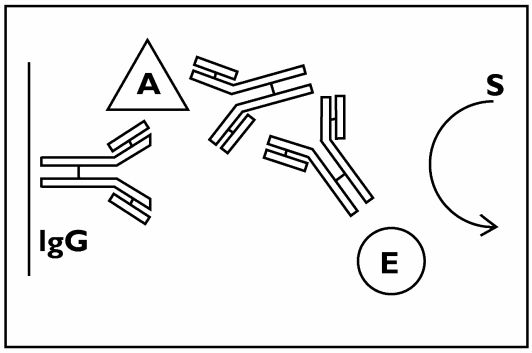
TAS-ELISA PROTOCOL (Mouse monoclonals)

Assay Principle



This triple antibody sandwich ELISA (TAS) uses polyclonal antibodies (IgG) which are bound to the surface of the microtitre plate to capture the antigen (A) of interest and a monoclonal antibody (Mab) which is raised in mice to detect the antigen. An anti-species antibody-enzyme conjugate (E) is used to detect the monoclonal antibody. The presence of enzyme (in this case alkaline phosphatase) is detected by a colorimetric substrate (S) reaction.

Reagents required for TAS ELISA

Coating antibody (codes in the ADGEN catalogue ending in -01/-02).
Probe antibody (ADGEN codes ending in -03/-04)
Anti-species conjugate (anti-mouse 04-004/005/006)
or Reagent set - coating antibody, probe antibody and anti-species conjugate (ADGEN codes ending in -05/-06/-07)
Coating buffer (ADGEN codes 02-001/02-002)
Phosphate buffered saline + Tween 20 (ADGEN code 02-003)
Extraction buffer (ADGEN codes 02-004 - 02-016 depending on antigen of interest)
Mab dilution buffer (ADGEN codes 02-006/02-007)
Conjugate buffer (ADGEN codes 02-008/02-009)
pNPP tablets (ADGEN codes 03-001/03-002)
Substrate buffer (ADGEN code 02-010/02-011)

Alternatively, for your convenience ADGEN supply a TAS ELISA buffer pack (02-019/02-020) and prepared liquid substrates which are stable, convenient and easy to use (03-003/03-004) and for enhanced detection in your assay choose ADGEN Blue liquid substrate system (03-005/03-006).

Additionally, positive and negative controls are available from ADGEN (codes ending in -11/-12) for a wide variety of pathogens, these allow you to have increased confidence in the performance of the assay.

Protocol (please read before starting assay)

1. Dilute coating (PAb) antibody as recommended on the bottle label in **coating buffer** and add 100µl to the required number of wells for your test.

2. Wrap the plate tightly in cling film or place in a plastic box with some damp paper towels and close the box. Incubate the plate at 37^0C for 4 hours.

3. Wash the plate three times with **phosphate buffered saline + Tween 20 (0.05%) - PBST.** To do this fill the wells of the plate with **PBST** and invert to remove the buffer repeat twice then pat the plate dry on paper towels.

4. Extract the samples by grinding 1g of tissue with 10ml of **general extraction buffer** in a mortar and pestle (or an alternative method of grinding). Then filter the sample through a layer of muslin (or similar fine cotton gauze). If this is not available then allow the plant material to settle and use the supernatant in the test. In some cases the recommended ratio of sample to buffer may have to be reduced to allow a clear signal to be obtained if the plant material is not highly infected.

5. Add 100µl of each sample, positive and negative control to the coated wells. ADGEN recommend that all samples and controls are tested in duplicate.
   Remember, **1 ADGEN UNIT = 2 TEST WELLS**
   ADGEN positive and negative controls are reconstituted by adding 2ml of distilled/deionised water and gently shaking. Any unused reconstituted control may be stored at -20^0C however, the performance of the positive controls may decrease when stored in this manner.

6. Wrap the plate as described in (2) above and incubate at 4^0C overnight (at least 16 hours).

7. Wash the plate as described in (3) above.

8. Dilute the antibody probe (Mab) as recommended on the bottle label in **Mab dilution buffer** and add 100µl to each test well.

9. Wrap as in (2) above and incubate at 37^0C for 2 hours.

10. Wash three times as described in (3) above.

11. Dilute the goat anti-mouse IgG-AP as indicated on the bottle with **conjugate buffer** and add 100µl to each well. ADGEN supply anti-species conjugate as part of **Reagent Sets** at the optimal dilution for the assay. However, if another conjugate is being used it should be titrated before use.

12. Wrap as in (2) above and incubate at 37^0C for 1 hour.

13. Wash the plate four times as described in (3) above. An extra wash step is included at this stage to ensure that all unbound conjugate is removed from the wells.

14. Prepare the substrate just before use - add pNPP at 1mg/ml to **substrate buffer** (one 5mg tablet in 5ml of buffer). Alternatively, use one of the ADGEN liquid substrates. All of these substrates may change colour when exposed to light and should be protected from light to prevent this occurring.

15. Add 100µl of prepared substrate to each test well.

16. Wrap the plate as in (2) above and incubate in the dark at room temperature for 1 hour.

17. Read the absorbance using a spectrophotometer at 405nm (for pNPP and ADGEN Yellow) or 595 - 650nm (for ADGEN Blue). Alternatively, positive and negative values may be scored visually although this may not be as accurate as using a spectrophotometer. A positive sample may be determined as one which gives an absorbance value which is greater than the absorbance value of the negative control. A negative sample is one which gives an absorbance value which is the same as, or less than, the negative control. Visually, a positive sample will give a darker colour than the negative control and a negative sample will give a similar, or lighter, colour to the negative control.

Notes

1. ADGEN antibodies and conjugates are sold in units of 500,1000 and 5000. When they are diluted as indicated in the protocol each unit provides sufficient for each test to be conducted in duplicate wells containing 100µl of each reagent.
   That is, **1 ADGEN UNIT = 2 TEST WELLS.**

2. ADGEN recommend that, whenever possible, positive and negative controls be run during all tests to ensure that all of The test components are working properly.

3. The antibodies/conjugates should be stored at 4 - 6^0C on receipt.

Recommended buffers for TAS ELISA

Coating Buffer (Carbonate Buffer)

Sodium carbonate	1.59g
Sodium hydrogen carbonate	2.93g

Make up to 1 litre with dH_20. The pH of this buffer is 9.6 and does not require to be adjusted.

Phosphate buffered saline (PBS)x10

Sodium chloride	80g
Potassium diHydrogen orthophosphate	2g
diSodium Hydrogen orthophosphate	11.5g
Potassium chloride	2g

Make up to 1 litre with dH_20. The pH of this solution when diluted to 1xs is 7.2

Wash buffer (PBS + Tween 20)

Phosphate buffered saline	1 litre
Tween 20	0.5 ml

General Extraction Buffer

Polyvinylpyrrolidone (PVP)	20g
Ovalbumin 2g	
Sodium sulphite (anhydrous)	1.3g
Sodium azide	0.2g
Tween 20	0.5ml
Sodium chloride	8g
Potassium diHydrogen orthophosphate	0.2g
diSodium Hydrogen orthophosphate	1.15g
Potassium chloride	0.2g

Make up to 1 litre with distilled/deionised water. This buffer can be difficult to get into solution and it is easier if the PVP is mixed into a "paste" with a small volume of water before adding the other components and the remainder of the water.

Mab dilution buffer/Conjugate buffer

Bovine serum albumin 0.2g	
PBST	100ml

Substrate buffer (Diethanolamine buffer 1M)

Diethanolamine	90.39g
Diethanolamine-HCl	19.82g
Magnesium chloride	0.1g

Make up to 1 litre with dH_20. The pH of this buffer is 9.8 and it does not require to be adjusted. (The diethanolamine and diethanolamine-HCl are liquids however, it is easier to weigh them out than to measure their volumes as they are extremely viscous.)

pNPP is added to the above buffer at 1mg/ml to make up the substrate for alkaline phosphatase.

Trouble Shooting Guide

A uniform high level of colour appears in all wells.

- The plate may not have been washed properly.
- The substrate solution may have been contaminated. pNPP tablets can be contaminated by touching them, using ADGEN Yellow removes this potential source of contamination.
- The substrate solution may have been exposed to bright light.
- One of the buffers may have been contaminated by conjugate.
- The plate may have been coated with antibody-enzyme conjugate.
- The plate may have been coated with coating antibody which has been contaminated with antibody-enzyme conjugate.
- The wrong anti-species conjugate may have been used (may be against the coating antibody not the probe antibody).

All wells are coloured but to different degrees

- The plate may not have been properly washed.
- The plate may have been exposed to bright light.

Colour appears in all of the outer wells of the plate

- The outer wells may not have been properly washed.
- You may be experiencing 'edge effects'. This is a problem associated with the cooling procedure employed when the plates are being moulded. Newer, high quality plates such as those used at, and supplied by, ADGEN do not cause this problem.

Some colour appears in a negative control well, while some of the other wells are clear.

- A positive sample may have been added to the negative control well.
- During washing some positive control sample may have been washed into the negative control well.
- Contamination of the negative control well may have occurred by carry over of a positive sample if the same pipette tip was used. New tips must be used for every sample.

All of the wells, including the positive control, are clear.

- The antibody-enzyme conjugate may not have been added to the conjugate buffer.
- The positive control may have gone off.
- The pNPP substrate tablet may not have been added to the substrate buffer.

The colour in the positive control wells is very low

- One of the buffers that have been used may be too old.
- The positive control may be starting to go 'off'.
- The substrate buffer may have been diluted with PBST and not water as recommended. Phosphate in PBST reduces the amount of colour produced.

If you still have a problem then prompt and comprehensive technical advice regarding ADGEN Phytodiagnostics products is always available from Neogen Europe Ltd.

Tel: ++44 (0) 1292 525275
Fax: ++44 (0) 1292 525477
e-mail: plant@neogeneurope.com
website: www.neogeneurope.com