ELISA Protocol for Begomovirus Detection

1. Plan the layout of the ELISA plate ensuring that the necessary buffer, positive and negative controls are added. Always use duplicate wells. Make sure you have allowed enough time for the colour development which can take upwards of two hours.

2. Prepare the pumpkin yellow leaf puckering virus (PYLPV) polyclonal antibody by diluting in coating buffer (1:5000 dilution). For one plate, use 2 µl of polyclonal antibody in 10 ml of coating buffer.

3. Coat the plates with 100 µl diluted PYLPV polyclonal. Incubate at 37°C for 2 hours.

While the plates are incubating, prepare samples.

4. **Prepare samples (0.2 g) in labelled plastic bags or in 1.5 ml eppendorf tubes. Avoid using stem or leaf vein material.**
   To each bag add 800 µl of extraction buffer. Grind the leaf material using a glass rod, pestle or similar smooth ended object ensuring that the plastic bag is not damaged. Make sure the leaf material is thoroughly homogenised. Maintain on ice or at 4°C.

   In the case of dried leaf material, use 0.1 g and up to 4 ml of extraction buffer.

5. **If the samples were extracted in plastic bags, transfer the samples to clean, labelled eppendorf tubes. Centrifuge the samples in the microfuge at 10,000 rpm for 5 minutes. While the tubes are centrifuging, label new tubes.**

6. **Transfer the supernatant to the newly labelled tubes.**

7. **To each tube, add an equal volume of 0.5% BSA buffer. Vortex.**

8. **Store samples on ice or 4°C.**

Returning to the coating plates.....

9. Pour out the polyclonal solution from the plate and wash 4 times with washing buffer using a wash bottle. Ensure that the wells are free from excess liquid but not totally dry. This can be done by holding the plate inverted in your hand and "slamming" it onto a dry paper towel on the bench.

10. Block the plate by adding to each well 100 μl of 2% BSA. Incubate at 37°C for 1 hour.

11. Wash plates as in step 9.

12. Add 100 μl of each sample to the pre-designated wells. Incubate at 37°C for 1 hour.

During the incubation time, prepare the primary antibody (either M1 – specific for TYLCV, or D2 – general begomovirus antibody) by diluting in 0.5% BSA buffer (1:200 dilution). For one plate, use 50 μl of antibody in 10 ml of 0.5% BSA buffer.

13. Wash plates as in step 9.

14. Add 100 μl of diluted primary antibody (M1 or D2) to each well. If using more than one antibody, take care in aliquoting them. Incubate at 37°C for 1 hour.

During the incubation time, prepare the secondary alkaline phosphatase conjugated goat anti-mouse antibody by diluting in 0.5% BSA buffer (1:2000 dilution). For one plate, use 5 μl of antibody in 10 ml of antibody dilution buffer.

15. Wash plates as in step 9.

16. Add 100 μl of diluted secondary alkaline phosphatase conjugated goat anti-mouse antibody to each well. Take care in aliquoting them. Incubate at 37°C for 1 hour.

During the incubation time, prepare the PNPP substrate by diluting 50 μl of PNPP stock in 10 ml of diethanolamine buffer. Store in the dark at 4°C until use.

17. Wash plates as in step 9.

18. Add 100 μl of PNPP to each well. Allow the colour to develop (in the case of PRSV this can be as quick as 5 or 10 minutes); for D2 or M1 this may take 1 to 2 hours or overnight. If overnight, seal the plate with parafilm and leave at 4°C. Read the plate by eye or using an ELISA plate reader (absorbance 405 nm). Stop the reaction by adding 50 μl of 3 M NaOH. The plates can be stored on top of a moist paper towel in a sealable plastic bag at 4°C.

Reagents

A. Coating buffer (0.05 M carbonate buffer, pH 9.6) 500 ml

Na_2CO_3	0.795 g
$NaHCO_3$	1.465 g
dH_20	480 ml

If need be, adjust the pH to 9.6 (although this should not normally be required) and make up to 500 ml. Autoclave and store at 4°C. If you don't autoclave the buffer, store for a maximum of 14 days at 4°C.

B. Modification of 5X PBS, Tween-20, 1000 ml

NaCl	146.1 g
Na_2HPO_4	4.6 g
KH_2PO_4	1 g
Tween-20	2.5 ml
dH_20	950 ml

Ensure that reagents are re-suspended and make up to 1000 ml. Autoclave and store at room temperature. After opening, store at 4°C.

Washing buffer (1X)

Modification of 5X PBS, Tween-20	200 ml
dH_20	800 ml

C. Diethanolamine buffer

Diethanolamine	48.5 ml
dH_20	400 ml

Adjust pH to 9.8 and make up to 500 ml. Wrap bottle in aluminium foil and store at 4°C. **DO NOT AUTOCLAVE.**

D. 3 M NaOH

NaOH	24 g
Make up to 200 ml with dH_20	

DO NOT AUTOCLAVE

E. Extraction buffer (0.05 M Tris-HCl, 0.06 M sodium sulphite, pH 8.5) (Ann. Appl. Biol. **121**:297-303 (1991))

Tris base	6.06 g
Sodium sulphite	7.56 g
dH_20	950 ml

Adjust pH to 8.5 and make up to 1000 ml. Autoclave and store at room temperature. After opening, store at 4°C.

F. Blocking solution (2% BSA in washing buffer (1X))

BSA*	0.4 g
Washing buffer (1X)	20 ml

DO NOT AUTOCLAVE. Best to make up when required. Can store at 4°C for up to 7 days.

* Note: An alternative to BSA is skim milk powder (SMP). A 5% SMP solution can be used, however, good quality brands from Australia or New Zealand are recommended. Full milk powder is NOT recommended.

G. 0.5% BSA buffer (0.5% BSA in washing buffer)**

Blocking solution (2% BSA in washing buffer (1X))	10 ml
Washing buffer (1X)	30 ml

DO NOT AUTOCLAVE. Best to make up when required. Can store at 4°C for up to 7 days.

** Note: If you are using the 5% SMP washing buffer, make up a 1.25% final solution.

H. Antibodies

(i) Goat anti-Mouse IgG (H+L) Alkaline Phosphatase Conjgate (ZYMED, Cat # 81-6522). Store at 4°C. Use at 1:2000 (or up to 1:10,000) dilution.

(iii) M1: TYLCV specific mouse ascites fluid (monoclonal). M1 is specific for TYLCV and, in Thailand, does not react with other known geminiviruses. Use at 1:200 dilution. Store at -20°C.

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(iv) D2: General Begomovirus (bipartite, dicot infecting geminivirus) specific mouse ascites fluid (monoclonal). D2 does not react with other known plant viruses. Use at 1:200 dilution. Store at -20°C.

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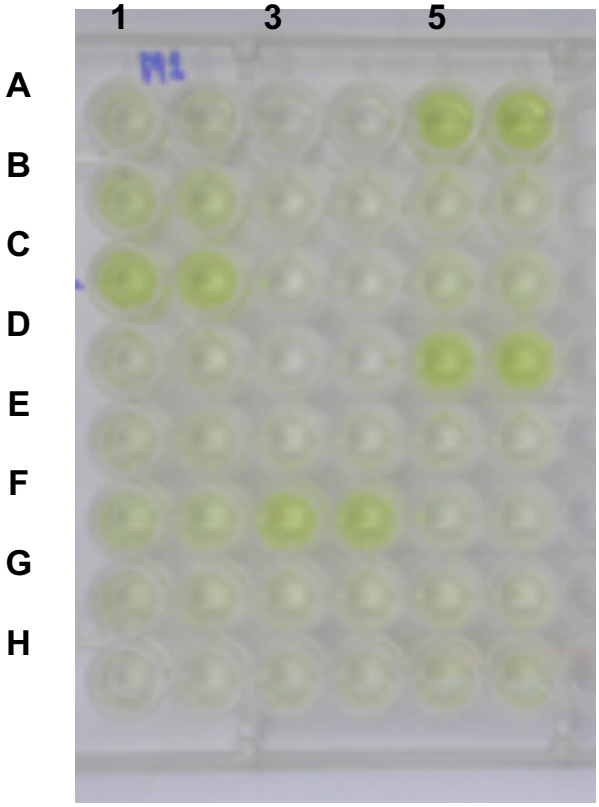
I. Nunc maxi-sorb flat bottom ELISA plates
OR
EIA/RIA flat bottom, high binding plates (Costar, Cat# 3590)

Appendix

Precautions

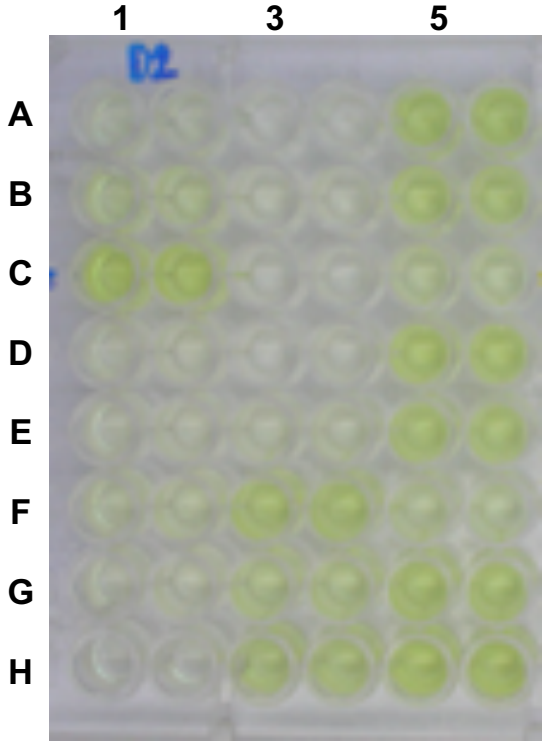
1. It is **ABSOLUTELY ESSENTIAL** that the blocking step (2% BSA after coating the plates with the polyclonal antisera) be done. Unless this is done the background is unacceptable.
2. Controls **MUST BE INCLUDED** – buffer, healthy and positive.
3. It may be necessary to allow the alkaline phosphatase reaction to proceed for more than 1 hour – 2 hours or overnight. This should be judged based on the background of the buffer and negative controls.
4. Always take readings regularly – don't wait for 2 hours as it might be too late.
5. An ELISA plate reader is not essential but nonetheless helps. At HGR we have a TECAN Sunrise Reader (#F039300) with Touch Screen (#B037302) and Magellan Software (#B017522). The plates are read at 405 nm.
6. In designing the plate, always do replicates (ie. 2 wells per sample)

Below are typical photos of ELISA plates prepared using M1 and D2.



Well	Sample	Well	Sample
A1	Bu	A3	To
B1	H	B3	To
C1	TYLCV	C3	To
D1	To	D3	To
E1	To	E3	To
F1	To	F3	To*
G1	To	G3	Pk
H1	To	H3	Pk
		A5	Pk**
		B5	Pk
		C5	Pk
		D5	Pk**
		E5	Pk
		F5	Pk
		G5	Pk
		H5	Pk

Figure 1. ELISA prepared using samples of tomato (To) and pumpkin (Pk) with monoclonal antibody M1. Buffer (Bu), healthy tomato (H) and positive controls (TYLCV) are in the uppermost left wells. Samples considered positive are indicated *. Samples indicated ** should be reconfirmed as M1 should be specific for TYLCV. TYLCV has not been reported to infect pumpkin, but may depending on the geographical isolate.



Well	Sample	Well	Sample
A1	Bu	A3	To
B1	H	B3	To
C1	TYLCV	C3	To
D1	To	D3	To
E1	To	E3	To
F1	To	F3	To#
G1	To	G3	Pk
H1	To	H3	Pk
		A5	Pk**
		B5	Pk
		C5	Pk
		D5	Pk**
		E5	Pk
		F5	Pk
		G5	Pk
		H5	Pk

Figure 2. ELISA prepared using identical samples of tomato and pumpkin to those in Figure 1 with monoclonal antibody D2. D2 should react with most known begomoviruses and so should react with TYLCV. Samples positive using M1 are also positive with D2 (indicated #).

Final word. There are many viruses referred to as TYLCV and Tomato Leaf Curl Virus (TLCV), so the applicability of both antisera needs to be assessed in each geographical location. The host range of some Begomoviruses, including those referred to as TYLCV, has been reported to be very broad, including cucurbits.

Both M1 and D2 are indicators of TYLCV and Begomoviruses, respectively, and their specificity or otherwise, like all antisera, needs to be used in association with other indicators including symptoms, local reports, host range etc. Unlike PRSV antisera, both M1 and D2 have not been exhaustively used throughout Asia, so we should collate accurately the information from all results.

	1	2	3	4	5	6	7	8	9	10	11	12
A	B	B										
B	SAP	SAP										
C	+ve	+ve										
D												
E												
F												
G												
H												