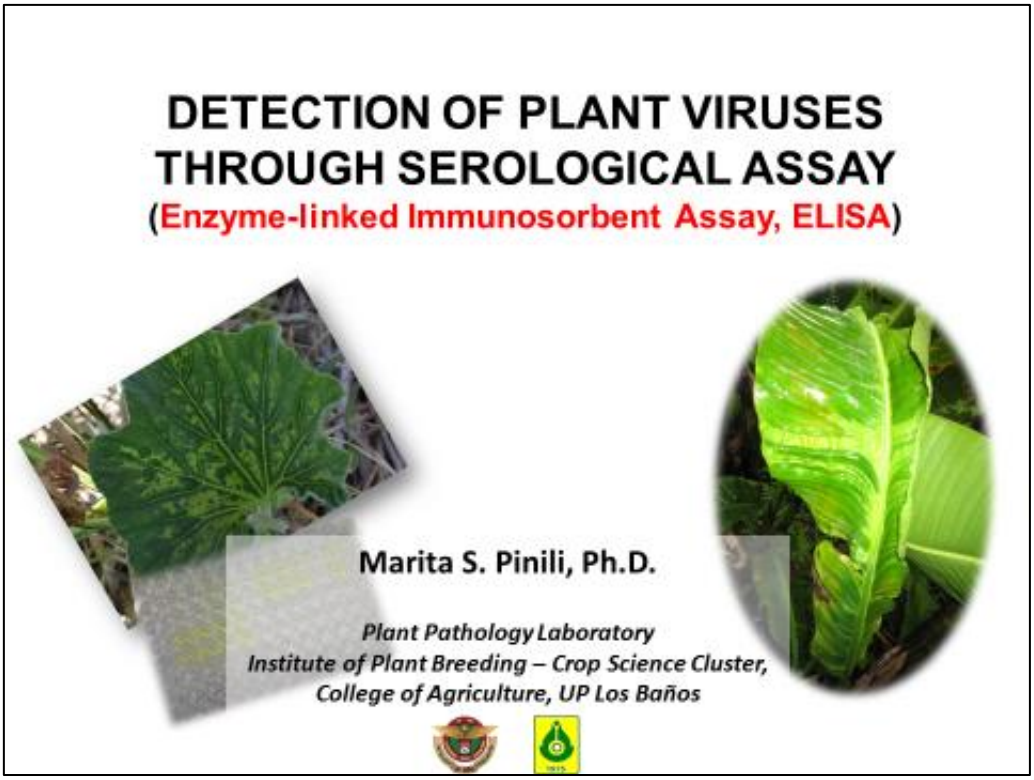
PCR Cocktail Preparation				
Components	**[Stock]**	**[Final]**	**1x=10µl**	
DEPC Water			2.2	
Reaction mix	2x	1x	5	
Primer F	10µM	0.5µM	0.2	
Primer R	10µM	0.5µM	0.2	
Superscript III RT			0.4	
RNA			2	
Total Reaction Volume			10	
Aliquot **8µl** to each DNA tube				

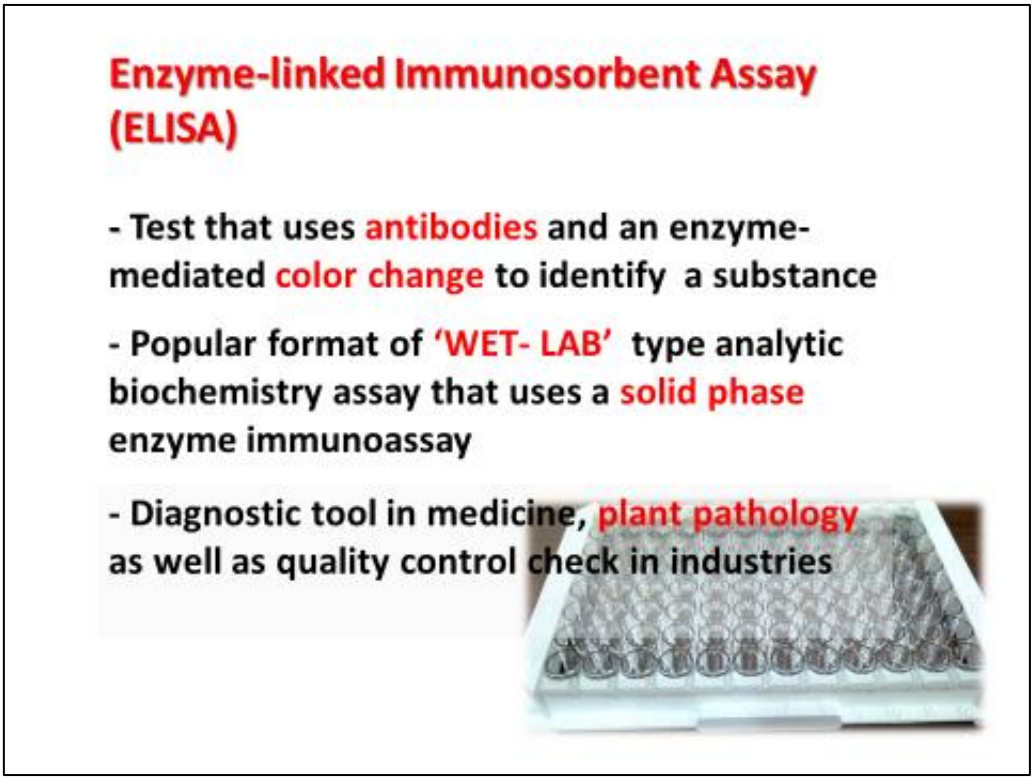
Gel Electrophoresis		**Notes/Remarks:**
%Gel:	Voltage:	
Run Time:	Staining Time:	

Documentation

Set-up by:

DETECTION OF PLANT VIRUSES THROUGH SEROLOGICAL ASSAY (Enzyme-linked Immunosorbent Assay, ELISA)





DETECTION OF PLANT VIRUSES THROUGH SEROLOGICAL ASSAY (Enzyme-linked Immunosorbent Assay, ELISA)

Enzyme-linked Immunosorbent Assay (ELISA)

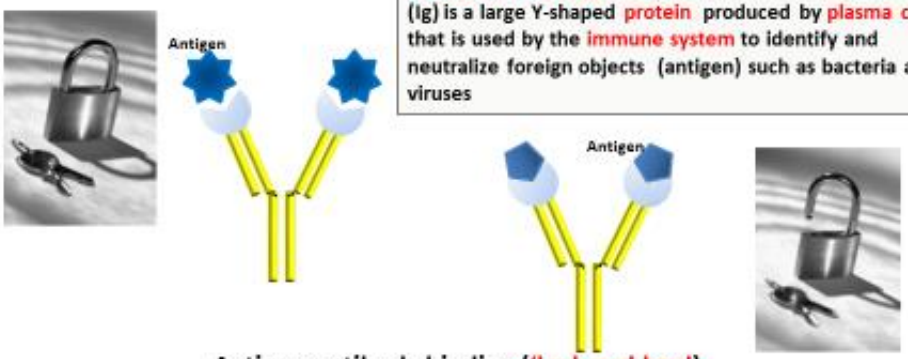
1960- Radioimmunoassay (RIA) first described by Yalow and Berson

1971 - Peter Perlman and Eva Engvall at Stockholm University, Sweden and Anton Schuurs and Bauke van Weemen in the Netherlands independently published papers that synthesized knowledge into methods to perform ELISA

Principles of ELISA

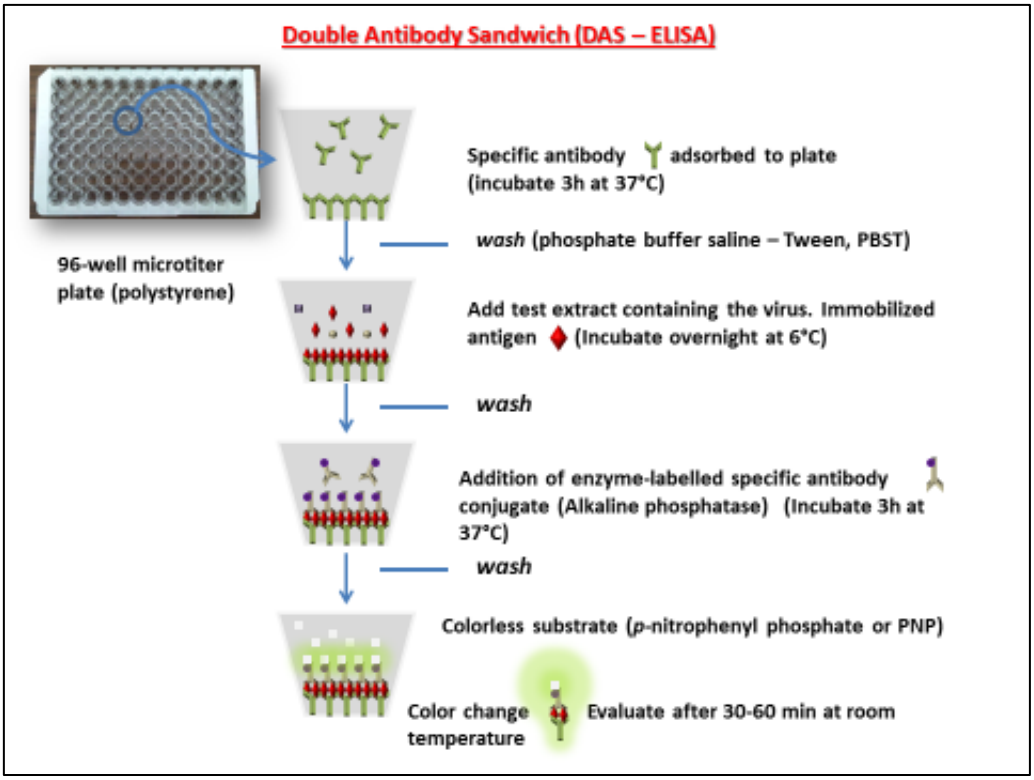
Antigen or antibody generator – Any substance which provokes an adaptive immune response. Ex. plant viruses

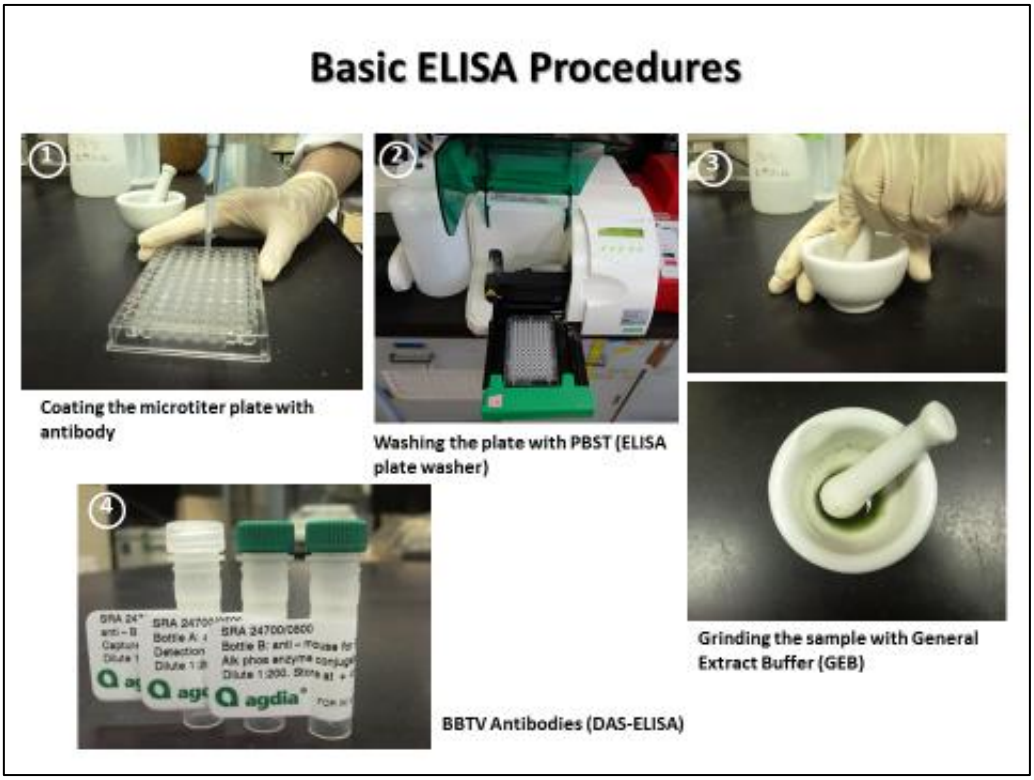
Antibody /Antiserum – also known as immunoglobulin (Ig) is a large Y-shaped protein produced by plasma cells that is used by the immune system to identify and neutralize foreign objects (antigen) such as bacteria and viruses



Antigen-antibody binding ('lock and key')

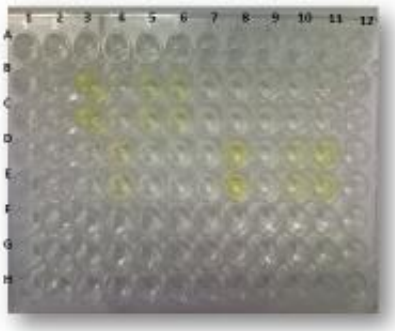
DETECTION OF PLANT VIRUSES THROUGH SEROLOGICAL ASSAY (Enzyme-linked Immunosorbent Assay, ELISA)





DETECTION OF PLANT VIRUSES THROUGH SEROLOGICAL ASSAY (Enzyme-linked Immunosorbent Assay, ELISA)

Basic ELISA Procedures



96-well polystyrene microtiter plate (yellow color indicates presence of BBTV on abaca samples)

Spectrophotometer

Advantages of DAS-ELISA

1. Extreme sensitivity
2. Applicability to large number of samples
3. Economy in use of high cost antisera
4. Semi-automatable
5. Quantitative
6. Independent of virus morphology
7. Independent of virus concentration