1. PRINCIPLE OF THE TEST – 3G MIX

This Triple Antibody Sandwich-Enzyme Linked Immuno Sorbent Assay (TAS-ELISA) uses antibodies which are bound to the surface of a microplate to capture the antigens. The presence of the antigen is detected using specific antibodies. Then, the immunological complex is revealed by addition of an anti-species antibody coupled with Alkaline Phosphatase (this antibody is specific for the species that the previous specific antibody is raised in). Finally, the addition of the substrate of the enzyme (pNPP) induces a yellow product, detectable at 405 nm, when the antigen is present.

Before opening the tubes containing the antibodies (coating and conjugate), please to **centrifuge** in order to collect correctly the content at the bottom of the tube.

2. PROTOCOL

COATING STEP — color code: blue

Prepare buffers:

· Prepare the volume of coating buffer 1X needed for the test. You will need 100 µL of coating buffer for each test well you are using (see recommendations for an example).
· Buffer formulation is provided on page 4.
· **SEDIAG** Coating buffer (ref: COAT-Buf) has to be diluted to the final concentration (1X) with deionised/distilled water.

Prepare capture antibody:

· Dilute coating antibodies in **Coating Buffer 1X** as recommended on the bottle label and on the test data sheet. Homogenize.
· Deposit **100** µl per well.
· Incubate the plate 2 hours at +37°C (*)

Wash plate:

· Empty the wells into a sink or waste container by inverting the plate. Fill the test wells to overflowing with washing buffer 1X (can be purchased directly from SEDIAG in 20X, ref: WASH-Buf). Repeat **2** more times (see recommendations for more details).

Note: Use freshly coated plates immediately

ANTIGEN STEP

Prepare buffers:

· Prepare the volume of Grapevine extraction buffer 1X needed for the test. You will need 100 µL of extraction buffer for each test well you are using.
· Buffer formulation is provided on page 4.
· **SEDIAG** Grapevine extraction buffer (ref: GNXTR-Buf) has to be diluted to the final concentration (1X) with deionised/distilled water.

(*) During incubation, plates should be covered with an adhesive plastic film or a lid.

Sample preparation:

- Crush 1 g of each sample in 10 ml of **Grapevine extraction buffer 1X** (see recommendations for more details). Homogenize. In some cases the ratio of sample to buffer may be reduced to obtain a cleared signal if the plant material is not highly infected.
- The controls should be diluted as indicated on labels (see the control datasheet).
- Following your loading diagram, carefully deposit **100** µl of each sample, positive and negative control into each well and 100 µL of extraction buffer in buffer-wells.
- Incubate the plate 16 hours (overnight) at +4°C (*)

Wash plate:

- Wash **5** times as before.

AP-CONJUGATE (BOTTLES A&B) STEP

color code: red

Prepare buffers:

- Prepare the volume of conjugate buffer 1X needed for the test. You will need 100 µL of conjugate buffer for each test well you are using.
- Buffer formulation is provided on page 4.
- **SEDIAG** conjugate buffer (ref: CONJ-Buf) has to be diluted to the final concentration (1X) with deionised/distilled water.

Prepare conjugate antibody:

- Dilute the alkaline phosphatase conjugate antibodies A & B in unique tube in the **Conjugate Buffer 1X** as recommended on the bottle label and on the test data sheet. Homogenize.
- Deposit **100** µl per well.
- For example : if the dilution given on tubes A and B of concentrated alkaline phosphatase conjugate is 1:100, and you are preparing 10 mL final concentration of enzyme conjugate, you should first dispense 10 mL of conjugate buffer. Then, add 100 µL from tube A and 100 µL from tube B to this buffer.
- Incubate the plate 2 hours at +37°C (*)

Wash plate:

- Wash **5** times as before.

SUBSTRATE STEP

Prepare buffers:

- Prepare the volume of substrate buffer 1X needed for the test. You will need 100 µL of substrate buffer for each test well you are using.
- Buffer formulation is provided on page 4.
- **SEDIAG** substrate buffer (ref: SUBST-Buf) has to be diluted to the final concentration (1X) with deionised/distilled water.

(*) During incubation, plates should be covered with an adhesive plastic film or a lid.

Prepare substrate:

- Each pNPP tablet (ref: TAB-Acc) will make 5 mL of pNPP solution (after reconstitution with room-temperature substrate buffer 1X), at a concentration of 1mg/mL, about enough for 45 wells.
- If you use powder, dissolve 1 mg of pNPP/mL of room-temperature **Substrate Buffer 1X** just before use.
- Deposit **100** µl per well.
- Incubate the plate at +37°C (*)
- Read the absorbance at 405 nm, using a spectrophotometer, after 1 hour and 2 hours of incubation. See recommendations for the results evaluation.

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

(*) During incubation, plates should be covered with an adhesive plastic film or a lid.

3. RECOMMENDATIONS

Storage : Store all the reagents and buffers at recommended temperature to assure their full shelf-life. Do not store prepared buffers from day to day.

Safety : Avoid the direct contact with eyes, skin or ingestion of the various compounds. Always wash hands thoroughly after using the products. If you have any queries about the use of this product, please contact SEDIAG. Safety datasheets are available on request.

Preparation of the Coating-Ab and Conjugate-Abs

Deposit the solution of antibodies immediately on the plate after this preparation. The volume of solution depends on the number of tests to be performed.
For example, if the dilution of the concentrate reagent (antibody or streptavidin) given is **1/100** and you are preparing 10 ml of antibody solution, you should mix 10 ml of buffer with 100 µl of the concentrate reagent.
It is imperative to use microtiter plate MaxiSorp Nunc ® and to follow a loading diagram established before the deposit of samples.

Washes

Wash the plates in the PBST (washing buffer) 3 - 5 times according to the recommendations of the protocol. Solutions in the plate wells can be removed by aspiration, or, more frequently, the plate is inverted rapidly with a quick shake of the hand and tapped firmly on paper towels. Inspect the testwells. All wells should be free of plant tissue or air bubbles. If tissue or bubbles are present repeat the washing and tap firmly on a paper towel.

Samples preparation

When possible select samples showing symptoms. Leaf tissue is often used in ELISA testing. Stem, seed and other tissue can also be tested.
To prepare the samples, you can use sample mesh bags (ref: SAC-Acc), a mortar and a pestle, or other grinding devices to grind samples. If you are using a mortar and a pestle, wash and rinse them thoroughly between each samples. After extraction, the material can be kept at +4°C during a maximum of 12 hours. The sensibility of the test can be altered because of a bad conservation, so it is better to deposit samples directly after their grinding.
Use of positive and negative controls is deeply recommended during the tests to be able to validate the results.

Evaluate Results

Wells in which color develops indicate positive results. Wells in which there is no significant color development indicate negative results.
Test results are valid only if positive control wells give a positive result and buffer wells remain clear.
Results may be interpreted after more than 60 minutes of incubation as long as negative wells remain virtually clear.

4. TECHNICAL SERVICE

If you have any question about using our reagents, do not hesitate to contact us for more details by phone (+33 3 80 67 49 42) or by email (info@sediag.com).

5. BUFFER FORMULATIONS

- **PBS 1X**

Dissolve in 1000 ml distilled water:

NaCl	8 g
Na_2HPO_4-12H_2O	2.9 g
KH_2PO_4	0.2 g
KCl	0.2 g
NaN_3	0.2 g

The pH of this buffer is 7.4

- **PBST 1X = Washing Buffer 1X**

Add to 1000 ml PBS:

Tween20	0.5 ml

The pH of this buffer is 7.4

- **Grapevine Extraction Buffer 1X**

Dissolve in 1000 ml distilled water:

Tris	24.2g
NaCl	8g
PVP (Mw 10,000-40,000)	20 g
Tween20	0.5 ml
NaN_3	.0.2g

Adjust the pH at 8.2 with HCl

- **Coating Buffer 1X**

Dissolve in 1000 ml distilled water:

NA_2CO_3	1.59 g
$NaHCO_3$	2.93 g
NaN_3	0.2 g
Bromocresol purple	5 mg

The pH of this buffer is 9.6.

- **Conjugate Buffer 1X**

Add to 1000 ml PBST 1X:

BSA	2 g
Congo Red	40 mg

The pH of this buffer is 7.4

- **Substrate (pNPP) Buffer 1X**

Dissolve in 1000 ml distilled water:

Diethanolamine	97 ml
NaN_3	0.2 g

Adjust the pH at 9.8 with HCl

Update : 08/25/2022