TECHNICAL SHEET No. 1

VIRUS DETECTION: *Grapevine virus A* (GVA)

METHODS: RT-PCR, and IC-RT-PCR

General

Virus detected: GVA from grapevine leaves, petioles, and stems.
General Methods are reverse transcription PCR (RT-PCR), Immuno-Capture reverse transcription PCR.

Developed by

Name of researchers: Omar Dar-Issa, and Naim Iraki, UNESCO Biotechnology Center at Bethlehem University.
Address (Email): **omari@bethlehem.edu**; niraki@behtlehem.edu

Date: Nov. 1, 2002

Goals

Sensitive methods for GVA detection.

Introduction

Grapevine, which belongs to Vitidaceae family, is considered the second important fruit crop after olives in Palestine. Based on the Palestinian Central Bureau of Statistics (1), the total fruit-trees cultivated area in Palestine is 1,118,075 dunums, of which 83,174dunums are grapevine yards (7.4%). About three quarters of the grapevine area is located in Bethlehem and Hebron districts (1).

Till recently, 44 different viruses have been identified in grapevines worldwide, not all of them cause serious diseases though (2). Viruses causing the most important grapevine diseases in Palestine belong to three genera; Nepovirus, Closterovirus, and Trichovirus. Another disease detected in Palestine (grapevine fleck) is caused by a virus that has not been grouped yet (2).

A preliminary work conducted in1995 (3) in an attempt to determine incidence of viral infections in viticulture in Palestine has shown that infection ranges between 50-98%. The most prevalent virus was GVA followed by GLRAV-1, V-3, grapevine fleck virus, GLRaV-2, GVB, GFLV, and GLRaV-7. The highest infection with most viruses was in Bethlehem, Jenin, and Hebron areas. The GFLV, and GLRaV-7, the least common viruses among those detected in Palestine, are restricted to Bethlehem, Jenin, and Hebron areas

Grapevine A is a single stranded RNA virus belonging to the Trichovirus genus. GVA is one of the most common causes of the Rugose wood disease, particularly the syndrom known as Kober stem grooving disease (3). This disease is

highly distributed in Bethlehem and Hebron areas where infections reach 85% (3). The virus was first reported in *Vitis vinifera*; from Taranto, Italy; by Ciccarone in 19614). The symptoms caused by GVA include pits and grooves in the trunk. GVA is transmitted by a vector (very rarely); an insect; Pseudococcidae. Virus also transmitted by mechanical inoculation (only to *Nicotiana glutinosa*); or by grafting but not transmitted by contact between plants; neither by seed or pollen. Grapevine A susceptible host species are *Nicotiana clevelandii*, *Nicotiana glutinosa*, *Vitis labrusca*, *Vitis rupestris, Vitis rupestris var. Rupestris, and Vitis vinifera* (4).

Materials and Methods

For both RT-PCR and IC-RT-PCR methods, the same primer set were used to detect GVA in the tested samples:

Primers

PCR Product: 430 bp of the coat protein

Primer 1, which is a complementary sense primer:
C995: 5 AAGCCTGACCTAGTCATCTTGG 3

Primer2, which is the antisense primer: H587: 5 GACAAATGGCACACTACG 3

RNA extraction from grapevine stems and petioles for RT-PCR.

1. Cut the sample (0.5-0.7g of the stems or petioles) into pieces by a scalpel and mortar in liquid nitrogen.
2. Add 5 volumes the plant weight of citric buffer (50mM, pH 5.6) containing 2% PVP and 20mM of DIECA.
3. Mortar the samples very well with carburandum till you get green liquid homogenate.
4. Transfer the homogenate into an epindorf tube and centrifuge at 10000rpm for 10 min at 4ºC.
5. Distribute the supernatant into aliquots and store at –80C till use.
6. Dilute the extract to 40% in distilled water containing 1% of Triton X-100.
7. Incubate at 65ºC for 15 min.

cDNA synthesis (final volume 25µl)

8. Per 5µl of the extracted RNA, add 15 µl of the RT mix (RT buffer 1X, dNTPs 0.4mM, Hexa primer 100ng, 0.1M DTT, qsp with dH2O).
9. Incubate at 100ºC for 2 min for denaturation.
10. Put on ice for 2 min.
11. Incubate 10 min at room temperature (annealing temperature).
12. Add 1 unit of the RT enzyme (M-MLV) per reaction (diluted in 5µl of H2O).
13. Incubate at 37ºC for 1hour.
14. Store at –20ºC till use in the PCR.

IC-RT-PCR:

Coating:
1. Dilute the polyclonal antibodies obtained from Sanofi Kit, (make dilutions as recommended by Sanofi) in the coating buffer (carbonate bicarbonate) and deposit 100ml in each well of the ELISA plate.
2. Incubate the plate at 37ºC for 2 hours.

Extaction: (all the steps should be done at 4ºC)

1. Grind the 0.5-0.7g of the petioles in liquid nitrogen.
2. Add 5 volumes of the extraction buffer (PBS containing 2% of PVP and 0.1% of Tween 20) and mortar in the presence of carborandum.
3. Collect the homogenate and centrifuge at 10000rpm for 10 min at 4ºC.
4. Take the supernatant into a new tube.
5. At the end of the coating period, deposit 100µl of the extract (supernatant) per each well.
6. Incubate the plate at 4ºC for overnight.

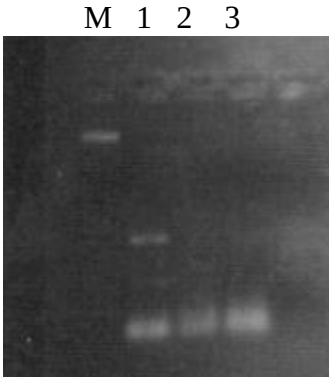
RT reaction:

7. Prepare the RT mix (RT buffer 1X, DNTPs 0.4mM, Hexa primer 100ng, 0.1M DTT, 1% Triton X-100, qsp with dH2O) and heat at 65ºC for few minutes.
7. Wash the plate 3 times with PBST.
8. Add 20ml of the heated RT mix per each well, then incubate the plate at 65ºC for 15 min.
9. Put the plate on ice and release the virus by 5-10 seconds pippitation in each well, then transfer the well content to an epidorff tube.
10. Incubate the tubes at 100ºC for 2 min, then at 4ºC (on ice) for 3 min, and finally at room temperature for 10 min.
11. Add 5µl of dH2O containing 1 unit of the RT enzyme (M-MLV) per each tube.
12. Incubate the tubes at 37ºC for 1hour.
13 Store at –20ºC till use for the PCR.

PCR conditions:
- 5µl of the cDNA, 0.2mM dNTPs, 1X Polymerase buffer including 2.5mM $MgCl_2$, 100ng of each primer, and 1 unit of the Taq Enzyme. Complete to 50µl with dH_2O. However, amounts less than 1.25 units of the Taq polymerase failed to detect GVA. Hence we used 1.25 units per reaction for detection GVA.

PCR cycles:
(Hot start: Add the Taq Polymerase while the reaction is heated at 94ºC): 94ºC for 5 min, then 5 cycles of 94ºC for 50 sec, 50ºC for 1 min, and 72ºC for 1 min. 30 cycles of 94ºC for 50 sec, 51ºC for 1 min, and 72ºC for 1 min. A final step of 72ºC for 5 min.

Results:



M: molecular weight marker.
1: IC-RT-PCR product of a tested sample.
2,3: RT-PCR products of tested samples.

Comments

The detection of GVA using the IC-RT-PCR method was much sensitive than the RT-PCR method. The results showed very faint bands in some of the tested sample using the RT-PCR method compared to the bands detected using the IC-RT-PCR method. Hence, we recommend the later technique for the detection of GVA especially in samples containing low titer of the virus which could be previously determined by ELISA or other tests..

References:

1. Palestinian Central Bureau of Statistics. Agricultural Statistics 1995/1996.

2. Hadidi ,A., H.,Khetarpal, R. and Koganezawa,H. (1998).Plant Virus Disease Control.
   Chapter: 20. APS Press. The American Phytopathological Society.St. Paul, Minnesota.

3. Ra'ed Al-Kowni MSc Thesis .1997. Institute agronomique Mediterraneen De Bari.

4. http://life.anu.edu.au/viruses/Ictv/

TECHNICAL SHEET No. 2

VIRUS DETECTION: *Grapevine Fanleaf virus* (GFLV)

METHODS: RT-PCR, and IC-RT-PCR

General

Virus detected: GFLV from grapevine leaves, petioles, and stems.
General Methods are reverse transcription PCR (RT-PCR), Immuno-Capture reverse transcription IC-RT-PCR.

Developed by

Name of researchers: Omar Dar-Issa, and Naim Iraki, UNESCO Biotechnology Center at Bethlehem University.
Address (Email): **omari@bethlehem.edu**; niraki@behtlehem.edu

Date: Nov. 1, 2002

Goals

Sensitive methods for GFLV detection.

Introduction

Degeneration is a disease in grapevines caused by Nepoviruses. This disease is spread in many grapevine-growing areas of the world. Incidence of GFLV in Palestine is about 2% (1). European Nepoviruses, referred to as, fanleaf include distorting strains that induce malformation of leaves and canes, and chromogenic strains, which cause a yellow mosaic in the plant leaves (2). A well-known example of such viruses is Grapevine Fanleaf (GFLV). The American Nepoviruses induce responses in grapevines that vary depending on the grapevine species and climatic condition. Grapevine decline, stunted growth, and low yield are the major symptoms of this disease (1). European and American Nepoviruses are both transmitted for long distance by infected propagating material and for short distance by nematodes (1).

Grapevine fanleaf is a single stranded RNA virus bleongs to the genus Nepovirus. Fanleaf disease was first reported in *Vitis vinifera*; from Austria; by Rathay in 1883 (3). The symptoms of this disease may vary according to different host species. For example, in *Vitis* spp. symptoms include green or yellow systemic mosaic, rings, line patterns and flecks, and leaf and nodal malformation. In other susceptible host species such as *Nicotiana clevelandii* the symptoms are systemic mottling and stunting. The virus is Transmitted by a vector; a nematode; *Xiphinema index* and *X. italiae* (3). Virus does not require a helper virus for vector transmission. It could also transmitted by mechanical inoculation or by grafting. The virus is neither transmitted by contact between plants; nor transmitted by pollen (although found in pollen of *Vitis*). Serology is the best tests used to identify fanleaf virus. Seedlings or

rooted cuttings of *V. vinifera* and *V. rupestris* are good bait plants in studies with nematode vectors.

Materials and Methods

For both RT-PCR and IC-RT-PCR methods, the same primer set were used to detect GFLV in the tested samples:

Primers

1. RNA polymerase coding gene, 749 bp of RNA1.

H27: 5 TTATTTGCACGCATCGGATGCGC 3
C28: 5 CGACATCAGAGAGTTCACCTAAGCC 3

2. Coat protein coding gene, 605 bp of RNA2

H: 5 GTGAGAGGATTAGCTGGTAGAGG 3
C: 5 AGCACTCCTAAGGGCCGTGACC 3

RNA extraction from grapevine stems and petioles for RT-PCR.

1. Cut the sample (0.5-0.7g of the stems or petioles) into pieces by a scalpel and mortar in liquid nitrogen.
2. Add 5 volumes the plant weight of citric buffer (50mM, pH 5.6) containing 2% PVP and 20mM of DIECA.
3. Mortar the samples very well with carburandum till you get green liquid homogenate.
4. Transfer the homogenate into an epindorf tube and centrifuge at 10000rpm for 10 min at 4ºC.
5. Distribute the supernatant into aliquots and store at –80C till use.
6. Dilute the extract to 40% in distilled water containing 1% of Triton X-100.
7. Incubate at 65ºC for 15 min.

cDNA synthesis (final volume 25µl)

8. Per 5µl of the extracted RNA, add 15 µl of the RT mix (RT buffer 1X, dNTPs 0.4mM, Hexa primer 100ng, 0.1M DTT, qsp with dH2O).
9. Incubate at 100ºC for 2 min for denaturation.
10. Put on ice for 2 min.
11. Incubate 10 min at room temperature (annealing temperature).
12. Add 1 unit of the RT enzyme (M-MLV) per reaction (diluted in 5µl of H2O).
13. Incubate at 37ºC for 1hour.
14. Store at –20ºC till use in the PCR.

IC-RT-PCR:

Coating:
1. Dilute the polyclonal antibodies obtained from Sanofi Kit, (make dilutions as recommended by Sanofi) in the coating buffer (carbonate bicarbonate) and deposit 100ml in each well of the ELISA plate.
2. Incubate the plate at 37ºC for 2 hours.

Extaction: (all the steps should be done at 4ºC)

1. Grind the 0.5-0.7g of the petioles in liquid nitrogen.
2. Add 5 volumes of the extraction buffer (PBS containing 2% of PVP and 0.1% of Tween 20) and mortar in the presence of carborandum.
3. Collect the homogenate and centrifuge at 10000rpm for 10 min at 4ºC.
4. Take the supernatant into a new tube.
5. At the end of the coating period, deposit 100µl of the extract (supernatant) per each well.
6. Incubate the plate at 4ºC for overnight.

RT reaction:

7. Prepare the RT mix (RT buffer 1X, DNTPs 0.4mM, Hexa primer 100ng, 0.1M DTT, 1% Triton X-100, qsp with dH2O) and heat at 65ºC for few minutes.
7. Wash the plate 3 times with PBST.
8. Add 20ml of the heated RT mix per each well, then incubate the plate at 65ºC for 15 min.
9. Put the plate on ice and release the virus by 5-10 seconds pippitation in each well, then transfer the well content to an epidorff tube.
10. Incubate the tubes at 100ºC for 2 min, then at 4ºC (on ice) for 3 min, and finally at room temperature for 10 min.
11. Add 5µl of dH2O containing 1 unit of the RT enzyme (M-MLV) per each tube.
12. Incubate the tubes at 37ºC for 1hour.
13 Store at –20ºC till use for the PCR.

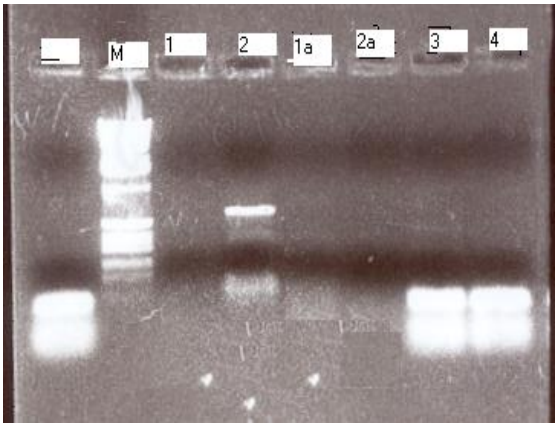
PCR conditions:
5µl of the cDNA, 0.2mM dNTPs, 1X Polymerase buffer including 2.5mM $MgCl_2$, 100ng of each primer, and 1 unit of the Taq Enzyme. Complete to 50µl with dH2O.

PCR cycles:
1. For RT-PCR
(Hot start): 94ºC for 5min then 5 cycles of 94ºC for 45seconds, 40ºC for 1 min, and 72ºC for 2 min, then 30 cycles of 94ºC for 45 seconds, 45ºC for 1 min, and 72ºC for 2 min. A final step of 72ºC for 10 min.

2. For IC-RT-PCR
(Hot start): 94ºC for 5 min, then 5 cycles of 94ºC for 50 sec, 40ºC for 1 min, and 72ºC for 1.5min. 30 cycles of 94ºC for 50 sec, 45ºC for 1 min, and 72ºC for 1.5min. A final step of 72ºC for 10 min.

Results:

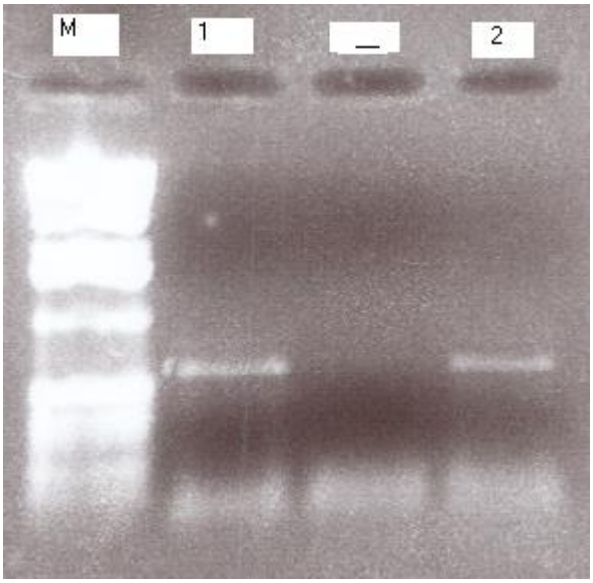
RT-PCR detection of GFLV



605

(-) : negative control, M: 1KB ladder, 1: leaf extract detected using the GFLV coat protein primer, 2: stem extract detected using GFLV coat protein primer, 1a: leaf extract detected using GFLV polymerase primer, 2a: stem extract detected using GFLV polymerase primer, 3 and 4: not related to the subject. The color of the GFLV leaf extract used for the RT-PCR was yellow, hence it might be the reason for the negative results some of symptomatic samples.

IC-RT-PCR for GFLV



M: 1KB ladder, 1 and 2: samples, (-): negative control.
Coat protien primers were used.

Comments

There was no big difference between the detection of GFLV using both methods when the coat protein primer were used. On the other hand, the Polymerase primer failed to detect the virus in RT-PCR reactions.

References:

1. Ra'ed Al-Kowni MSc Thesis .1997. Institute agronomique Mediterraneen De Bari.

2. Hadidi ,A., H.,Khetarpal, R. and Koganezawa,H. (1998).Plant Virus Disease Control. Chapter: 20. APS Press. The American Phytopathological Society.St. Paul, Minnesota.

3. http://life.anu.edu.au/viruses/Ictv/

TECHNICAL SHEET No. 3

VIRUS DETECTION: *Grapevine Leafroll Virus 1,3* (GLRV1,3)

METHODS: RT-PCR, and IC-RT-PCR

General

Virus detected: GLRV1,3 from grapevine leaves, petioles, and stems.
General Methods are reverse transcription PCR (RT-PCR), Immuno-Capture reverse transcription IC-RT-PCR.

Developed by

Name of researchers: Omar Dar-Issa, and Naim Iraki, UNESCO Biotechnology Center at Bethlehem University.
Address (Email): **omari@bethlehem.edu**; niraki@behtlehem.edu

Date: Nov. 1, 2002

Goals

Sensitive methods for GLRV1,3 detection.

Introduction

Leafroll is a widespread disease caused by Closteriviruses. Seven viruses of this genus have been detected in grapevines with leafroll symptoms (1). These viruses are called Grapevine LeafRoll associated Viruses 1-7 (GLRaV1-7). Leafroll is one of the most spread viral diseases in the Palestinian viticulture. Leafroll viruses are transmitted by infected propagating material and some of them by mealybugs (2). Since leafroll associated viruses multiply in the grapevine phloem, the leaves of such infected yard are thicker than normal with discolored margins rolled downwards.

Grapevine LeafRoll associated Viruses 1-7 which belong to the genus Closterovirus constitute of a filamentous particle and a single stranded RNA genome. The disease caused by these viruses is a widely distributed and affecting grapevine in all viticulture. It causes significant reduction in yield and quality of the crop. Preliminary survey in Palestine showed that about 30% of the trees is infected by leafroll viruses (2). The major symptoms include down rolling of leaves and inter-veinal chlorosis. Leaf rolling begins at the base of the cane and

spreads to younger leaves during midsummer. Discoloration of leaves (reddish-purple in red-fruited, see figure 3, and yellowish in white-fruited cultivars) is conspicuous in late summer and early autumn in intolerant cultivars (3). Leafroll is symptomless in some American *Vitis* spp. and hybrids (3). This disease is transmitted by mealy bugs and grafting (1).

Materials and Methods

For both RT-PCR and IC-RT-PCR methods, the same primer set were used to detect GLRV1,3 in the tested samples:

Primers

1. Designed by Hadidi and Minafra for part of the viral RNA polymerase gene, amplified fragment 340 bp:

C547 (22 bases): 5 ATTAACTTGACGGATGGCACGC 3
H229 (20 bases): 5 ATAAGCATTCGGGATGGACC 3

2. We use another primer set which was design by the Tunisian team for the coat protein gene. Amplified fragment 945 bp.

C50 (27 bases) 5 CGTAGGCTACTTCTTTTGCAATAGTTGG 3
H49 (25 bases) 5 ATGGCATTTGAACTGAAATTAGGGC 3

RNA extraction from grapevine stems and petioles for RT-PCR.

1. Cut the sample (0.5-0.7g of the stems or petioles) into pieces by a scalpel and mortar in liquid nitrogen.
2. Add 5 volumes the plant weight of citric buffer (50mM, pH 5.6) containing 2% PVP and 20mM of DIECA.
3. Mortar the samples very well with carburandum till you get green liquid homogenate.
4. Transfer the homogenate into an epindorf tube and centrifuge at 10000rpm for 10 min at 4ºC.
5. Distribute the supernatant into aliquots and store at –80C till use.
6. Dilute the extract to 40% in distilled water containing 1% of Triton X-100.
7. Incubate at 65ºC for 15 min.

cDNA synthesis (final volume 25μl)

8. Per 5µl of the extracted RNA, add 15 µl of the RT mix (RT buffer 1X, dNTPs 0.4mM, Hexa primer 100ng, 0.1M DTT, qsp with dH2O).
9. Incubate at 100ºC for 2 min for denaturation.
10. Put on ice for 2 min.
11. Incubate 10 min at room temperature (annealing temperature).
12. Add 1 unit of the RT enzyme (M-MLV) per reaction (diluted in 5µl of H2O).
13. Incubate at 37ºC for 1hour.
14. Store at –20ºC till use in the PCR.

IC-RT-PCR:

Coating:
1. Dilute the polyclonal antibodies obtained from Sanofi Kit, (make dilutions as recommended by Sanofi) in the coating buffer (carbonate bicarbonate) and deposit 100ml in each well of the ELISA plate.
2. Incubate the plate at 37ºC for 2 hours.

Extaction: (all the steps should be done at 4ºC)

1. Grind the 0.5-0.7g of the petioles in liquid nitrogen.
2. Add 5 volumes of the extraction buffer (PBS containing 2% of PVP and 0.1% of Tween 20) and mortar in the presence of carborandum.
3. Collect the homogenate and centrifuge at 10000rpm for 10 min at 4ºC.
4. Take the supernatant into a new tube.
5. At the end of the coating period, deposit 100µl of the extract (supernatant) per each well.
6. Incubate the plate at 4ºC for overnight.

RT reaction:

7. Prepare the RT mix (RT buffer 1X, DNTPs 0.4mM, Hexa primer 100ng, 0.1M DTT, 1% Triton X-100, qsp with dH2O) and heat at 65ºC for few minutes.
7. Wash the plate 3 times with PBST.
8. Add 20ml of the heated RT mix per each well, then incubate the plate at 65ºC for 15 min.
9. Put the plate on ice and release the virus by 5-10 seconds pippitation in each well, then transfer the well content to an epidorff tube.
10. Incubate the tubes at 100ºC for 2 min, then at 4ºC (on ice) for 3 min, and finally at room temperature for 10 min.
11. Add 5µl of dH2O containing 1 unit of the RT enzyme (M-MLV) per each tube.
12. Incubate the tubes at 37ºC for 1hour.
13 Store at –20ºC till use for the PCR.

PCR conditions:
5µl of the cDNA, 0.2mM dNTPs, 1X Polymerase buffer including 2.5mM $MgCl_2$, 100ng of each primer, and 1 unit of the Taq Enzyme. Complete to 50µl with dH2O.

PCR cycles:

1. For RT-PCR

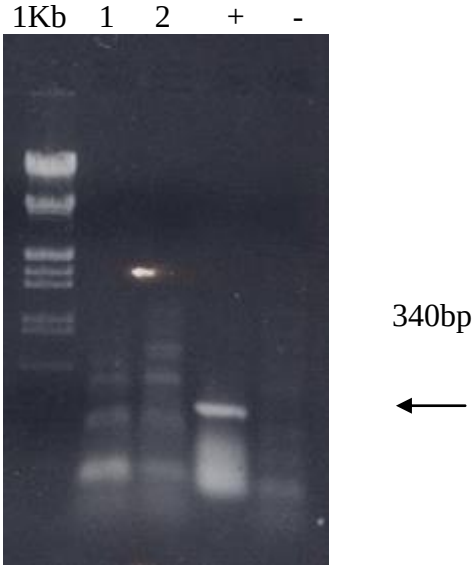
1. 1.Conditions using the Polymerase primer showed faint bands: : (Hot start): 94ºC for 5 min, then 10 cycles of 94ºC for 50 sec, 48ºC for 50 sec, and 72ºC for 1 min. 25 cycles of 94ºC for 50 sec, 54ºC for50 sec, and 72ºC for 1 min. A final step of 72ºC for 5 min.
2. Conditions using the Polymerase primer producing non specific products together with the specific one: (Hot start): 5 min at 94ºC, then 35 cycles of 40 sec at 94ºC, 50 sec at 48ºC, and 1 min at 72ºC. A final step of 5 min at 72ºC. The annealing temperature of the primer is 54, so when we reduce it to 48 by mistake, we get many non specific bands together with the specific one.

2. For IC-RT-PCR

1. Using the coat protein primer: (Hot start): 94ºC for 5 min, then 15 cycles of 94ºC for 50 sec, 45ºC for 1 min, and 72ºC for 1 min. 20 cycles of 94ºC for 50 sec, 48ºC for 1 min, and 72ºC for 1 min. A final step of 72ºC for 10 min.
2. Using the Polymerase or the coat protein primer: (Hot start):): 94ºC for 5 min, then 15 cycles of 94ºC for 50 sec, 54ºC for 1 min, and 72ºC for 1 min. 20 cycles of 94ºC for 50 sec, 46ºC for 70 sec, and 72ºC for 1 min. A final step of 72ºC for 10 min.

Results:

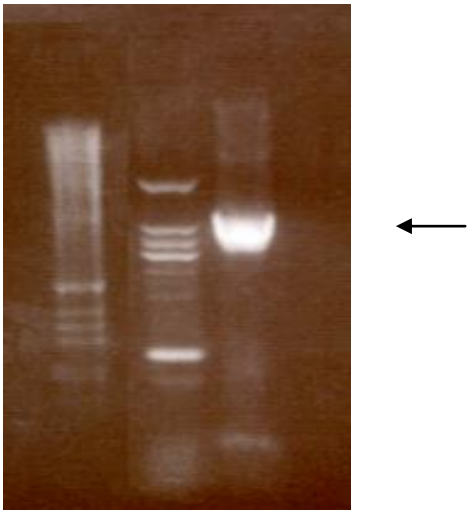
RT-PCR detection of GLRV1,3



L: Lambda ladder, 1,2: Samples amplified with the polymerase primers. +: positive control, -: negative control.

IC-RT-PCR for GLRV

1Kb 1 2



IC-RT-PCR of a grapevine sample. 1Kb: ladder, 1: the sample amplified using the polymerase primer, 2: the same sample amplified using the coat protein primer .

Comments

The virus was detected by both sets of primers. However, the coat protein primer was better since it doesn't produce non specific bands. Both the RT-PCR and the IC-RT-PCR were successfully applied for the detection of GLRV1,3 in total RNA extracts of the tested samples.

References:

5. Hadidi ,A., H.,Khetarpal, R. and Koganezawa,H. (1998).Plant Virus Disease Control. Chapter: 20. APS Press. The American Phytopathological Society.St. Paul, Minnesota.

6. Ra'ed Al-Kowni MSc Thesis .1997. Institute agronomique Mediterraneen De Bari.

7. http://life.anu.edu.au/viruses/Ictv/

TECHNICAL SHEET No. 4

VIRUS DETECTION: *Tomato Yellow Leaf Curl Virus* (TYLCV)

METHODS: Non-radioactive hybridization using the Enhanced Chemi Luminescence labeling system (ECL).

General

Virus detected: TYLCV from tomato leave and whiteflies.

Developed by

Name of researchers: Omar Dar-Issa, and Naim Iraki, UNESCO Biotechnology Center at Bethlehem University.
Address (Email): **omari@bethlehem.edu**; niraki@behtlehem.edu

Date: Nov. 10, 2002

Goals

Sensitive method for TYLCV detection.

Introduction

Tomato (*Lycopersicon esculentum)* is one of the most important vegetable crops of the family Solanaceae ,which includes about 1500 tropical and subtropical species . The genus *Lycopersicon* consists of only eight species and it is subdivided into two subgenera : *Eulycopersicon* and *Eriopersicon.* Fruits of plants of *Eulycopersicon* are usually red or yellow in color when ripe. This genus includes the cultivated tomato (1). Fruits of the *Eriopersicon* on the other hand , remain green or purple green throughout development . Tomato fruits normally contain 5-10% dry matter, of which 1% is skin and seeds. Reducing sugars such as glucose and fructose constitute nearly 50% of the dry matter . In addition to these sugars, tomato fruit also contains potentially toxic glyco-alkaloids, such as tomatine and solanine. These alkaloids protect the tomato plant against microorganisms and pests. Tomato is native to South America and Mexico (1). Large-scale cultivation of tomato did not begin until about a century ago then it became generally cultivated only after World War I . Now it is consumed all over the world and is the second largest vegetable crop in terms of dollar value (1). A report from Food and Agriculture Organization (FAO) 1993, shows that the highest production of tomato is in the U.S.A. The percentages of total world production were 15% , 0.62% for U.S.A. and the Middle East, respectively.

The most serious viral disease infecting tomatoes in the Mediterranean region is the Tomato Yellow Leaf Curl Virus (TYLCV) disease, which is the major factor limiting tomato production , during Summer, Fall and Winter cultivations. When necessary precautions are not taken (using nets or insecticides) infection may reach

100% depending on the age of plants and the time of infection. TYLCV is a monoparatite , circular , single stranded DNA genome of approximately 2.8 Kb (2). It belongs to the Geminiviridae family. Affected tomato plants are stunted, the shoots have short internodes (2). The leaves are small, curled, leathery and chlorotic (2) .The most significant effect of TYLCV infection is flower abscission. Usually, fewer than one in ten flowers set fruit, thus severely reducing the yield.

This tomato virus attacks a great variety of hosts including lentil and tobacco. TYLCV is transmitted by sweet potato whitefly (*Bemisia tabaci)* or silverleaf whitefly (*Bemisia argentifolii)*. A single whitefly is able to transmit the virus and the rate of transmission increases as the population density of the vector increases (3). No other means of transmission, such as mechanical were observed for this virus. The widespread occurrence of epidemics associated with TYLCV and it's potential threat to tomato production, make it essential to develop procedures for TYLCV detection in both *B. tabaci* and plants for disease management. Serological methods have met limited success with the whitefly -transmitted Geminiviruses. But recently, nucleic acid hybridization techniques and polymerase Chain Reaction (PCR) provide a sensitive technique for the detection and identification of TYLCV in infected plants, or the whitefly vector.

Materials and Methods

Tomato Yellow Leaf Curl Virus was detected by enhanced chemiluminescence system (ECL direct nucleic acid labeling and detection system-Amersham. RPN 3000) . Infected tomato leaves and frozen whiteflies, squashed onto a dry nylon membrane . Cloned viral template labeled with enzyme horseradish peroxidase. Positively charged peroxidase attached loosely to completely denatured negatively charged template. Addition of glutaraldehyde strengthens the attachment through formation of chemical cross-links, so that the probe becomes covalently attached to the enzyme. In hybridization the probe hybridized with target DNA immobilized on the membrane. Viral infection is detected by oxidation reduction reactions, using detection reagents which produced blue light. The light output is increased and prolonged by the presence of an enhancer , so that it can be detected on a blue-light sensitive film (see kit leaflet for more details).

Preparation of TYLCV specific non-radioactive probe:

Denature 100 ng of cloned replicative form of TYLCV-cp (available at the Hebrew university/Rehovot) at 100°C for 10 min. Cool in ice for 5 min. Add 10µl of DNA labeling reagent and 10µl of glutaraldehyde solution to the cold DNA . Incubate the probe at 37°C for 1h. Hybridize the squash blots with the labeled probe for 13h. Washed the blot twice with (1X SSC and 0.1%SDS) for 15 min.

Detection procedure :

Cover the blots with equal volumes of both detection reagents (supplied with the kit) . Place the blots in the film cassette , Expose top of the blots to autoradiography film for 1 min. Finally , Remove and develop the film.

Results

Autoradiographic detection of TYLCV-DNA with TYLCV - specific non-radioactive probe , in squashes of tomato leaf tissues and single insect (*Bemisia tabaci*). Healthy: squashes of healthy tomato leaf. Infected: squashes of TYLCV infected tomato leaf. V.W.: squash of viruliferous whitefly.

References:

1. Nelson, P. 1996. Quality attributes of processed tomato products: A review. Food Rev. Int. 12(3) , 375- 401.

2. http://hammock.ifas.ufl.edu/new/pg08400.htm

3. Mansour, A., and Al-Musa , A. 1992. Tomato yellow leaf curl virus: host range and virus-vector relationship. Plant Pathol. 41: 122 -125.

http://www.fas.usda.gov/htp/Hort_Circular/2002/02-07/Stats/Proc%20Tom.prn.pdf

TECHNICAL SHEET No. 5

VIRUS DETECTION: *Grapevine virus A (GVA)*

METHODS: Non- radioactive hybridization (Digoxigenin labeled probes)

General

Virus detected: GVA from grapevine leaves.

Developed by

Name of researchers: Omar Dar-Issa, and Naim Iraki, UNESCO Biotechnology Center at Bethlehem University.
Address (Email): **omari@bethlehem.edu**; niraki@behtlehem.edu

Date: Nov. 10, 2002

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 hexanucleoties mixture, dNTP labeling mixture with Dig-dUTP, and Klenow enzyme, H_2O, and EDTA. The hexanucleotides 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 hybridizing the target DNA template. The Probed template is detected by adding Anti-Dig-Alkaline Phosphotase followed by the specified substrate. Color is precipitated in few minutes.

Materials and Methods

Extraction of nucleic acids with PEX:

1. Wight 0.1g of grapevine leaf and froze at -80°C .
2. Incubate the frozen leaf with 500µl of 70% ethanol for 10 min..
3. Discard the ethanol and add 500µl of PEX solution (PEX :6.25mM potassium ethyl xanthogenate, 100mM Tris-HCL, 700mM NaCl , 10mMEDTA pH8) then incubate at 65°C for 5 min.

4. Centrifuge the samples at 14000 rpm for 5 minutes.
5. Remove the tissue by toothpicks, then add 1 ml of ethanol and incubate at -80°C for 30 min.
6. Centrifuge the mixture at 14000 rpm for 10 min. Dry the pellet at 65°C, then dissolve it with 25µl d.d.water.
7. Incubate the Dissolved pellet at 65°C for 15 min.

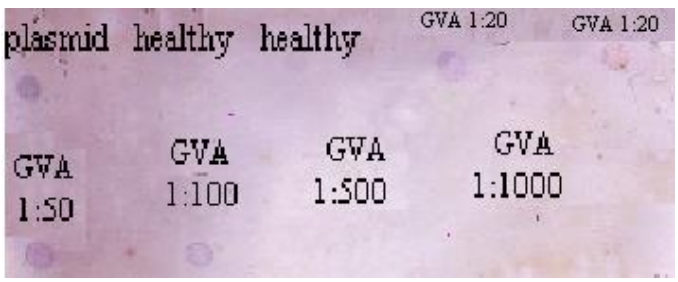
Probe preparation :

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

Molecular Hybridization and chemiluminescent detection

1. Dot blot 1µl 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 2h with pre-hybridization solution : (2.5 ml 50X SSC, 2.5ml 10%SDS, 3.24 ml 1M NaH_2PO_4, 1.76ml 1M Na_2HPO_4, 1µl of Denhards solution and adjust the volume to 40µl with d.d.water. Add 0.7ml 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 16h at 42°C.
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 0.1SSC containing 0.1%SDS.
5. Detection procedure: Incubate the blots at 42°C for 1h with blocking solution: (100mM NaCl , 50mM 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 1h.
8. Incubate the membrane in fresh detection buffer: (0.1M Tris-HCl, 0.1M NaCl, 50mM $MgCl_2$, PH 9.5, and mixed with 200µl NBT/BCIP).

Results:



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 done, but clear signals observed in GVA 1:20 1:50, 1:100 dilutions.

Comments

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 dellaporte extraction buffer and Tri-reagent RNA extractor. This is probably due to low titer of the virus in extracted tissue.

References:

8. 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, Trivandum, India.

9. Saldarelli, P., Guglielmi-Montano, H. and Martelli, G.P. (1994). Detection of three grapevine closterolike viruses by non-radioactive molecular probes. Vitis, 33, 157-352.

10. 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.

TECHNICAL SHEET No. 6

VIRUS DETECTION: *Grapevine virus A* (GVA), and *Grapevine Leaf Roll associated Virus 1,3* (GLRaV1,3)

METHOD: DAS-ELISA for the detection of the detection of GVA, GLRaV1,3

General

Virus detected: GVA and GLRaV1,3 from grapevine leaves or stems.
General Method: Double Antibody Sandwich - Enzyme Linked Immuno Sorbent Assay (DAS-ELISA).

Developed by

Name of researchers: Omar Dar-Issa, and Naim Iraki, UNESCO Biotechnology Center at Bethlehem University. According to Sanofi Diagnostics Pasteur and BIO-RAD Service Phytodiagnostics Plantest ELISA kits.
Address (Email): **omari@bethlehem.edu**; niraki@behtlehem.edu

Date: Nov. 12, 2002

Goals

Rapid method for large scale detection of GVA and GLRaV1,3.

Introduction

The Double Antibody Sandwich ELISA method involve a capturing step of the virus to a specific antibody in an ELISA well, then the virus is further captured by another antibody conjugated with an Enzyme (e.g. Alkaline Phosphotase), and a detection step using a specific substrate of the used enzyme. The developing color is finally read by an ELISA Reader. This method was successfully applied in our lab for large scale detection of GVA and GLRaV1,3. The results of the surveyed grapevine fields were compatible to those obtained when part of the samples were retested for the mentioned viruses using other detection methods such as Dig – hybridization and IC-RT-PCR.

Description of the protocol

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- <u>Sample preparation and deposition</u>:

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. Grind leaf samples 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 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. However, For GVA the positive control must be diluted in 5 ml of the extraction buffer. If your sample size is small, you could rehydrate part of the controls in the appropriate volume of distilled water.
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 additional two times 3 minutes each.

C- <u>Deposition of conjugated antibodies:</u>

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 of/ well of washing buffer (1-2 min. each).

D- <u>Deposition of substrate</u>.

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. <u>Important</u>: 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)

PBS 20X
Tween-20 (1%)
NaN3 <1g/l

Extraction buffer, pH 8.0 (X10)

Tris 2M
PVP 20%
Nacl 1.3M
Tween-20 0.5%
NaN3 <1g/l

Coating buffer, pH 9.6 (X5)

Na_2CO_3
$NaHCO_3$
NaN3 <1g/l

Conjugate buffer, pH 7.05 (X5)

PBS – Tween
BSA
NaN3 <1g/l

Substrate buffer, pH 9.8 (X5)

Diethanolamine
NaN3 <1g/l

Dealing with the results:

Read the Optical Density at wavelength 405: 30 minutes, 1hour, and 2 hours after substrate deposit.
Readings at times longer than two hours might be needed for better discrimination of the treatments especially of poorly infected samples. Sometime a reading after an overnight incubation period provided a good result. However, if the OD reading was good after two hours, there will be no need for extra readings.

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

Detection threshold:

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

References:

SANOFI Diagnostics Pasteur, S.A
BIO-RAD Service phytodiagnostics
3, Boulevard Raymond Poincare
92430 Marnes la Coquette – France.
Tel: (33) 1 47956263/6265
Fax: (33) 1 47956264

TECHNICAL SHEET No. 7

VIRUS DETECTION: *Citrus Tristeza Virus* (CTV)

METHOD: DAS-ELISA for the detection of the detection of CTV

General

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

Developed by

Name of researchers: Omar Dar-Issa, and Naim Iraki, UNESCO Biotechnology Center at Bethlehem University. According to Sanofi Diagnostics Pasteur and BIO-RAD Service Phytodiagnostics Plantest ELISA kits.
Address (Email): **omari@bethlehem.edu**; niraki@behtlehem.edu

Date: June. 28, 2004

Goals

Rapid method for large scale detection of CTV.

Introduction

Citrus is a major fruit in Palestine. It is mainly cultivated in 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 threats citrus industry in the area . One of the first quarantine measure towards prevention of the spread of this disease would be 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 involve a capturing step of the virus to a specific antibody in an ELISA well, then the virus is further captured by another antibody conjugated with an Enzyme (e.g. Alkaline Phosphotase), and a detection step using a specific substrate of the used enzyme. The developing 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.

Description of the protocol

A- Coating wells with coating antibodies:

7. Prepare the coating buffer: dilute the 5x stock solution 1:5 in distilled water.
8. Dilute the antibodies 1: 100 in the 1x coating buffer and mix thoroughly.
9. Load the diluted antibodies in the plate wells (100 µl/ well) and cover with adhesive film.

10. Incubate the plate at 37 °C for two hours. (At the end of this incubation, plates can be stored at 4 °C if necessary).
11. 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.
12. After the third wash, hit the plate gently on several layers of tissue paper until you remove all drops of liquid.

B- <u>Sample preparation and deposition</u>:

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

7. Dilute the 20x extraction buffer to 1x with distilled H_2O.
8. 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.
9. A clear extract could be obtained by either spinning the extract at 2000 rpm for 5 minutes or by incubating it for few hours at 4°C.
10. 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.
11. Cover the plate with adhesive film and incubate at 2- 8 °C for overnight.
12. Wash the plate twice with 1x washing buffer (200 µl /well), then wash the plate additional two times 3 minutes each.

C- <u>Deposition of conjugated antibodies:</u>

7. Dilute the 5x conjugate buffer to 1x in distilled water.
8. Dilute the conjugated antibodies to 1/ 100 with the 1x conjugate buffer.
9. Mix thoroughly before deposition.
10. Deposit the diluted conjugate 100 µl/ well.
11. 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).
12. Wash three times with 200 µl of/ well of washing buffer (1-2 min. each).

D- <u>Deposition of substrate</u>.

3. Dilute the 5x pNPP buffer to 1x with distilled water.
4. 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. <u>Important</u>: 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)

PBS 20X
Tween-20 (1%)
NaN3 <1g/l

Extraction buffer, pH 8.0 (X10)

PBS X20
PVP 20%
Tween-20 1%
NaN3 <1g/l

Coating buffer, pH 9.6 (X5)

Na_2CO_3
$NaHCO_3$
NaN3 <1g/l

Conjugate buffer, pH 7.05 (X5)

PBS – Tween
BSA
NaN3 <1g/l

Substrate buffer, pH 9.8 (X5)

Diethanolamine
NaN3 <1g/l

Dealing with the results:

Read the Optical Density at wavelength 405: 30 minutes, 1hour, and 2 hours after substrate deposit.
Readings at times longer than two hours might be needed for better discrimination of the treatments especially of poorly infected samples. However, if the OD reading was good after two hours, there will be no need for extra readings.

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

Detection threshold:

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

References:

SANOFI Diagnostics Pasteur, S.A
BIO-RAD Service phytodiagnostics
3, Boulevard Raymond Poincare
92430 Marnes la Coquette – France.
Tel: (33) 1 47956263/6265
Fax: (33) 1 47956264