NCM-ELISA detection of sweetpotato viruses - OP21

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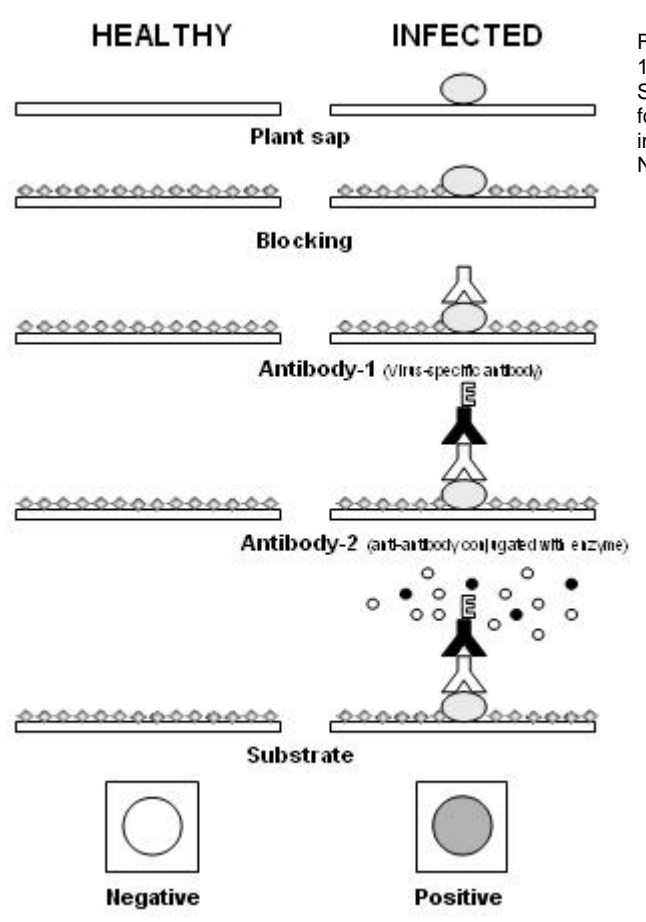

Version	Date	Author	Comment
56	Feb 02, 2010 16:12	Alison Light	
55	Jan 28, 2010 15:32	Alison Light	"Dip dry" in the Serological test section - is this correct I think you mean "drip dry" please amend accordingly
54	Nov 12, 2009 16:41	Maria Elena Vargas	Header formatting
53	Oct 18, 2009 16:27	Segundo Fuentes	Additions included according to external auditing, October 14th 2009
52	Oct 18, 2009 16:16	Segundo Fuentes	
51	Oct 15, 2009 13:11	Edwin Rojas	
50	Sep 17, 2009 09:46	Maria Elena Vargas	
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INTRODUCTION

NCM-ELISA is an immuno-enzymatic test that uses nitrocellulose membranes instead of the polystyrene microtitration plates as a support for the reagents used in the serological reaction. The test is as sensitive as the direct double antibody sandwich ELISA (DAS-ELISA), easier to perform, and can be completed in a shorter period of time. It also has another very important advantage: the samples can be dotted onto the nitrocellulose membrane and stored for several weeks before continuing with the test; or they can be sent to another laboratory for development. All the steps are done at room temperature. The test consists of:

- Blotting a very small amount of the test sample (2 to 30 µl)onto the membrane.
- Blocking areas not utilized by the samples.
- Reacting the virus particles with specific antibodies (IgG).
- Detecting the virus specific antibodies with enzyme-labeled antibodies by means of an appropriate substrate (see Figure 1). The intensity of the coloration which is proportional to the virus concentration is stable for a long period of time. The membrane can be easily stored. As in DAS-ELISA, several viruses can be detected at the same time on one membrane by using a mixture of antibodies which are specific for the virus to be tested. This is especially useful for seed production programs where it is generally important to learn only if the sample is infected with virus(es) or not.

HEALTHY   INFECTED

Plant sap

Blocking

Antibody-1 (Virus-specific antibody)

Antibody-2 (anti-antibody conjugated with enzyme)

Substrate

Negative   Positive

SCOPE

The NCM-ELISA is capable of detecting the following sweetpotato viruses : SPFMV, SPMMV, SPLV, SPMSV, SPVG, SPCFV, C-6 virus, SPCSV, SPCaLV, CMV.

A controlled list of virus detection methodology being used is maintained at the link : List of pathogens   Factors that could affect reliability for virus detection in sweet potato

SAFETY

A laboratory coat should be worn at all times.
Do not touch the nitrocellulose membrane with bare hands. Wear dry and clean gloves and/or use forceps

MATERIALS

1. Buffers stocks

NAME	CHEMICAL COMPOSITION	STORAGE CONDITIONS
TBS pH 7.5 (2,000 ml)	Tris Base 4.84 g (0.02 M) NaCl 58.44 g (0.5 M)	Dissolve in 1,990 ml distilled water and adjust to pH 7.5 with concentrated HCl (37%). Complete to 2,000 ml with distilled water.

TTBS (2,000 ml)	TBS 2,000 ml Tween-20 1.0 ml (0.05 %)	Store at 4 - 6°C.
Extraction buffer	TBS buffer containing 0.2 % sodium sulfite	Store at 4 - 6°C.
Blocking solution	TBS + 2 % milk + 2 % TRITON X-100	Prepare fresh for each test.
Substrate buffer pH 9.5 (500 ml)	Tris Base 6.05 g (0.1 M) NaCl 2.92 g (0.1 M) $MgCl_26H_2$ 0 0.51 g (0.005 M)	Dissolve Tris Base, NaCl and $MgCl_2.6H_20$ in 450 ml distilled water. Adjust pH to 9.5 with concentrated HCl (37%). Complete to 500 ml with distilled water.
Antibody and conjugate solution	TBS buffer containing 2% milk	Prepare fresh for each step.
NBT Stock solution	NBT 40 mg N,N-dimethylformamide (70%)1.2 ml	Mix well and store at 4 - 6°C, protected from light (dark bottle or cover it with foil).
BCIP Stock solution	BCIP 20 mg N,N-dimethylformamide 1.2 ml	Mix well and store at 4 - 6°C, protected from light (dark bottle or cover it with foil).
Substrate solution	Substrate buffer 30ml NBT stock solution 90 ul BCIP stock solution 90 ul	Add the NBT stock solution in 30 ml of substrate buffer. Afterwards add the BCIP stock solution drop by drop while stirring (it is NBT/BCIP substrate solution).

2.Other reagents

NAME	STORAGE
GAR : Goat anti-rabbit IgG alkaline phosphate conjugate (BIO-RAD).	Store in a dark and cold place at 4°C. Do not freeze
NBT : Nitro blue tetrazolium (BIO-RAD).	Store in a dark and cold place at -20°C.
BCIP : 5 Bromo-4-chloro-3-indolyl phosphate (BIO-RAD).	Store in a dark and cold place at -20°C.
DMF : N,N-dimethylformamide (SIGMA).	Store at room temperature.
HCl : Hydrochloride acid 37% (MERCK).	Store at room temperature.
MILK : Powdered cow milk	Store at room temperature.

3. Materials and equipment

Name	Material	Storage Conditions / Use
Membranes (BIO-RAD)	Nitrocellulose	Store in a dry place at room temperature.
Filter papers (Whatman # 4)	Filters	Store at room temperature.
Sample bags	Polyethylene (4x6x6)	Use one for each sample.
Small tubes of 1 cm diam.	Glass	Use to cut disks from leaf samples.
Thick test tube or piece of round wood		Use in sample maceration.
Micropipettes (20, 200 and 1000 μl)		Check according to Equipment Control procedure.
200 and 1000ul tips		
Surgical forceps		Use them when manipulating the membranes.

2000, 1000, 500, 250ml containers or glass bottles		
1000, 500, 250, 100, 50ml graduated cylinders		
Distilled water		
Refrigerator	2 - 8°C	
pH meter		
digital and analytical balances		
rotary shaker		
vacuum pump		
Milli-Q water purification apparatus		
Magentic stirrer		

PROCEDURE

Sample preparation

Collect and identify the samples in the plastic bags (this must be done on the same day the test is performed). Make a composite sample from each plant to be tested by taking one leaf from the top, middle and bottom levels. Select, if possible, those showing symptoms.

From each leaf sample cut a disk approximately 1 cm in diameter. To perform this, position each leaf within the upper part of the plastic bag and, using a small test tube, cut a leaf disk by exerting pressure on the outside of the plastic bag. Eliminate the remaining part of the leaves and grind the leaf disks with 3 ml of extraction buffer (1 ml of extraction buffer per leaf disk). Grind the tissue completely using a large test tube or a piece of round wood. Final sap dilution is approx. 1/50 (lower dilutions could give nonspecific reaction or interfere with the final developed reaction because of higher concentration of polysaccharide components in sap).

Let the bag stand in an upright position for 30-45 minutes at room temperature until the plant sap phases out (this can be achieved by placing the bags in a large beaker).

Sample application to nitrocellulose membrane

Previously, cut the nitrocellulose membranes to the size needed.

Identify the membranes by writing the name of the virus on the top and pre-wet the membranes in TBS for at least 5 minutes prior to use.

In the meantime, connect the dot blotting apparatus to a vacuum pump.

Place a pre-wet piece of No. 4 Whatman paper over the dot blot manifold and place the pre-wet nitrocellulose membrane over the filter paper.

When necessary use a piece of parafilm to block the remaining area of the manifold not covered by the nitrocellulose membrane. Carefully apply a vacuum (200 to 230 mm of mercury) by turning the pump on.

Pipette 30 µl sample (plant sap) into each well formed on the nitrocellulose membrane by the vacuum. Take care not to pipette plant tissue. Using a clean tip for each sample, repeat the process until all the samples have been spotted.

Remove the nitrocellulose membrane from the apparatus and transfer the membrane onto a dry piece of filter paper and let it dry for 15-30 minutes.

Record the order/number of the samples as they were spotted onto the membrane on the NCM-ELISA record sheet.

Serological test

Dip dry nitrocellulose membrane in blocking solution for 1 hour at room temperature with gentle shaking (50 rpm).

Add the 1st antibody in TBS + 2 % milk. Incubate overnight at room temperature with gentle shaking (50 rpm).

Wash nitrocellulose membrane in TTBS three times for 3 minutes each with rapid shaking (100 rpm).

Add the 2nd antibody (GAR) in TBS + 2 % milk. Incubate for 1 hour at room temperature with gentle shaking (50 rpm).

Wash nitrocellulose membrane in TTBS four times for 3 minutes each with rapid shaking (100 rpm).

When ready to use, prepare the NBT/BCIP substrate solution (prepare immediately before use).

Incubate the nitrocellulose membrane for 30 minutes (1 to 1.5h for SPCSV) in NBT/BCIP substrate solution at room temperature with gentle shaking (50 rpm).

Stop the color development by discarding the NBT/BCIP substrate solution and immersing the nitrocellulose membrane in distilled water. Wash nitrocellulose membrane in tap water three times for 3 minutes each.

Dry the membranes before recording reactions on the NCM-ELISA record sheet. Transfer the NCM-ELISA results to the corporative server through wireless LAN using a hand help computer.

Internal quality control

Make 90 dots on the nitrocellulose membrane with processed samples and separate the last six spaces in the membrane for dotting the controls (two spaces for buffer, two for negative and two for positive controls, respectively).

Positive reactions are those showing different grades of purple color. Record the reactions on the NCM-ELISA record sheet along with the responses of the samples. Record the positive reaction with a "+" and the mild reaction with a "±".

Positive controls are extracted sap from the plant tissue (*Ipomoea setosa*, *Nicotiana benthamiana*, *Nicotiana tabacum*) infected with viruses. Virus infected plants are grown in a greenhouse at 18-28°C with 10,000 - 15,000 lux light intensity for at least 8 hours.

Negative controls are produced from sap from virus-free sweet potato plants grown in an isolated greenhouse at the same conditions as above.

Both negative and positive controls are spotted on the nitrocellulose membranes every time a NCM-ELISA test is performed. Batch of plants used as controls are renewed at one-month intervals.