TECHNICAL SHEET No. 2

Virus Detection: *Citrus Tristeza Virus* (CTV)

Method: DAS-ELISA

General

Virus detected: CTV from citrus stems.
General Method: Double Antibody Sandwich - Enzyme Linked Immuno Sorbent Assay (DAS-ELISA).

Developed by

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Goals

To develop a rapid method for large scale detection of CTV.

Introduction

Citrus is a major fruit in Palestine. It is mainly cultivated in the Gaza strip, Qalqylia, Tulkarm, and Jericho. The major citrus cultivars in Palestine include; Valencia, Shammoty, Lemon, and Clement. One of the major constrains facing the citrus industry in Palestine is the Tristeza, a viral disease caused by Citrus Tristeza Virus (CTV), which threatens the citrus industry in the area. One of the first quarantine measures taken to prevent the spread of this disease should be the survey of nurseries that supply propagation material locally and to other countries. The technique of ELISA could be used for such large scale surveys.

The Double Antibody Sandwich ELISA method involves a capture step of the virus to a specific antibody in an ELISA, then the virus is further captured by another antibody conjugated with an enzyme (e.g. Alkaline Phosphotase), and then a detection step uses a specific substrate of the chosen enzyme. The developed color is finally read by an ELISA Reader. This method was successfully applied in our lab for large scale detection of CTV and other viruses.

Methods

A- Coating wells with coating antibodies

1. Prepare the coating buffer: dilute the 5x stock solution 1:5 in distilled water.
2. Dilute the antibodies 1:100 in the 1x coating buffer and mix thoroughly.
3. Load the diluted antibodies in the plate wells (100 µl/ well) and cover with adhesive film.
4. Incubate the plate at 37 °C for two hours. (At the end of this incubation, plates can be stored at 4 °C if necessary).
5. Wash the wells three times with 1x washing buffer. For each wash, use 200 µl/ well of the washing solution. When pouring out the washing solution, shake the plate.
6. After the third wash, hit the plate gently on several layers of tissue paper until you remove all drops of liquid.

B- Sample preparation and deposition

Samples can be prepared during the incubation period of the coating step.

1. Dilute the 20x extraction buffer to 1x with distilled H_2O.
2. Peel the epidermis layer of the stem and discard it. Then take the tissues under the epidermis that include the phloem cells and grind them in the 1x extraction buffer at a ratio of: 1gram/ 5 ml extraction buffer. This ratio may be changed according to the season and the nature of the sample. Follow the recommended ratios described in the kit manual.
3. A clear extract could be obtained by either spinning the extract at 2000 rpm for 5 minutes or by incubating it for a few hours at 4°C.
4. Deposit 100 µl of the extract per each coated ELISA well (prepared in section A). Also, deposit 100 µl/well from the negative and positive controls (kit) in the appropriate wells. Controls should be rehydrated in 1ml of distilled water, stored at 4°C and used within 5 days.
5. Cover the plate with adhesive film and incubate at 2-8 °C for overnight.
6. Wash the plate twice with 1x washing buffer (200 µl /well), then wash the plate an additional two times, 3 minutes each.

C- Deposition of conjugated antibodies

1. Dilute the 5x conjugate buffer to 1x in distilled water.
2. Dilute the conjugated antibodies to 1:100 with the 1x conjugate buffer.
3. Mix thoroughly before deposition.
4. Deposit the diluted conjugate 100 µl/ well.
5. Cover plates with adhesive film and incubate at 37°C for two hours (At the end of the incubation period samples can be stored at 4°C if necessary).
6. Wash three times with 200 µl/ well of washing buffer (1-2 min. each).

D- Deposition of substrate

1. Dilute the 5x pNPP buffer to 1x with distilled water.
2. For a plate dissolve one tablet of pNPP substrate in 12 ml of the 1x pNPP buffer (vortex for 30 seconds). Make sure that the material is dissolved.

Important: Do this step immediately before use (fresh preparation).

3. Deposit 100 µl/well of the prepared substrate.
4. Incubate wells at 37°C for 15 minutes. Leave at room temperature till reading O.D.s .

Buffers used-Dilute buffers as described in the protocol. The composition of the buffers as provided by the kit manufacturers:

Washing buffer, pH 7.4 (X20)	**Extraction buffer, pH 8.0 (X10)**	**Coating buffer, pH 9.6 (X5)**
PBS 20X	PBS X20	Na_2CO_3
Tween-20 (1%)	PVP 20%	$NaHCO_3$
NaN3 <1g/l	Tween-20 1%	NaN_3 <1g/l
	NaN_3 <1g/l	

Conjugate buffer, pH 7.05 (X5)	**Substrate buffer, pH 9.8 (X5)**
PBS – Tween	Diethanolamine
BSA	NaN_3 <1g/l
NaN_3 <1g/l	

Dealing with the results

Read the Optical Density at wavelength 405: 30 minutes, 1 hour, and 2 hours after substrate deposit. Readings at times longer than two hours might be needed for better discrimination of the treatments, especially for poorly infected samples. However, if the OD reading was good after two hours then extra readings are unnecessary.

Samples OD = crude OD reading – average OD of substrate wells.

Detection threshold

It is recommended OD of the infected sample be set to twice that of the negative control. Samples of readings above this threshold are infected. Readings which are equal to the threshold are suspect. Also, a sample of a reading close to that of the positive control is infected.

References

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