TECHNICAL SHEET No. 10

Virus Detection: *Grapevine virus A* (GVA)

Methods: Non-radioactive hybridization (Digoxigenin-labeled probes)

General

Virus detected: GVA from grapevine leaves
Methods: Non-radioactive hybridization (Digoxigenin-labeled probes)

Developed by

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Goals

Sensitive method for GVA detection

Introduction

Several methods have been developed for the detection of grapevine viruses. These methods include the use of monoclonal and polyclonal antibodies, i.e ELISA (1), molecular hybridization (2) and PCR (3). The hybridization methods involved either radioactive or digoxignin-labeled probes. The Digoxignin system is an easy, safe, and effective non-radioactive method for labeling and detecting nucleic acids. This method involve the use of Digoxigenin-labeled probes for Southern, Northern, and dot-blot hybridization. In the case of Dig DNA labeling, Digoxigenin-11-dUTP could be incorporated by the random-primed labeling method. This labeling reaction contains a template DNA, random hexa-nucleotides mixture, dNTP labeling mixture with Dig-dUTP, and Klenow enzyme, H_2O, and EDTA. The hexa-nucleotides are randomly annealed to the denatured template DNA. Then the Klenow enzyme catalyzes the labeling reaction, which is then terminated by addition of EDTA. The resultant labeled probe is then used for hybridization with the target DNA template. The probe template is detected by adding Anti-Dig-Alkaline Phosphatase antibody followed by the specified substrate. Color is precipitated in few minutes.

Materials and Methods

Extraction of nucleic acids with PEX

1. Weigh 0.1 g of grapevine leaves and freeze at -80 ºC.
2. Incubate the frozen leaf with 500 µl of 70% ethanol for 10 min.
3. Discard the ethanol and add 500 µl of PEX solution (PEX: 6.25 mM potassium ethyl xanthogenate, 100 mM Tris-HCL, 700 mM NaCl, 10 mM EDTA, pH 8) then incubate at 65 ºC for 5 min.
4. Centrifuge the samples at 14,000 rpm for 5 min.
5. Remove the tissue by toothpicks, then add 1 ml of ethanol and incubate at -80 ºC for 30 min.
6. Centrifuge the mixture at 14,000 rpm for 10 min. Dry the pellet at 65ºC, then dissolve

it with 25 µl d.d. water.

7. Incubate the dissolved pellet at 65 ºC for 15 min.

Probe Preparation

Heat 10 µl of GVA coat protein clone at 100 ºC for 10 min, then put on ice. Add the following components to the heated clone: (2 µl of hexa-nucleotide mixture, 2 µl of dNTPs, and 1 µl Klenow enzyme) and mix them. Incubate the mixture at 37 ºC for 20 h.

Molecular Hybridization and chemiluminescent detection

1. Dot blot 1 µl of the extracted RNA on nylon-membrane, and then expose the membrane to U.V. light for 5 min.
2. Prehybridize the dot blots for 2 h with pre-hybridization solution: (2.5 ml 50X SSC, 2.5 ml 10% SDS, 3.24 ml 1 M NaH_2PO_4, 1.76 ml 1 M Na_2HPO_4, 1 µl of Denhards solution and adjust the volume to 40 µl with d.d.water. Add 0.7 ml of boiled Salmon Sperm to the previous components, then boil the whole mixture for 10 min).
3. Remove the pre-hybridization solution and incubate blots with boiled probe for 16 h at 42 ºC.
4. Washing: Wash the blots twice for 5 min each with 2X SSC containing 0.1% SDS at room temperature. Then another two washes for 15 min each in 0.1 SSC containing 0.1% SDS.
5. Detection procedure: Incubate the blots at 42 C for 1 h with blocking solution: (100 mM NaCl, 50 mM Tris-HCl pH 7.5, 4% blocking reagent). We use this solution as substitute for the malic acid buffer indicated in the kit manual.
6. Wash the blots with previous washing buffers as in step 4.
7. Incubate blots with 1:5000 diluted anti-dig conjugate in blocking solution for 1 h.
8. Incubate the membrane in fresh detection buffer: (0.1 M Tris-HCl, 0.1 M NaCl, 50 mM $MgCl_2$, pH 9.5, and mixed with 200 µl NBT/BCIP).

Results

Figure 1. Sensitivity of DIG-DNA probe was assessed by dot blot hybridization. Total nucleic acids extracted with PEX from grapevines infected with grapevine leaf roll virus, and probed with GVA-cp. Serial dilutions had been done, but clear signals were observed in GVA 1:20, 1:50, 1:100 dilutions.

Discussion

Dig-labeled probes successfully detect GVA in PEX extracts. The sensitivity of the method for the detection of GVA in total nucleic acid extracts using PEX reagent is higher than using Dellaporta extraction buffer and Tri-reagent RNA extractor. This is probably due to low titer of the virus in extracted tissue.

References

1. Martelli, G.P., Saldarelli, P. and Boscia, D. (1997). Filamentous viruses of the grapevine: closteroviruses. In P.L.Monette (Ed.) Filamentous viruses of woody plants. Research Signpost, Trivandpmax, India.

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3. Minafra, A. and Hadidi, A. (1994). Sensitive detection of grapevine virus A, B and Leafroll-associated III from viruliferous mealybugs and infected tissue by cDNA amplification. J. Virol. Methods 47, 175-188.