Minimum Virus Testing Protocols for Sweetpotato Plants in the National Clean Plant Network – Sweetpotato

First Draft – December, 2015
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Target List of Sweetpotato Viruses

September 21, 2015

The NCPN-SP virus working group held meetings in Glenn Dale/Beltsville, Maryland on September 16-17, 2015. By consensus, the group felt the viruses listed below are those that should be specifically targeted in our efforts to provide clean sweetpotato planting materials in the U.S.

Potyvirus complex

Four viruses in the genus potyvirus are found essentially wherever sweetpotatoes are grown in the United States. Although they spread at different rates in the field and may occur at different incidence, they all are very common. They share similar symptoms and are transmitted by many species of aphid non-persistently. While each of these four viruses has minimal impact on sweetpotato yields when they infect plants by themselves, as plants become infected by two, three, or all four of the viruses, yields may be reduced by up to 25-40%. These thus represent a chronic problem that our clean plant programs try to overcome. Three tests are available for sensitive detection of these viruses: two PCR tests that amplify a single product for any member of the genus, and a multiplex polymerase chain reaction (PCR) testing procedure developed by Li et al. (2012) that allows sensitive, specific detection of all four viruses in one test. The viruses in descending order of frequency of detection are:

Sweet potato feathery mottle virus (SPFMV) – SPFMV is the most common sweetpotato virus and can re-infect clean plants rapidly in the field, in many cases re-infecting 100% of plants in one season when a source of SPFMV is near clean plantings. Several strains of SPFMV have been recognized: ordinary (O), East African (EA), and russet crack (RC). The RC strain has been shown to cause the russet crack disease on storage roots of the cultivar Jersey and it has been presumed but not definitively proven that the same strain causes russet crack on contemporary cultivars such as Beauregard.

Sweet potato virus G (SPVG) – although it occasionally spreads more rapidly than SPFMV, SPVG is overall second most common. It is not known to have multiple strains.

Sweet potato virus C (SPVC) – was considered to be a strain of SPFMV until 2010 but is now recognized as a separate species. Information on spread of SPVC and its effect on yield is lacking.

Sweet potato virus 2 (SPV2) – is found less frequently than the other three potyviruses above. Isolates from Louisiana have not been transmitted by aphids under experimental conditions, but isolates from other countries and possibly other regions of the U.S. have been transmitted by aphids experimentally.

Geminiviruses

This is a group of viruses that has proliferated on several important crop plants over the past 25 years. Since the first description of *Sweet potato leaf curl virus* in 1999, between 10 and 23 species of geminivirus have been found in sweetpotato, but SPLCV is the only one for which there is significant biological information. Since the Li et al. 2004 PCR test has so far reliably detected a broad range of geminiviruses, it is not clear that the distinctions among the numerous species is biologically meaningful, thus we intend to treat these viruses as a group. SPLCV does not cause foliar symptoms on most sweetpotato genotypes, but can reduce yield of Beauregard by about 30% despite the lack of symptoms. In some growing conditions, storage roots may have darker than normal skin color and appear lobed or fluted. These viruses are transmitted by whiteflies and can thus be potentially difficult to control in greenhouses where whiteflies are often hard to manage. Although geminiviruses are not known to occur commonly in commercial production of table stock sweetpotatoes, there is a lack of recent survey data and their true incidence has not been reliably determined. They are common in older cultivars of purple ornamental sweetpotatoes. Because they represent a significant risk for contamination, especially in greenhouses, and a risk for yield reduction, and because it would be difficult to recognize contamination because there are no consistent foliar symptoms, geminiviruses represent a group for which we should maintain vigilance.

Sweet potato chlorotic stunt virus

SPCSV has only been detected on three isolated occasions in the U.S. since serological or molecular methods became available to specifically identify it. However, historical reports of symptoms suggest the unproven possibility that it may have occurred more commonly in the U.S. before these methods became available. SPCSV is thought to have originated in Africa, where it interacts with SPFMV to cause a devastating disease known as Sweetpotato virus disease (SPVD) that caused 80-90% yield reductions when American cultivars were grown there. By itself, SPCSV causes very mild symptoms on sweetpotato that could easily be confused with nutrient deficiency (especially phosphorus) symptoms. There are two major strains of this virus, the West African, which has been found in the U.S., and the East African which is more severe but has not been found in the U.S. They are transmitted by whiteflies. Since sweetpotatoes growing in the greenhouse during winter months often show symptoms similar to those caused by SPCSV, and since it is whitefly transmitted, it represents a potential problem that could become economically devastating and thus requires vigilance.

NCPN-SP Minimum Protocol for Testing Sweetpotatoes for Viruses
2015

The following protocol is the result of discussions at the Sweetpotato Virus Workshop held in Beltsville and Greenbelt, Maryland, September 17-18, 2015 and subsequently among members of the virus working group of NCPN-SP. It emphasizes testing for viruses on the target list above. It is considered a minimal scheme for testing sweetpotatoes for viruses and may be augmented with additional testing at the discretion of individual centers.

Biological indexing

Vines will be grafted as scions to an *Ipomoea setosa* stock seedling following the procedures in Dennien et al., 2013. The *I. setosa* seedling should be grown out to about 10 nodes (approx. 4-6 weeks after planting) and should be grafted with 2 two-node scions from the test plant, one from the basal portion of the vine and one from near the apex of the vine. A wedge graft can be made at about 3 nodes above the cotyledonary node and a side veneer graft just below the cotyledonary node. The *I. setosa* indicator plant should be allowed to grow after grafting for 3-4 weeks and observed continually for symptom development. It should then be cut back to just above the graft site and allowed to regrow for an additional 3-4 weeks, continually observing for symptom development. Symptoms typical of different viruses are illustrated in Clark et al., 2013 and Dennien et al., 2013.

Nucleic acid tests

Sweetpotatoes for virus testing under this protocol will be established in a greenhouse with rigorous insect management to prevent exposure to known insect vectors such as aphids, whiteflies, and thrips. Each test should include a virus-tested negative control plant and a positive control. The positive control will be derived from the 'GWB' Beauregard sweetpotato held at the USDA, ARS, NGRL in Beltsville and to be backed up as tissue culture plants at the NCSU MPRU and the LSU AgCenter NCPN-SP centers. This source plant is infected with each of the target viruses: SPFMV, SPVG, SPVC, SPV2, SPCSV, and SPLCV. The NGRL will provide total nucleic acid extracts to NCPN-SP centers requesting them and plants to NCPN-SP centers that obtain the requisite PPQ 526 permit. Plants should be grown in a sandy potting medium to allow easier root collection with minimal nitrogen fertilization so as not to suppress virus symptom development. Plants should be grown out until they have 10 nodes with unfolded leaves. Leaf samples should be collected from upper, middle, and lower nodes and should utilize the basal portion of the leaf lamina (near the junction with the petiole). Root samples – collect routinely or only when leaf samples are negative or ambiguous?? Process together with leaf samples or separately?

Total nucleic acids should be extracted without nuclease treatment using either the Qiagen RNEasy Plant Mini Kit protocol as modified by Halibi and FPS lab (Appendix 1) or by the CTAB protocol of Li et al. (2008) as provided in Appendix 2. Crude lysate pellets from extracts of standards will be preserved at each location at –70 to -80^{o}C such as in step 21 in Appendix 2.

Potyvirus testing

To test for the presence of potyviruses, the degenerate primers of Ha et al. (2008) for sequences within the CI region (CIFor/CIRev) and the HC-Pro-coding region (HPFo/HPRev) will be used in PCR according to the work instructions in Appendix 3. Alternatively, the multiplex PCR for four sweetpotato potyviruses of Li et al. (2012) may be used according to the work instructions in Appendix 4, or the NIb2F/NIb3R primers of Zheng et al. (2010) may be used following the work instructions in Appendix 5.

SPCSV testing

To test for the East African and West African strains of Sweet potato chlorotic stunt virus, the multiplex one-step quantitative real time PCR protocol developed by Wei and Nakhla in the USDA, APHIS National Plant Germplasm and Quarantine Lab in Beltsville, MD will be followed according to the work instructions in Appendix 6.

Geminivirus testing

To test for the complex of sweepoviruses known to infect sweetpotato, the PCR method of Li et al., 2004 will be used according to the work instructions in Appendix 7. Alternatively, the quantitative real-time PCR method of Ling et al. may be used following the work instructions in Appendix 8.

[We did not discuss how many times each of the above tests would be conducted on each test plant or whether we would test only the sweetpotato source or also the *I. setosa* indicator plants. Currently, we do three successive grafts to *I. setosa*.]. Plants being indexed will only be considered 'virus-tested' when they have had negative tests for each of the tests indicated above.

LITERATURE

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Appendix 1 – Modified Qiagen nucleic acid extraction protocol
(Provided courtesy Maher Al Rwahinih)

RNeasy Plant Mini Kit

Protocol for purification of total RNA from plants (modified by Habili and FPS lab)

Pre-protocol set up:
- Set up the water bath at 70°C.
- Label purple and pink QIAshredder columns for the number of samples to be processed.
- Prepare fresh Guanidine isothiocyanate buffer and 20 % lauroyl sarkosyl solution.
- Label 2 sets of eppendorf tubes
- Add 70 µl 20% lauroyl sarkosyl solution to one set of eppendorf tubes.

1. Weigh out 0.3 g tissue into a BIOREBA sample bag, and add 3 ml guanidine isothiocyanate buffer. **(Prepare small amounts of Guanidine buffer fresh, based on the number of samples you will process.) See buffer composition below.**

2. Add a few crystals of sodium metabisulfite salt (on tip of small spatula) into sample bag, and grind the samples**.** (sodium metabisulfite replaces the 2-mercaptoethanol (1% vol/vol) which was added in previous protocols.

Action: Sodium metabisulfite is added as a reducing agent

Intracellular RNases are released during the lysis step of the RNA isolation procedure and must be rapidly and thoroughly inactivated to obtain high-quality RNA. Sodium metabisulfite is a reducing agent that will irreversibly denature RNases, therefore preserving the integrity of the RNA.

3. Transfer 0.5-0.7 ml of ground plant extract to Eppendorf tubes filled with 70 µl 20% lauroyl sarkosyl solution (wt/vol), using a disposable transfer pipette.

4. Mix well by inversion and incubate at 70 °C for 10 minutes (in water bath). Mix by inversion every 2-3 minutes during the incubation.

5. Mix and transfer extract by pipetting to **purple** QIAshredder columns. Spin at 14000g (or maximum speed) for 5 minutes in tabletop centrifuge.

6. Being careful not to disturb the pellet, transfer 350µl of flow-through to a clean 1.5ml tube containing 315µl of ethanol (95-100%). Mix well by pipetting, and then transfer all contents to **pink** RNeasy column. Spin for 45 seconds at 14,000g. Discard flow-through. RNA is now bound on the pink column.

This step can be repeated twice if you have excess flow –through. (optional)

7. Add 700µl of RW1 buffer to column and spin 10,000g for 15 seconds. Discard flow-through.

8. Wash with 500µl of RPE buffer (15 sec, 10,000g) and discard flow-through wash. Repeat.

9. Elute all RPE buffer by final centrifugation at 14,000g for 5 minutes.

10. Transfer columns onto clean 1.5 ml tubes provided with kit and elute RNA using 100µl of RNase-free water provided with kit (spin for 1 minute at 14,000g).

11. Store RNA at –80 °C. RNA may be aliquotted into strip tubes for easier use prior to freezing (recommend aliquots of 20 µl per sample to reduce damage to RNA from multiple freeze-thaw cycles).

Guanidine Buffer for Extractions with the RNeasy Kit*

	500 ml	250 ml	100 ml	25 ml
4M guanidine isothiocyanate	236.4 g	118.2 g	47.28 g	11.82 g
0.2 M sodium acetate (pH 5.0)	8.203 g	4.102 g	1.6401 g	0.41 g
25 mM EDTA	4.625 g	2.313 g	0.925 g	0.232 g
2.5% (w/v) PVP-40	12.5 g	6.25 g	2.5 g	0.625 g

1. In approximately half the total volume of water, stir all chemicals until dissolved
2. Solution gets cold after adding guanidine isothiocyanate – stir on low heat or stir overnight at room temp.
3. pH to 5.0 using glacial acetic acid.
4. Bring up to total volume.
5. Wrap bottle in aluminum foil and store in dark cabinet, as buffer is light-sensitive.

* Alternatively, buffer RLT from the Qiagen RNeasy kit may be used as the extraction grinding buffer; however for grapevine tissue, it is recommended that 2.5% (w/v) PVP-40 be dissolved in the buffer before using.

20% Lauroyl Sarkosyl Solution

Dissolve 20 g of N-lauroyl sarkosyl in 100 ml (total volume) of sterile water. Warm up to a maximum of 65 ºC. Filter sterilize with 0.22 μm bottle top filter (Nalgene) or syringe filter (Millipore).

Updated 1/26/2010

Appendix 2 - Li et al. 2008 CTAB extraction protocol

(Provided courtesy Ruhui Li)

CTAB-based isolation of total nucleic acids from plants for PCR/RT-PCR detection

The time of year and the growth stage of the host are critically important for virus testing. In general, matured plant tissues contain higher concentration of phenolic compounds and polysaccharides which will affect the extraction efficiency and inhibit PCR/RT-PCR reaction. Therefore, detection of viruses is best done in early season when plant tissues are succulent and temperatures are mild. Samples, usually young leaves, are collected from different shoots of a plant and pooled for isolation of total nucleic acids.

Materials

CTAB buffer (extraction buffer; see recipe)

2-mercaptoethanol

24:1 (v/v) chloroform/isoamyl alcohol

Isopropanol, room temperature

70% (v/v) ethanol

20 mM Tris-HCl, pH 8.0

2.0-ml microtubes (special type, 02-681-364 of Fisher Scientific)

Screw caps (02-681-344 of Fisher Scientific)

1.7-ml microtubes

Razor blades (optional)

1/4" Steel shot (Slingshot AMMO, Crosman Corp.)

Wooden coffee stirrer

QuickPrep 24TM instrument (MP Biomedicals)

Freezer

Water bath, dry-bath incubator or hybridization oven

Method

1. Label a set of 2.0-ml microcentrifuge tube with sample numbers and add two steel shots to each of them.

2. Tear five leaves or shoots into small pieces by hand, or slice stems or root tips into small pieces with a razor blade.

3. Weigh 0.1 g pooled plant tissue of each sample and transfer it to the labeled 2.0-ml microtube using a wooden coffee stirrer. Change gloves, razor blade (if used), weighing paper and wooden coffee stirrer between samples.

4. Add 1.2 ml CTAB buffer (add 2-mecaptoethanol immediately before use to a 0.5% final concentration) to each tube, close it with a screw cap, and label the cap.

Add the 2-mecaptoethanol under a chemical exhaust hood.

5. Cool the tube in a -20°C freezer for 10-15 min until the tubes are partially frozen.

Cooling time may vary. Make sure the buffer in the tubes is half frozen. This step is critical to reduce RNA degradation during homogenization.

6. Process the cooled tube with a QuickPrep 24® bead-mill homogenizer at speed setting 4.5 for 30 sec two times.

If a QuickPrep® bead-mill homogenizer or similar bead mill is not available, a mortar and pestle cooled to 4°C can be used to grind the sample. However, this method is not as efficient as using the FastPrep® homogenizer.

7. Incubate the microcentrifuge tubes in a water bath, a dry-bath incubator, or a hybridization oven at 65°C for 15-60 min (usually 30 min).

8. Centrifuge at 10,000 *g* in a microcentrifuge for 5 min.

9. Label a set of 1.7-ml microcentrifuge tubes with sample numbers and add 750 µl of chloroform/isoamyl alcohol (24:1).

10. Transfer 750-µl supernatant to each of the labeled tubes, close the lids well and vortex vigorously for 30 sec.

The supernatant may contain pieces of plant debris. The tubes may leak if not closed well.

11. Centrifuge at 12,000 *g* in a microcentrifuge for 10 min.

12. Label two sets of 1.7-ml microtubes with sample number, name and date and add 70% vol of isopropanol (210 µl) to the tubes.

Split each sample to two microtubes, one for test and one for storage in case.

13. Transfer 2x300 µl of the upper (aqueous) phase carefully to each of two labeled tubes for each sample and mix well.

14. Leave on bench for 10 min or store in a fridge for the next-day process if needed.

15. Centrifuge at 12,000 *g* in a microcentrifuge for 10 min.

16. Note the position of the pellet and carefully remove the supernatant by pouring. Be careful not to disturb the pellet. Dry the open edge of the tube on a paper towel. Touch different spot to avoid contamination.

17. Add 0.5 ml of 70% ethanol to the tube, and centrifuge at maximum speed in a microcentrifuge for 5 min.

18. Note the position of the pellet and carefully pour the ethanol.

Make sure not to lose the pellet.

19. Carefully remove the remaining ethanol by pipetting and air-dry the pellet on the bench for 10-15 min.

20. Close the tubes and store the samples in a freezer until use.

21. Resuspend the pellet in 100 µl of 20 mM Tris-HCl, pH 8.0, and leave the tube on ice for 10-15 min before testing.

1) This will soften the pellet.

2) One tube only, and keep another one as pellet.

22. Vortex and centrifuge the tubes to collect the extracts. Store the extracts on ice (testing) and in a freezer (storage).

CTAB buffer (500 ml)

-Add 10 g of cetyltrimethylammonium bromide (CTAB, 2%) into 400-ml distilled water

-Dissolve with stirring and a little heat (60ºC), if necessary. It takes 15-30 min.

-Add 10 g of polyvinylpyrollidone 40,000 (PVP 40,000, 2%) and 40.9 g of NaCl (1.4 M) to the solution and stir to dissolve.

-Add 20 ml of 0.5M EDTA, pH 8.0 (20 mM solution) and 50 ml of 1 M Tris-HCl, pH 8.0 (100 mM) to the solution.

-Transfer the solution to a 500-ml graduated cylinder and add distilled water to make final volume.

1) Add 2-mecaptoethanol to 0.2% immediately before use.

2) CTAB can be stored 2 to 3 months at room temperature.

Reference

Li R, Mock R, Huang Q, Abad J, Hartung J, Kinard G (2008) A reliable and inexpensive method of nucleic acid extraction for the PCR-based detection of diverse plant pathogens. J Virol Methods 154:48-55.

Appendix 3 – One-Step RT-PCR for *Potyviridae* Detection

(Provided courtesy Christie Almeyda)

Multiplex One-Step Reverse Transcriptase (RT) Conventional PCR for Potyviridae using SuperScript III One-Step RT-PCR with Platinum Taq Kit. Invitrogen. Catalog. No. 12574-026.

Master Mix	1X
Water	8.5 ul
2x Reaction mix	12.5 ul
5 uM CI-F and CI-R primer mix	2 ul
5 uM PNad5f and PNad5mr primer mix	0.5 ul
Superscript III Taq mix	0.5 ul
Total Master Mix	24 ul
RNA sample	2 ul
Final Total	26 ul

Primer	Sequence
CI-F	5'-GGIVVIGTIGGIWSIGGIAARTCIAC-3'
CI-R	5'-ACICCRTTYTCDATDATRTTIGTIGC-3'
PNad5f	5'-GATGCTTCTTGGGGCTTCTTKTT-3'
PNad5mr	5'-ATCTCCAGTCACCAACATTRGCATAA-3'

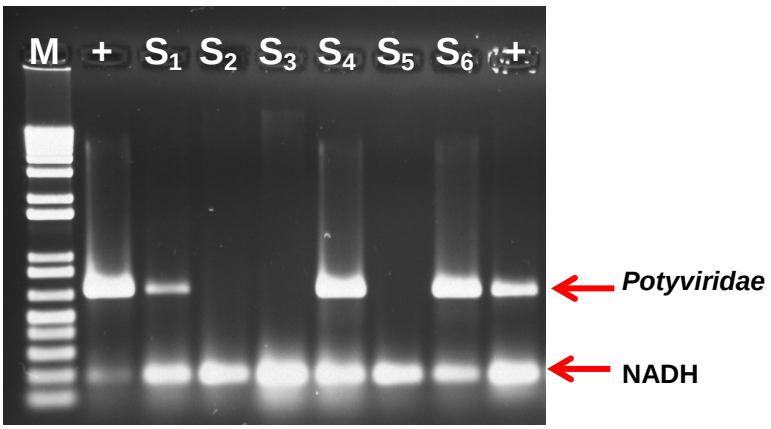
Thermocycler conditions:

48 °C x 45 min
94 °C x 2 min
[94 °C x 30 sec
45 °C x 30 sec
72 °C x 30 sec] 35 X
72 °C x 2 min
12 °C ∞

Expected Products:

Potyviridae IC primers: 700 bp
NADH internal control: 180 bp

From gel:
M: Marker
S_x: Sample1, Sample2….
+: Positive Control



Multiplex RT-PCR
Potyviridae and NADH internal control

Appendix 4 - Simultaneous Detection and Differentiation of Four Potyviruses by RT-PCR in Sweet Potato

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Sweet Potato Virus Disease (SPVD) is the most devastating disease of sweet potato (Tairo et al., 2005; Untiveros et al., 2007). The disease is usually caused by mixed infection of *Sweet potato chlorotic stunt virus* (SPCSV) with *Sweet potato feathery mottle virus* (SPFMV), *Sweet potato virus G* (SPVG), *Sweet potato mild mottle virus* (SPMMV) or other related potyviruses. Single infection of these viruses usually shows mild or no symptoms, but co-infection of SPFMV, SPVG and/or other viruses with SPCSV causes severe symptoms such as general chlorosis, stunting, leaf strapping and leaf crinkling and reduces yields by 56–100% (Milgram et al., 1996; Gibson *et al*., 1998; Karyeija *et al.*, 1998; Njeru et al., 2003; Kokkinos & Clark, 2006b; Mukasa et al., 2006; Rännäli et al., 2008).

SPFMV is a species of genus *Potyvirus* in the family *Potyviridae*. Its virions are flexuous filaments of 805-880 nm long with a single-stranded RNA of 10.5 kb (Sakai et al., 1997). The virus can be transmitted mechanically or by aphids and is widespread (Karyeija et al., 1998; Di Feo et al., 2000; Loebenstein et al., 2003; Valverde et al., 2004). Based the symptom and serological differences as well as phylogenetic analyses of the coat protein gene sequences, SPFMV can be divided into four phylogenetic lineages or strains, East Africa (EA), constituted by East African samples; Russet Crack (RC), comprising samples from Australia, Africa, Asia and North America; Ordinary (O) containing samples from Japan, China, Korea, Niger, Nigeria and Argentina; and Common (C) including samples from USA, China, Australia, East Africa and Argentina (Untiveros et al., 2008). RC, O and EA are closely related to each other but are phylogenetically distant from C. Strain EA has a much more restricted geographical distribution than the others (Kreuze et al ., 2000; Mukasa et al ., 2003; Souto et al ., 2003). The Common strain has been recently recognized as a distinct virus (Untiveros et al., 2010).

SPVG was first described as a potyvirus distinct from SPFMV in a sweet potato clone from China (Colinet et al., 1994) and has been reported in countries of Africa, Asia, America, Europe and Oceania (Souto et al., 2003; IsHak et al., 2003; Trenado et al., 2007; Rännäli et al., 2008; Untiveros et al., 2008). Phylogenetic analysis based on CP aa sequences of 22 isolates revealed a limited genetic diversity among the SPVG isolates (Rännäli et al., 2008, our unpublished data).

SPV2 was the second potyvirus described from diseased sweet potato collected from Taiwan based on its biological and serological distinctions from SPFMV (Rossel and

Thottappilly, 1988). Sequence analysis of the 3' partial genome of 2006 bp of SPV2 confirmed that it was a distinct potyvirus closely related to SPFMV, SPVG and a sweet potato potyvirus isolate from Zimbabwe (SPV-Zw). Together with SPV-Zw and two Ipomoea vein mosaic virus isolates (LSU-2, LSU-5) from the United States, SPV2 was renamed as Sweet potato virus Y (SPVY) (Ateka et al., 2004), which has been used in some literature. SPV2 occurs in most sweet potato production areas (Souto et al., 2003; Tairo et al., 2006; Ateka et al., 2007; Trenado et al., 2007; Untiveros et al., 2008). Sequence analyses of the CP aa sequences revealed identities of 81-99% among the SPV2 isolates and partial correlation of the geographic origin of the SPV2 isolates and their phylogenetic clustering (Ateka et al., 2007).

Molecular studies have shown that co-infection of SPCSV enhances SPFMV RNA viral titers by at least 600-fold, whereas SPCSV titers remain equal or are reduced as compared to single infection (Gutierrez et al., 2003; Karyeija et al., 2000; Kokkinos & Clark, 2006a; Mukasa et al., 2006). The severity of SPVD, and the degree of SPFMV titer increase, depends on the strain of SPFMV involved in the double infection. Besides SPFMV, several other potyviruses including SPVG and other unrelated viruses can cause synergistic diseases when co-infecting with SPCSV (Kokkinos & Clark, 2006b; Mukasa et al., 2006; Untiveros et al., 2007).

Detection methods including grafting, nitrocellulose membrane ELISA, membrane immunobinding and PCR-based techniques have been applied to detect SPFMV and other related potyviruses (Moyer et al., 1989; Gibb et al., 1993; Colinet et al., 1998; Dje & Diallo, 2005; Kokkinos & Clark, 2006a; Jones& Dwyer, 2007). Graft inoculation of sweet potato clones such as TIB8 infected with SPCSV requires the SPCSV-infected plants and greenhouse space and takes several months for symptom observation. Serological detection of SPFMV and related potyviruses requires antiserum or antisera that are not always available. Sequence data of many sweet potato viruses are available in GenBank database for designing primers and reagents are for easily accessible and relatively inexpensive for their RT-PCR/PCR detection. RT-PCR using potyvirus group-specific primers has broad detection spectrum but might miss the target when virus titer is low due to its reduced specificity. For RT-PCR detection of genetically diverse SPFMV and SPVG, a common application is to use primers based on conserved regions aligned from known sequences, which allows detection of most, if not all, viral strains/isolates (Colinet al., 1998; IsHak et al., 2003; Jones& Dwyer, 2007). Additional RT-PCR assays using strain-specific primers can be used to differentiate strains (Jones& Dwyer, 2007).

We describe here a one-step RT-PCR for detection and differentiation of the four closely related potyviruses in sweet potato. The assay was validated to be rapid, sensitive and reliable using the samples from our collections and from fields. It allows differentiation of these potyviruses and is cost-effective.

2. Materials and Methods

2.1. Virus sources

Seven sweet potato accessions, Q44439, Z01001, Z01015, Z01019, NH, and GWB and RC were determined to be infected by virus/viruses after graft inoculation onto the clone TIB8 and other detection assay(s) and maintained in a certificated BARC greenhouse under permit P526P-09-01564. The viruses were verified by RT-PCR/PCR and sequencing. Q44439 was infected by SPFMV; Z01001 and Z01019 by SPFMV, SPVC and SPV2; Z01015 by SPFMV, *Sweet potato leaf curl virus.* GWB is a diseased sweet potato plant (cv. Beauregard) infected with SPFMV, SPVC, SPVG, SPV2, SPCSV and SPLCV. NH is infected with SPFMV, SPVG, SPV2, SPCSV and SPLCV. RC, originally from Louisiana, is infected with SPFMV. Infection of SPV2 in Z01015 and infection of SPVC in Z01015, GWB and NH was determined by the quadruplex RT-PCR in this study, respectively.

2.2. Extraction of nucleic acid

Two extraction methods, the CTAB method (Li et al., 2008) and the Qiagen RNeasy Plant Mini Kit, were first evaluated for sample preparation in a RT-PCR assay. Since the CTAB worked very well, the method was selected for use throughout the study.

Caution should be taken to avoid contamination at every step. Wear gloves in the whole process. Use of new gloves, razor blades, wooden coffee stirrers and tips between samples is important during sample collection and weighing. Use of tips with filter barriers is also important to avoid contamination.

2.2. Primers

In initial tests, RT-PCR assays using different published primers including those described by Kreuze et al. (2000) were tested to detect SPFMV. Three pairs of PMB primers described by Jones and Dwyer (2007) were then tested in the RT-PCR assays (Table 1). New primers were also designed based on alignments of available sequences of target viruses during progress of the study.

Table 1. Primers tested in this study*

Name	Direaction	Sequence	Size	Target	Reference
NIB1536+	Sense	TAATGAAATGTAYGATGATAG	1051 bp	SPFMV	Kreuze et al., 2000
3NTR34-	Antisense	TTAAAGGCATACTAAAGATAA			
PMB-17	Sense	GARCCAGARCAGTWTGARG	530 bp	Non-RC strain	Jones & Dwyer, 2007
PMB-18	Sense	CCTGAGCAACATAACATTGG	520 bp	RC strains	
PMB-19	Sense	GGYGTKTGGACDATGATGGAYGG	361 bp	All SPFMV strains	
PMB-20	Antisense	GTGTGCCTCTCCGTATCYTCTTCTTGCG			
FMV3	Sense	AAYGGAYTRATGGTWTGGTGCAT	~470 bp	All SPFMV strains	This study
FMV4	Antisense	TGCACACCCCTCATTCCYAAGAG			
SPGF	Sense	GTATGAAGACTCTCTGACAAATTTTG	1191 bp	SPVG	
SPCF	Sense	GTGAGAAAYCTATGCGCTCTGTT	836 bp	SPVC	
SPFF	Sense	GGATTAYGGTGTTGACGACACA	589 bp	SPFMV	
SP2F	Sense	CGTACATTGAAAAGAGAAACAGGATA	369 bp	SPV2	
SPFCG2R	Antisense	TCGGGACTGAARGAYACGAATTTAA			

* Not all tested primers were listed.

2.3. RT-PCR

The protocol presented here has been optimized for the the Invitrogen One-Step RT-PCR System. One-Step RT-PCR kits from Qiagen and TaKaRa were used in a comparison study.

2.3.1. Materials

SuperScript™ III One-Step RT-PCR System

PCR strip or PCR plate

1.5 ml microcentrifuge tubes

Thermal Cycler

2.3.2. Procedure

1. Thaw 2x reaction buffer, Q primers and nucleic acid extracts on ice at RT. Centrifuge briefly to collect liquid to the bottom soon after melting and keep them on ice.

2. Label a set of PCR strips or a PCR plate (if more than 60 samples).

3. Prepare the RT-PCR master mix (19 µl per reaction):

Sterile water 0.7 µl

2X Reaction buffer 10.0 µl 10 µM SPGF primer 2.5 µl

10 µM SPCF primer 0.4 µl

10 µM SPFF primer 2.0 µl

10 µM SP2F primer 0.2 µl

10 µM SPFCG2R 2.0 µl

Enzyme Mix * 1.2 µl

*Do not add Enzyme Mix until immediately before usage

4. Add 1 µl of total nucleic acid extract to a labeled PCR tube or a well of the PCR plate.

5. Add the enzyme mix to the PCR master mix tube, and mix well by pipetting. Centrifuge briefly to collect droplets.

6. Add 19 µl of the above RT-PCR master mix to the tube or the well, and mix well by pipetting. Centrifuge briefly to collect droplets.

7. Run the PCR reaction with the following thermal cycling conditions:

50°C/30 min, 95°C/2 min, 1 cycle

94°C/30 sec, 60°C/30 sec, 65°C/1 min, 30 cycles

72°C/5 min, 1 cycle

These conditions are optimized using MJ Research PTC-200, MJ Research and a Bio-Rad C1000 thermal cycler.

8. Electrophorese 8 µl of the amplified PCR product through a 1.2% agarose gel containing 0.1 µg/ml ethidium bromide.

GelRed strain can be used to replace ethidium bromide. If a PCR plate is used for thermal cycling, the 96-well E-Gel (Invitrogen) can be used for electrophoresis.

9. View the gel under UV light to determine if the expected PCR product is present.

2.4. Cloning and sequencing

The selected PCR products were cloned onto pGEM-TV easy (Promega) and sequenced. At three clones from each PCR product were sequenced.

3. Results

3.1. Evaluation of primers

Primers PMB19/20 was designed to detect all four SPFMV strains and worked to amplify the expected PCR product from five out of seven infected accessions (Fig. 1A). Primers PMB18/20 were designed to detect only RC isolates and worked to amplify the expected product from four accessions but not our RC isolate (Fig. 1B). Primers PMB17/20 was designed to detection all non-RC strains and worked to amplify five accessions but the amplicons were not strong (Fig. 1C). Based on the RT-PCR results using all PMB three primer pairs, only five of tested accessions were infected with SPFMV, and four of these accessions were co-infected with RC and non-RC strains. However, sequence data obtained from RT-PCR clones indicated that primer pair PMB17/20 also amplified both RC and non-RC strains, and our RC strain is a true RC strain (98% identical) based on PCR clones obtained from amplicons of primer pairs PMB17/20 and PMB18/20. Therefore, two primer pairs should be used with caution.

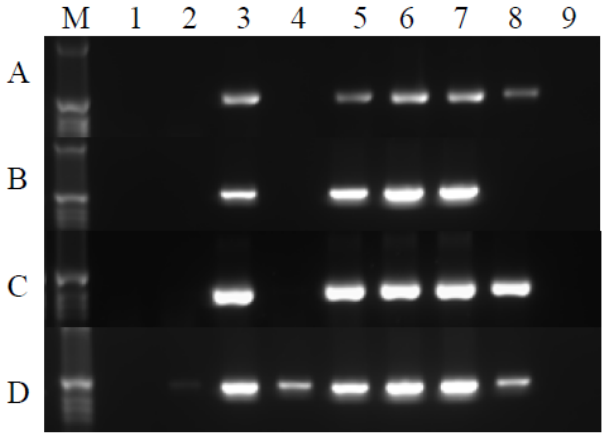

Fig. 1. Comparison of different primer pairs for RT-PCR detection of *Sweet potato feathery mottle virus* in infected sweet potato plants. Primer pair used: A) PMB17/20; B) PMB18/20; C) PMB19/20; D) FMV3/4. Lanes: M) 1 kb DNA ladder; 1) Q44429; 2) Z010001 (SPVG); 3) Z01015 (SPVC, SPFMV and SPV2); 4) Z01019 (SPVG); 5) Q44439* (SPFMV); 6) NH (SPVG, SPFMV and SPV2); 7) GWB (SPVG, SPVC, SPFMV and SPV2); 8) RC-1 (SPFMV); 9) water.
* Virus was lost in the later clone.

To expand primer detection range, more primers were designed based on alignment of available sequences of SPFMV, SPVG, SPVY and other related potyviruses and tested in RT-PCR assays. Primer pair FMV3/4 worked to amplify expected products from all seven accessions (Fig. 1D). Analyses of the obtained sequences indicated two infected accessions missed by primers PMB19/20 but detected by primers FMV3/4 were actually infected by SPVG (Table 2). The two isolates were 98% identical in 467 nt sequence to those of SPVG-Egypt 1 and two LSU isolates. Amplification of SPFMV by primers FMV3/4 was efficient but amplification of SPVG was not.

One problem with the RT-PCR using FMV3/4 was that it could not differentiate SPFMV and other three closely related potyviuses (SPVC, SPVG and SPV2). The amplicon has to be sequenced to identify the virus detected, which is not practical when a large number of samples are tested . To develop a RT-PCR for simultaneous detection and differentiation of the four viruses, several sets of four virus-specific primers and one conserved reverse primer were designed based on an alignment of all available sequences of the four viruses in the GenBank database. Only one set of primers, SPGF, SPCF, SPFF, SP2F and SPFCG2R (Table 1), amplified all four viral targets with similar efficiencies in quadruplex RT-PCR (Figs. 2, 3, 4 & 5). The identity of each amplicon was confirmed by sequencing.

3.2. Optimization of the quadruplex RT-PCR

Different concentrations and ratios of the primer set were first tested to determine the optimal system which allows similar amplification efficiencies of all four targets (Fig. 2). The

optimal concentrations of the primer set are 2.5 µl (1.25 µM) for SPGF, 0.4 µl (0.2 µM) for SPCF, 2.0 µl SPFF (1.0 µM), 0.2 µl (0.1 µM) for SP2F in the reaction volume of 20 µl.

isolates. Amplification of SPFMV by primers FMV3/4 was efficient but amplification of SPVG was not.

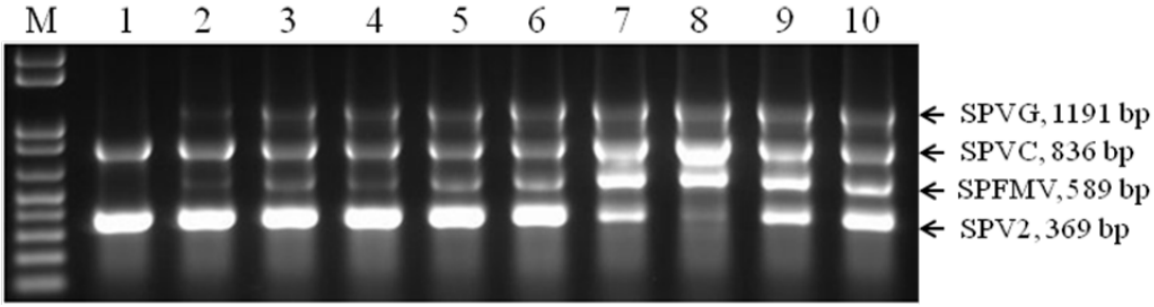

Fig. 2. Influence of primer concentrations and ratios on efficiency of the quadruplex RT-PCR. The extract from GWB was used as template in the test. M) 1 kb plus DNA ladder; primer concentration in total reaction volume of 20 µl: 1) SPGF (0.5 µl), SPCF (0.5 µl), SPFF (0.5 µl), SP2F (0.5 µl) and SPFCG2R (2.0 µl); 2) SPGF (1.0 µl), SPCF (0.5 µl), SPFF (1.0 µl), SP2F (0.5 µl) and SPFCG2R (2.0 µl); 3) SPGF (2.0 µl), SPCF (0.5 µl), SPFF (2.0 µl), SP2F (0.5 µl) and SPFCG2R (2.0 µl); 4) SPGF (2.5 µl), SPCF (0.5 µl), SPFF (2.0 µl), SP2F (0.5 µl) and SPFCG2R (2.0 µl); 5) SPGF (2.5 µl), SPCF (0.5 µl), SPFF (2.0 µl), SP2F (0.3 µl) and SPFCG2R (2.0 µl); 6) SPGF (2.5 µl), SPCF (0.4 µl), SPFF (2.0 µl), SP2F (0.3 µl) and SPFCG2R (2.0 µl); 7) SPGF (2.5 µl), SPCF (0.5 µl), SPFF (2.0 µl), SP2F (0.2 µl) and SPFCG2R (2.0 µl); 8) SPGF (2.5 µl), SPCF (0.5 µl), SPFF (2.0 µl), SP2F (0.1 µl) and SPFCG2R (2.0 µl); 9) SPGF (2.5 µl), SPCF (0.4 µl), SPFF (2.0 µl), SP2F (0.2 µl) and SPFCG2R (2.0 µl); 10) SPGF (2.0 µl), SPCF (0.4 µl), SPFF (2.0 µl), SP2F (0.2 µl) and SPFCG2R (1.2 µl).

Different cycling conditions were tested (Fig. 3), and the optimal conditions are one cycle of 50ºC for 30 min and 95 ºC for 2 min; 30 cycles of 94ºC for 30 sec, 60ºC for 30 sec and 65ºC for 1 min; and one cycle of 72ºC for 5 min (Fig. 3, lane 3).

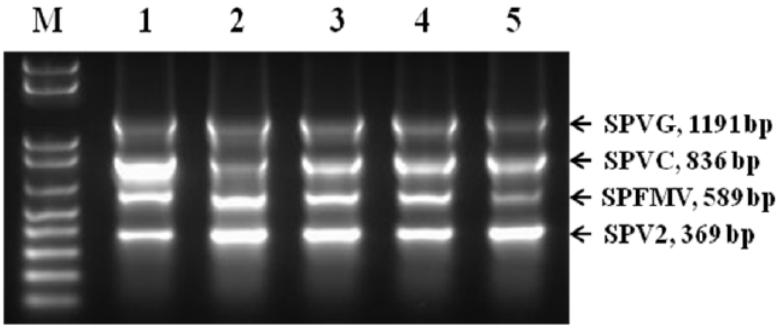

Fig. 3. Influence of thermal cycling conditions on efficiency of the quadruplex RT-PCR. The extract from GWB was used as template in the test. M) 1 kb plus DNA ladder; cycling conditions: 1) 1 cycle of 50ºC for 30 min and 95 ºC for 2 min; 30 cycles of 94ºC for 30 sec, 60ºC for 30 sec and 68ºC for 1 min; and 1 cycle of 72ºC for 5 min; 2) 1 cycle of 50ºC for 30 min and 95 ºC for 2 min; 45 cycles of 94ºC for 30 sec, 60ºC for 30 sec and 68ºC for 1 min; and 1 cycle of 72ºC for 5 min; 3) 1 cycle of 50ºC for 30 min and 95 ºC for 2 min; 30 cycles of 94ºC for 30 sec, 60ºC for 30 sec and 65ºC for 1 min; and 1 cycle of 72ºC for 5 min; 4) 1 cycle of 50ºC for 30 min and 95 ºC for 2 min; 30 cycles of 94ºC for 30 sec, 60ºC for 30 sec and 68ºC for 3 min; and 1 cycle of 72ºC for 5 min; 5) 1 cycle of 50ºC for 30 min and 95 ºC for 2 min; 45 cycles of 94ºC for 30 sec, 60ºC for 30 sec and 65ºC for 3 min; and 1 cycle of 72ºC for 5 min.

3.3. Sensitivity of the quadruplex RT-PCR

The sensitivity of the quadruplex RT-PCR was compared with those of each simplex RT-PCR (Fig. 4). The results showed that the detection limit of 10-4 of the quadruplex RT-PCR was very similar to those of the simplex ones, indicating the quadruplex is sensitive in detection of the four viruses in sweet potato.

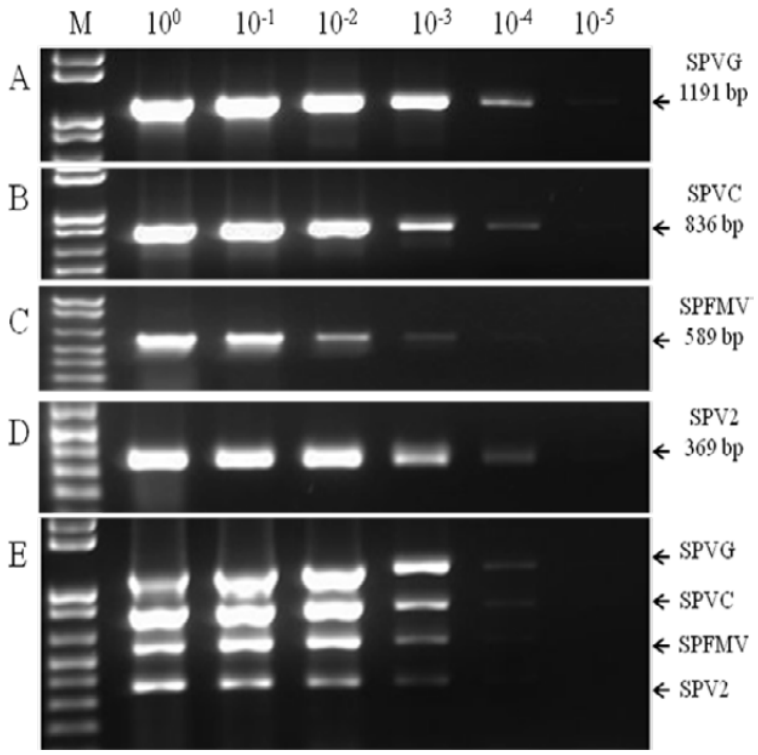

Fig. 4. Comparison of sensitivities of the simplex RT-PCR for SPVG (A), SPVC (B), SPFMV (C) and SPV2 (D) and quadruplex RT-PCR (E). The extract from GWB was used as template in the test. M) 1 kb plus DNA ladder; 1) undiluted extract; 2) 1:10 dilution; 3) $1:10^2$ dilution; 4) $1:10^3$ dilution; 5) $1:10^4$ dilution; 6) $1:10^5$ dilution.

3.4. Primer interactions

Interaction of different primers greatly affects the efficiency of multiplex RT-PCR. All possible combinations of primers were tested in RT-PCR to check the influence of the primer interactions on the amplification efficiency for each target (Fig. 5). Results showed that the efficiency for amplification of SPV2 was not affected by presence of other primers in most combinations, while the efficiency for SPVG was reduced in all combinations. Presence of SP2F affected the efficiency for both SPVC and SPFMV. Presence of all primers had affected the amplification efficiencies for all four viruses (Fig. 5, lanes 9, 10 & 12-15), however, the impact was limited (Fig. 4).

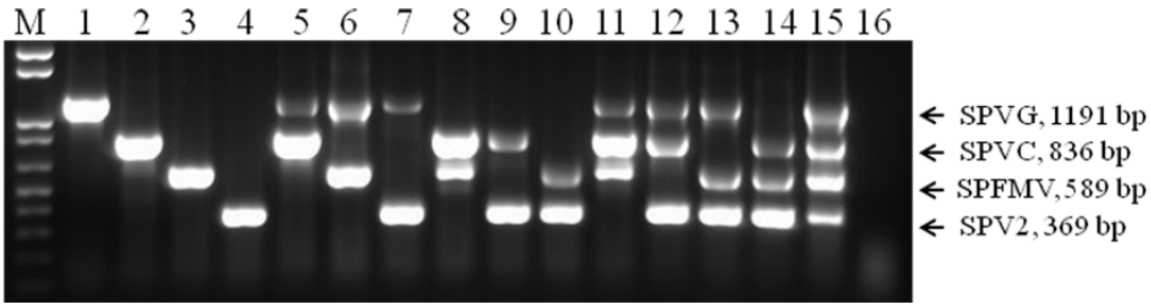

Fig. 5. Influence of different primer combinations on efficiencies of the RT-PCR assays. The extract from GWB was used as template in the test (lane 1-15). M) 1 kb plus DNA ladder; primers used: 1) SPGF/SPFCG2R; 2) SPCF/SPFCG2R; 3) SPFF/SPFCG2R; 4) SP2F/SPFCG2R; 5) SPGF+SPCF/SPFCG2R; 6) SPGF+SPFF/SPFCG2R; 7) SPGF+SP2F/SPFCG2R; 8) SPCF+SPFF/SPFCG2R; 9) SPCF+SP2F/SPFCG2R; 10) SPFF+SP2F/SPFCG2R; 11) SPGF+SPCF+SPFF/SPFCG2R; 12) SPGF+SPCF+SP2F/SPFCG2R; 13) SPGF+SPFF+SP2F/SPFCG2R; 14) SPCF+SPFF+SP2F/SPFCG2R; 15) SPGF+SPCF+SPFF+SP2F/SPFCG2R; 16) SPGF+SPCF+SPFF+SP2F/SPFCG2R for healthy control.

3.4. Effects of One-Step RT-PCR Kits

The Invitrogen One-Step RT-PCR System was compared with the Qiagen OneStep RT-PCR Kit and TaKaRa RNA PCR Kit to test the reagent specificity. The results showed that both Invitron and TaKaRa kits worked, but not the Qiagen kit (Fig. 8). The same Qiagen kit worked in triplex RT-PCR for detection of three pathogens infecting stone fruit trees, indicating the reagents of the kit were valid.

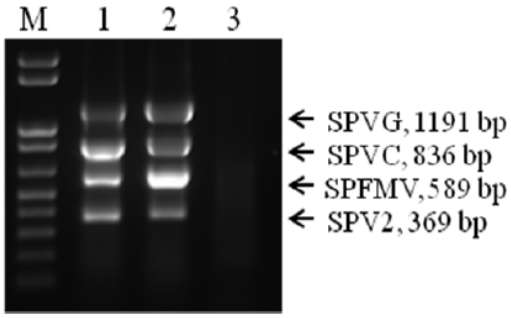

Fig. 7. Comparison of commercial one-step RT-PCR kits for the quadruplex RT-PCR. The extract from GWB was used as template in the test. M) 1 kb plus DNA ladder; 1) Invtirogen; 2) TaKaRa; 3) Qiagen.

3.5. Validation of the quadruplex RT-PCR

To confirm the reliability of this quadruplex RT-PCR, samples from our positive collection were tested (Fig. 6 & Table 2). Results not only confirmed the infections of SPFMV, SPG and SPV2 in the known positive controls, but infection of SPVC in three plants, Z01015, Q44432 and GWB. Viral identities in each plant were confirmed by sequencing.

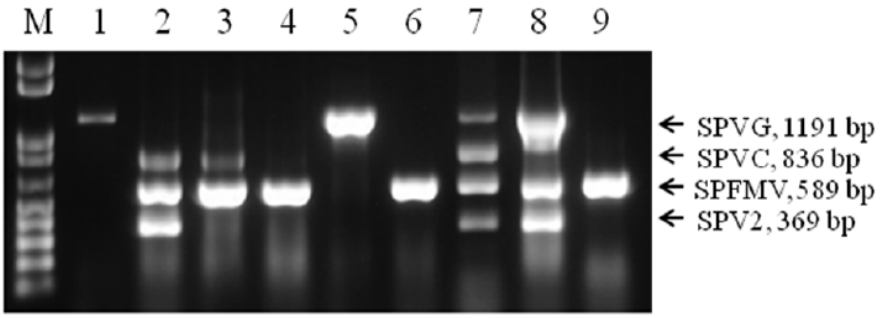

Fig. 6. Simultaneous detection and differentiation of four potyviruses in the positive collection of NGRL-PDRU by a quadruplex RT-PCR. Lanes: M) 1 kb DNA ladder; 1) Z010001; 2) Z01015; 3) Q44432; 4) Q44446; 5) Z01019; 6) RC-95; 7) GWB; 8) NH; 9) Yunnan SP230 (extract only).

Table 2. List of and primers used in this study and viruses detected in positive collection of sweet potato in our greenhouse

	Primers					
Sample Name	NIB1536+/ 3NTR34-	PMB19/ PMB20	PMB18/ PMB20	PMB17/ PMB20	FMV3/ FMV4	SPFF/SPCF/SPGF /SP2F/SPFCG2R
Q44429	-	-	-	-	-	-
Z01000	nd	nd	nd	nd	nd	-
Z01001	-	-	-	-	+	SPVG
Z01015	-	+++	+++	+	+++	SPFMV, SPVC, SPV2
Z01019	nd	-	-	-	++	SPVG
Z01045	nd	nd	nd	nd	nd	-
Z01046	nd	nd	nd	nd	nd	-
Z01057	nd	nd	nd	nd	nd	-
Q44419	nd	nd	nd	nd	nd	-
Q44432	nd	nd	nd	nd	nd	SPFMV, SPVC
Q44437	nd	nd	nd	nd	nd	-
Q44439	nd	+++	+++	+	+++	-(virus lost)
Q44446	nd	nd	nd	nd	nd	SPFMV
NH	nd	+++	+++	+	+++	SPFMV, SPVG, SPV2
GWB	nd	+++	+++	+	+++	SPFMV, SPVC, SPVG, SPV2

RC-95 nd +++ - + +++ SPFMV

A total of 279 sweet potato field samples from 16 counties of Yunnan Province, Southwestern China were also tested (Fig. 7, partial data). SPFMV, SPVC, SPVG and/or SPV2 could be detected from 123 samples (Fig. 7, partial data). In some cases, bands with medium or low intensity were observed for individual PCR products; nevertheless, all fragments were clearly identified and assigned to the respective viruses. Specifically, SPFMV was detected in 75 samples (26.88%), SPVC in 69 samples (24.73%), SPVG in 109 samples (39.07%) and SPV2 in 13 samples (4.66%). SPVG appears to be the most prevalent virus infecting sweet potatoes in Yunnan. Mixed infection is common for field samples, and 72.36% (89/123) of the positive samples were co-infected with 2 to 4 viruses. Eight of the infected samples (2.87%) were quadruplicate infected with SPFMV, SPVC, SPVG and SPV2. Thirty five of triple infected samples (12.54%) were infected with SPFMV, SPVC and SPVG, while three (1.08%) were infected with SPFMV, SPVG and SPV2, and one (0.36%) with SPVC, SPVG and SPV2. Twenty one of double infected samples (7.53%) were infected with SPVC and SPVG, while seventeen (6.09%) were infected with SPFMV and SPVG, two (0.72%) with SPFMV and SPVC, one (0.36%) with SPVC and SPV2. Twenty four (8.60%) samples were single infected with SPVG, while nine (3.23%) infected with SPFMV, and only one (0.36%) with SPVC. No SPV2 single infection was detected. Viral identities of selected samples were confirmed by sequencing.

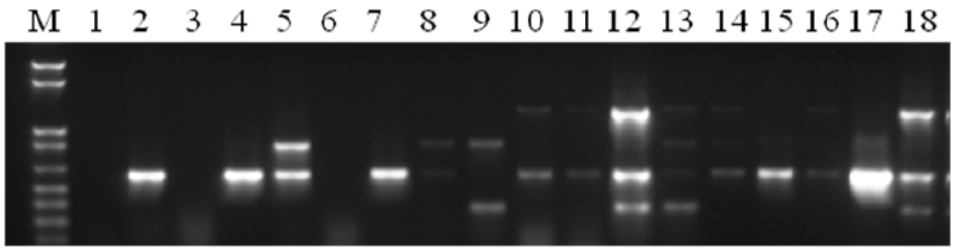

Fig. 7. Simultaneous detection and differentiation of four potyviruses in the field samples by a quadruplex RT-PCR. Lanes: M) 1 kb DNA ladder; 1-18) field samples.

3.6. Seasonal effect

It was observed that virus titer of SPVG in singly infected sweet potato is low in summer when temperature is elevated, and sometimes it is under detectable levels. However, the SPVG was detectable in other seasons.

4. RECOMMENDATION

This document presents results on a quadruplex RT-PCR for simultaneous detection and differentiation of four closely related potyviruses in sweet potato. It has been proven to be sensitive, reliable and cost-effective, and can be used in quarantine and certification programs as well as virus surveys. Both the CTAB and Qiagen extraction methods can be used to prepare the

samples. Either the Invitrogen One-Step RT-PCR Kit or TaKaRa RNA PCR Kit can be used as reagents in the test.

5. ACKNOWLEDGEMENTS

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Valverde, R.A., Lozano, G., Navas-Castillo, J., Ramos, A., Valdes, F. 2004. First report of *Sweet potato chlorotic stunt virus* and *Sweet potato feathery mottle virus* infecting sweet potato in Spain. Plant Dis. 88:428

Appendix 5 – Work instructions for the NIb2F/NIb3R PCR of Zheng et al. (2010)

The purpose of this work instruction is to describe a reverse-transcription (RT)-PCR assay for the detection of potyviruses in plant tissue. This assay can detect a wide assortment of potyviruses including *Sweet potato feathery mottle virus* (SPFMV) and is based on the protocol of Zheng *et al*. (2010)

I. Introduction

Sweet potato (*Ipomea batatas*) is host to at least six members of the plant virus genus *Potyvirus*. Four of these viruses, *Sweet potato feathery mottle virus* (SPFMV), *Sweet potato virus C* (SPVC), *Sweet potato virus G* (SPVG), and *Sweet potato virus 2* (SPV2), are present the US. Although they spread at different rates and may have different incidences in the field, they all are common in US sweet potato production areas. They share similar symptoms and are transmitted by many species of aphids in a non-persistent manner. While each of these four viruses has minimal impact on sweet potato yields when they infect plants as a single infection, as plants become infected by two, three, or all four of the viruses, yields may be reduced by up to 25-40%. Two additional members of the genus *Potyvirus* that infect sweet potato, *Sweet potato latent virus* (SPLV) and *Sweet potato mild speckling virus* (SPMSV), have not been reported in the US. SPLV is present in Africa and Asia, whereas SPMSV has been reported in Africa, South America, southeast Asia and New Zealand.

II. Related Work Instructions

Appendix 1
Appendix 2

III. Equipment, Materials and Reagents

A. Equipment

1. Conventional PCR thermal cycler
2. PCR workstation or dedicated PCR enclosure
3. Microcentrifuge
4. Minicentrifuge
5. Vortex
6. Freezer, manual defrost capable of -20^{o}C
7. Microwave
8. Analytical balance
9. Dedicated PCR pipettes, annually calibrated
10. Gel electrophoresis and documentation units
11. Dedicated gel-loading pipette(s)

B. Materials

1. Sterile filter (barrier) pipette tips
2. Microcentrifuge tubes (sterile, nuclease-free)
3. Thin-wall PCR tubes appropriate for conventional PCR thermal cycler
4. Gloves
5. Ice bucket and ice
6. Disposable lab bench mats

C. Reagents

1. SuperScript III one-step RT-PCR kit (Thermofisher Scientific Cat. 12574-018 or -026)
2. Primers (see Table 1)
3. Molecular grade (MG) water
4. 100 base pair ladder
5. 1X TAE gel running buffer
6. Agarose
7. Ethidium bromide
8. 6X gel loading dye

IV. Primers

Order primers as described in Table 1.

Table 1. Primers for Universal Potyvirus Detection

Primer name	Sequence 5'-3'	Working Concentration
NIb2F	GTITGYGTIGAYGAYTTYAAYAA	10 µM
Nib3R	TCIACIACIGTIGAIGGYTGNCC	10 µM

N=A+T+C+G, Y=C+T, I=deoxyinosine

A. Preparation

Note: Primer stock and working solutions should be prepared after receipt of new reagents. Reagent solutions are stored in small aliquots in the freezer until needed. It is recommended that new reagents be tested (using controls) as a quality control activity, prior to testing samples.

1. Tubes with lyophilized primers are centrifuged briefly (10-20 seconds at 10,000-14,000 rpm) before opening to ensure that the lyophilized material is in the bottom of the tube.

The following steps must be done in a decontaminated PCR hood/enclosure:

2. Concentrated freezer stock solutions (**100 µM**) of primers: Primers are re-hydrated to a **100µM** stock solution in MG water. Mix well by vortexing for 30 seconds and incubating on ice for 5 min. Aliquot and store at -20ºC.

   Note: If your rehydrated primers are > 12 months old, it is strongly suggested that you reorder them since degradation is one of the major reasons for failure or low efficiency of PCR

3. Working solution of primer mix (10 µM)
   For the NIb2F & NIb3R primer mix, add 10 µl of NIb2F stock solution and 10 µl of NIb3R stock solution to 80 µl of MG water. Mix well by vortexing (5-10 seconds) at setting 7-10, then centrifuge briefly (10-20 seconds) at 10,000-14,000 rpm. Store small aliquots (i.e. 25 µl) of working solution at -20ºC in 1.5 ml microfuge tubes.

V. Preparation of Master Mix

1. Remove all reagents from -20°C and thaw. Once frozen reagents are thawed, vortex briefly (5-10 seconds) at speed setting 7, and centrifuge briefly (10-20 seconds at 10,000-14,000 rpm) to settle the liquid to the bottom of the tube. Place tubes in ice.

2. In a separate ice bucket thaw prepared RNAs (if frozen) including a positive control. Vortex the tubes briefly (3-5 seconds) at a speed setting of 7 to thoroughly mix, then centrifuge 10-20 seconds at 10,000-14,000 rpm to settle the liquid at the bottom of the tube. Place tubes in ice.

3. Calculate the volume of master mix needed. Add three to the number of test samples; one for a positive control, one for a negative control, and one for pipetting error. Label a set of thermal cycler tubes for the samples determined above plus one 1.5 ml microcentrifuge tube for the master mix.

4. Prepare the master mix (see Table 3) inside a decontaminated PCR workstation, on top of a new disposable lab mat. Keep master mix on ice once prepared.

Table 3. One-Step RT-PCR Master Mix for Potyvirus Detection

Reagent	**1 Reaction** (µl)	**10 Reactions** (µl)
MG water	5.7	57
2X Reaction Mix	10	100
NIb2f/NIb3R primer mix (10 µM)	2.5	25
SuperScript III RT/Platinum Taq Mix	0.8	8
Total	*19 µl*	*190 µl*

5. Mix master mix well by pipetting up and down several times, then pipette 19 µl into each thermal cycler tube.

VI. Adding Samples and Controls

Note: Do not add control or sample RNA while working inside a PCR workstation.

1. Take the labeled master mix tubes to the sample loading area and place all items on a new disposable lab mat.

2. **Test samples**: Add 1 µl of undiluted test sample RNA to the corresponding thermal cycler tube for a total reaction volume of 20 µl.

3. **Controls**: Add 1 µl of control to the appropriate tube. Each diagnostic run should include at least two controls:
   a) Potyvirus-positive plant RNA control
   b) Non-template control (NTC) - MG water

   Note: Do not use 20 µl of master mix as your NTC. The negative control indicates contamination of master mix reagents or contamination introduced by the analyst during sample addition so it should be prepared in a similar manner including the addition of 1 µl of MG water instead of RNA.

4. Close the caps and spin the tubes in a minicentrifuge for 10 seconds. Place the tubes in the thermal cycler.

VII. Loading the Thermal Cycler and Starting the Run

1. Turn on the thermal cycler and allow the machine to run through its self-testing procedures.

2. Program Set-up:

   Program the following settings for RT-PCR reactions into the machine or select the correct saved program

   - 48°C for 45 minutes
   - 95°C for 2 minutes
   - 35 cycles of the following:
     - 95°C for 45 seconds
     - 45°C for 45 seconds
     - 72°C for 45 seconds
   - 72°C for 5 minutes
   - hold at 4°C

3. Start the run.

VIII. Gel Electrophoresis

1. Prepare a 1.5-2% (w/v) agarose gel with 1X TAE buffer. Gels should be sufficient size to accommodate the number of samples and controls, as well as two flanking DNA ladders. Sample wells must be large enough to accommodate 15 µl.

2. Load samples and controls. Mix 10 µl of PCR reaction with approximately 2 µl of gel loading dye. Mix by pipetting and load into the appropriate well.

3. Add the appropriate volume of 100 bp DNA ladder to wells flanking the sample and control wells.

4. Run the gel at 5-7 V/cm in 1X TAE buffer until the bands are adequately resolved.

5. Stain the gel for 10-15 minutes in 1.17 µg/ml ethidium bromide solution. Destain for 10-15 minutes in distilled water.

6. Document the gel using an imaging system and dispose of gloves and waste in an appropriate waste receptacle.

IX. Assessment of Results

This assay amplifies a ~350 bp product from plant tissues infected with members of the genus *Potyvirus*. To assess the results:

1. The DNA ladders must be visible and well-resolved. If yes, proceed to 2. If not, repeat the gel electrophoresis.

2. The NTC (MG water control) must not have a band present. If no bands are present, proceed to 3. If a band is present, the RT-PCR must be repeated.

3. The positive control (potyvirus positive sample) must have a distinct ~350 bp band present. If present, proceed to 4. If not present, re-evaluate the positive control; RT-PCR must be repeated.

4. Test samples with a distinct ~350 bp product are presumed positive for potyvirus. Test samples lacking this product are presumed negative

Note: no internal control is available for this assay, making it susceptible to false negative results due to i) poor RNA recovery during the extraction procedure, or ii) failure to add RNA to the RT-PCR reaction).

5. Presumed positive samples can be further evaluated by i) cloning or direct sequencing of the PCR product or ii) performing a second, independent assay to confirm its positive status.

Note: this assay cannot distinguish between species of potyviruses.

Reference:

Zheng, L, Rodoni, B.C., Gibbs, M.J., and Gibbs, A.J. 2010. A novel pair of universal primers for the detection of potyviruses. Plant Pathology 59:211-220

Appendix 6 - Work instructions for SPCSV detection

The purpose of this work instruction is to describe a multiplex, one-step quantitative reverse transcription PCR (qRT-PCR) for the detection of *Sweet potato chlorotic stunt virus* (SPCSV) – East African (EA) and West African (WA) groups. This assay can be used for SPCSV-EA and WA groups identification in quarantined plant Germplasm.

I. Introduction

Sweet potato chlorotic stunt virus (SPCSV) typically stunts sweet potato plants and causes vein yellowing or sunken veins on leaves. Symptoms may be very mild or even absent. This virus is commonly found in combination with other viruses and synergism with many of these viruses may occur (Untiveros, et al., 2007). For instance, in the case of co-infection of SPCSV with *Sweet potato feathery mottle virus* (SPFMV) there is an interaction that causes sweet potato virus disease (SPVD), a severe synergistic disease (Alicai, et al., 1999).

SPCSV is a phloem-associated virus transmitted by the whitefly *Bemisia tabaci*. It is a positive single-stranded RNA virus with a bipartite genome, being classified in the genus *Crinivirus* of the family *Closteroviridae* (Kreuze et al. 2002). Serological studies and phylogenetic analysis of the SPCSV isolates indicate that there are two distinct genetic groups: East African (EA) and West African (WA) groups (Tairo et al. 2005). This work instruction is intended to be used for identifying both the SPCSV-EA and SPCSV-EA groups.

The work instruction is for a multiplex one-step qRT-PCR using two sets of primers and probes in detection of SPCSV-EA and WA groups.

As to detection of SPCSV-EA group, this work instruction is based on SPCSV-EA strains as represented by sequences available in NCBI Nucleotide database [GB Accession #: NC_004124, AJ010754 to AJ010769, AJ811970 to AJ811972, DQ864334 to DQ864356, and HQ291260 (Peruvian isolate)]. The SPCSV-EA assay described here targets a segment of the coat protein (CP) gene on RNA2 using SPCSV-EA specific primers (SPCSVf1 and SPCSVr1) and a TaqMan FAM labeled probe (SPCSVp1). These primers and probe are encompassed by the primers used in the One-Step conventional RT-PCR assay (WI-B-T-G-xx).

As to detection of SPCSV-WA, this work instruction is based on SPCSV WA strains as represented by sequences available in NCBI Nucleotide database (GB Accession #: EU124487, AF260321, AJ278652, AJ278653, AJ515381, and EF667069). The SPCSV-WA assay described here targets a segment of the heat shock protein 70 homologue (Hsp 70h) on RNA2 using SPCSV-WA specific primers (SPCSVf3 and SPCSVr3) and a TaqMan ROX labeled probe (SPCSVp3). These primers and probe are encompassed by the primers used in the One-Step RT conventional PCR assay (WI-B-T-G-xx).

We multiplexed these two sets of virus-specific primers and probes with plant gene (Nad5) specific primers and TaqMan TET labeled probe (Nad5p1as an internal control) in a One-step RT-qPCR.

II. Related Work Instructions

WI-B-T-G-1 RNA Extraction for Potyvirus that Infect Germplasm

WI-B-T-G-xx Detection of *Sweet potato chlorotic stunt virus* (SPCSV) - East African (EA) group using a Multiplex One-Step Reverse Transcription (RT) conventional PCR

WI-B-T-G-17 Detection of *Sweet potato chlorotic stunt virus* (SPCSV) - West African (WA) group using a Multiplex Reverse Transcription (RT) quantitative PCR (qPCR) in a Cepheid SmartCycler

WI-B-T-G-19 Detection of *Sweet potato chlorotic stunt virus* (SPCSV) - West African (WA) group using a Multiplex Reverse Transcription (RT) Conventional PCR

WI-B-T-G-xx Detection of *Sweet potato chlorotic stunt virus* (SPCSV) - West African (WA) group using a Multiplex One-Step Reverse Transcription (RT) Conventional PCR

WI-B-T-G-xx Detection of *Sweet potato chlorotic stunt virus* (SPCSV) - East African (EA) group using a Multiplex One-Step Reverse Transcription (RT) quantitative PCR (qPCR) in a Cepheid SmartCycler

WI-B-T-G-xx Detection of *Sweet potato chlorotic stunt virus* (SPCSV) - West African (WA) group using a Multiplex One-Step Reverse Transcription (RT) quantitative PCR (qPCR) in a Cepheid SmartCycler

III. Equipment, Materials and Reagents

A. Equipment

1. PCR set-up hood or dedicated PCR enclosure (any vendor)
2. Cepheid SmartCycler® II
3. Cepheid SmartCycler® bench-top centrifuge (Cepheid #900-00200)
4. Cepheid cooling block (Cepheid #900-0028)
5. Freezer, non-frost free, -20ºC + 2ºC (any vendor)
6. Vortex (any vendor)
7. Dedicated, annually-calibrated pipettors (P10, P50, P200, P1000, any vendor)
8. Microcentrifuge, bench-top, capable of >10,000 rpm (any vendor)
9. Thermomixer (i.e.: Eppendorf #5350)

B. Materials

1. Sterile filter (barrier) pipette tips (P10, P50, P200, P1000, any vendor)
2. SmartCycler® tubes - 25µl (Cepheid # 900-0022)
3. Microcentrifuge tubes, 1.5-1.7 ml (pre-sterilized, certified DNase & RNase free, any vendor)
4. Microcentrifuge tubes 1.5ml Amber-colored, autoclaved (any vendor)
5. Microcentrifuge tube openers (any vendor) See note below on use
6. Ice
7. Gloves (any vendor)
8. Paper mat or towels, absorbent (any vendor)
9. Disposable, absorbent bench under pads (any vendor)

C. Reagents

Critical Reagents:

1. Invitrogen SuperScript III Platinum One-Step qRT-PCR System (Cat. No. 11732-020); *Note: Kit includes SuperScript III RT/Platinum Taq Mix, 2X Reaction Mix (a buffer containing 0.4 mM of each dNTP, and 6 mM $MgSO_4$), and 50 mM Magnesium Sulfate ($MgSO_4$)*
2. Primers and probes, see Tables 1, 2 and 3.

Non-critical reagent:

1. Molecular Grade (MG) Water (any vendor)

Note: All flip-cap microcentrifuge tubes are to be opened using a decontaminated tube opener. Tube openers and reusable tube holders/racks are decontaminated by soaking 30 min to 2 hours in 10% bleach solution, followed by two rinses in deionized water to remove bleach residue.

IV. Primers and Probes

Order primers and probes described below. *Please note that probes may take up to 14 days for delivery*

Table 1. SPCSV-EA-specific Primers and Probe

Primer or Probe *	**Sequence 5'-3'** (Synthesized by Integrated DNA Technologies, Inc.; Purification - Standard Desalting for the primers & HPLC Purification for the probe)	**Working Concentration**
Primer SPCSVf1	5' – TTT GAC TCT GAC TCC GAT GTA GG - 3'	2 µM
Primer SPCSVr1	5' – AAC CTC GCA AGA GCC AGT T - 3'	2 µM
Probe SPCSVp1	5'-/FAM/ TGT GGG AAG AAG AGA CAT GGA G /BHQ1/-3'	1 µM

**f=forward primer, r=reverse primer, p=probe*

Table 2. SPCSV-WA-specific Primers and Probe

Primer or Probe *	**Sequence 5'-3'** (Synthesized by Integrated DNA Technologies, Inc.; Purification - Standard Desalting for the primers & HPLC Purification for the probe)	**Working Concentration**
Primer SPCSVf3	5' – CGG AAT TTA TCC CAA CGT GTT TAT C - 3'	2 µM
Primer SPCSVr3	5' – GTT GAG AAG CAT CTA ACA CTT GTG - 3'	2 µM
Probe SPCSVp3	5'-/ROX/ AGC ACC ACC GAC TAT TAC ATC ACC /IAbRQSp/-3'	1 µM

**f=forward primer, r=reverse primer, p=probe*

Table 3. Internal Control Nad5 Primers and Probe (specific for plant genome)

Primer or Probe*	**Sequence 5'-3'** (Synthesized by Integrated DNA Technologies, Inc.; Purification - Standard Desalting for the primers & HPLC Purification for the probe)	**Working Concentration**
Primer Nad5f	5' - GAT GCT TCT TGG GGC TTC TTK TT - 3'	2 µM
Primer Nad5RT1r	5' – ACA TAA ATC GAG GGC TAT GCG GAT C - 3'	2 µM

Probe Nad5p1	5′-/TET/ CAT AAG TAG CTT GGT CCA TCT TTA TTC CAT /BHQ2/-3′	1 µM

** f=forward primer, r=reverse primer, p1=probe*

A. Preparation

Note: Primer and probe freezer and working solutions should be prepared after receipt of new reagents. Reagent solutions are stored in small aliquots in the freezer until needed. It is recommended that new reagents be tested (using controls) as a quality control activity, prior to testing samples.

1. Tubes with lyophilized primers and probes are centrifuged briefly (10-20 seconds at 10,000-14,000 rpm) before opening to ensure that the lyophilized material is in the bottom of the tube.

The following steps must be done in a decontaminated PCR hood/enclosure:

2. Concentrated freezer stock solutions (**100 µM**) of primers and probes: Primers and probes are re-hydrated to a **100µM** stock solution in MG water. Mix well by shaking at 800-1000 rpm speed in a thermomixer for 10 min at room temperature and store at -20ºC.

   Optional: If no thermomixer is available add MG water to the lyophilized primers or probes and vortex for 30 seconds at setting 10 after addition of MG water and then incubate on ice for 30 minutes. Vortex the tubes for 30 seconds at setting 10 before storing them at -20°C.

   Note: If your rehydrated primers or probes are > 12 months old, it is strongly suggested that you reorder them since degradation is one of the major reasons for failure or low efficiency of PCR

3. Working solutions of primer and probe mixes (**2µM** of primers and **1µM** of probe):
   a) For SPCSVf1 / SPCSVr1 / SPCSVp1 specific primers & probe mix (**2µM** working solution of each primer and **1µM** working solution of probe), mix 20 µl of the 100 µM SPCSVf1 concentrated stock, 20 µl of the 100 µM SPCSVr1 concentrated stock, and 10 µl of 100 µM SPCSVp1 concentrated stock with 950 µl of MG water.

   b) For SPCSVf3 / SPCSVr3 / SPCSVp3 specific primers & probe mix (**2µM** working solution of each primer and **1µM** working solution of probe), mix 20 µl of the 100 µM SPCSVf3 concentrated stock, 20 µl of the 100 µM SPCSVr3 concentrated stock, and 10 µl of 100 µM SPCSVp3 concentrated stock with 950 µl of MG water.

   c) For Nad5f / Nad5RT1r / Nad5p1 internal control primers & probe mix (**2µM** working solution of each primer and **1µM** working solution of probe), mix 20 µl of the 100 µM Nad5f concentrated stock, 20 µl of the 100 µM Nad5RT1r concentrated stock, and 10 µl of 100 µM Nad5p1 concentrated stock with 950 µl of MG water.

   Mix each solution well by vortexing (5-10 seconds) at setting 7-10, then centrifuge briefly (10-20 seconds) at 10,000-14,000 rpm. Store small aliquots (i.e. 25 µl) of working solutions of primers and probe mixes at -20ºC in 1.5 ml amber-colored microfuge tubes to protect the probe from degradation. Small aliquots, suitable for one-day use, of primers and probe mix

are desirable since probes are sensitive to light and freeze/thaw effects. When an assay is being conducted, the analyst should use a 'working' tube of primers and probe mix.

V. Preparation of Master Mix

1. Remove all reagents from -20°C and thaw. Since SuperScript III RT/Platinum Taq Mix does not require thawing, place immediately on ice. Once frozen reagents are thawed, vortex briefly (5-10 seconds) at speed setting 7, and centrifuge briefly (10-20 seconds at 10,000-14,000 rpm) to settle the liquid to the bottom of the tube. Place tubes in ice.

2. In a separate ice bucket thaw prepared RNAs (if frozen) including controls (See Section VI-3). Vortex the tubes briefly (3-5 seconds) at a speed setting of 7 to thoroughly mix, then centrifuge 10-20 seconds at 10,000-14,000 rpm to settle the liquid at the bottom of the tube. Place tubes in ice.

3. Calculate the volume of Master Mix needed. Include the number of RNA test samples and controls. To ensure sufficient volume prepare approximately 10% extra reaction mix (i.e.: 1-2 extra reactions for every 10-20 reactions). Label a set of Cepheid tubes for the number determined above plus one 1.5 ml microcentrifuge tube for the Master Mix.

4. Prepare the Master Mix (see Table 3) inside a decontaminated PCR workstation, on top of a new disposable lab mat. Keep Master Mix on ice once prepared.

Table 3. SPCSV-EA & WA Multiplex One-Step Quantitative RT-PCR Master Mix

Reagent	**1 Reaction** (µl)	**10 Reactions** (µl)
MG water	0.5	5.0
2 X Reaction Mix	12.5	125.0
$MgSO_4$ (50mM)	0.5	5.0
SPCSV-EA fpr Mix (2µM SPCSVf1 & 2µM SPCSVr1 & 1µM SPCSVp1 with FAM)	3.0	30.0
SPCSV-WA fpr Mix (2µM SPCSVf3 & 2µM SPCSVr3 & 1µM SPCSVp3 with ROX)	3.0	30.0
Nad5fpr Mix (2µM Nad5f & 2µM Nad5RT1r & 1µM Nad5p1 with TET)	3.0	30.0
SuperScript III RT/Platinum Taq Mix	0.5	5.0
Total	*23.0 µl*	*230.0 µl*

5. Mix Master Mix well by pipetting up and down several times, then pipette 23.0 µl into each Cepheid tube sitting in the Cepheid cold block. Lightly close the caps on the Cepheid tubes to prevent contamination during transportation to sample addition area.

VI. Adding Samples and Controls

1. Take the Cepheid cold block containing Master Mix tubes to the Cepheid cycler station and place all items on a new disposable lab mat. (Tubes should be labeled.)

2. **Test samples**: Add **2 µl** of **undiluted** test sample RNA to the corresponding Cepheid tube for a total reaction volume of 25 µl.

   Note: Do not add control or sample RNA while working inside a PCR enclosure.

3. **Controls**: Add **2 µl** of control to the appropriate tube. Each diagnostic run should include at least three controls:
   a) SPCSV-EA positive plant RNA control (NPGBL uses undiluted positive control)
   b) SPCSV-WA positive plant RNA control (NPGBL uses undiluted positive control)
   c) Healthy sweet potato plant RNA control
   d) Non-template control -MG water control (NTC-water) for the qPCR reaction

   Note 1: Controls should be tested prior to being used with samples to ensure they produce acceptable Cts for both TET and FAM (SPCSV-EA) or ROX (SPCSV-WA). If Ct values are not within specification, retest to determine the control is valid. If not, then re-extract new RNA control. Retest to confirm quality and acceptability of the new control.

   Note 2: Do not use 25 µl of Master Mix as your NTC. The negative control indicates contamination of master mix reagents or contamination introduced by the analyst during sample addition so it should be prepared in a similar manner including the addition of 2 µl of MG water instead of RNA.

4. Close the caps and spin the Cepheid tubes in the Cepheid centrifuge for 10 seconds. Make sure no bubbles remain on the sides of the flat diamond-shaped area of the Cepheid tube. Place the tubes in the SmartCycler® in the appropriate I-core modules.

VII. Loading Smart Cycler® and starting the run

1. Turn on the SmartCycler® Block first, followed by the SmartCycler® software. If not done in this order you will get an error message.

2. Program Set-up:
   - Stage 1: Hold at 55°C for 900 seconds with optics OFF
   - Stage 2: Hold at 95°C for 120 seconds with optics OFF
   - Stage 3: repeat 40 times and 2-Temperature Cycle.
     i. the first temperature cycle, set 95ºC for 15 second with optics OFF
     ii. the second temperature cycle, set **61ºC** for 30 seconds with optics ON.

   Use a temperature ramping rate of 3.0 degrees per second.

3. To begin amplification: (instructions contained in the SmartCycler® manual)
   i. Select “Create Run”
   ii. Give the run a name (i.e.: date and protocol)
   iii. Select dye set (for this qPCR it is “FTTC25”)
   iv. Select “add/Remove Sites”

v. Select the protocol
vi. Select the sites with the samples and click on ► symbol to add sites to the right column, and click on OKAY.
vii. Select "Start Run" found in the lower left-hand corner of the screen

4. To analyze data please refer to the thermocycler Manufacturer's instructions to view results table and curves.

VIII. Assessment of Results

The TET dye is specific for the internal control Nad5 reaction. The FAM dye is specific for the SPCSV-EA target reaction and the ROX dye is specific for the SPCSV-WA target reaction.

A. Control Reaction Assessment

All controls must be determined to be valid prior to test sample evaluation.

1. **Non-template control (MG water) for the qPCR reaction**
If the non-template control for the qPCR reaction tests positive (FAM Ct > 0.00 and/or ROX Ct > 0.00 and/or TET Ct > 0.00), then the entire run is invalid and all samples must be retested. (This indicates contamination of the qPCR run.)

2. **Healthy plant RNA control.** This control is to detect Nad5 gene from the host plant.
a. Nad5 internal control reaction:

i. If the Nad5 internal control of the Healthy plant RNA control is $17 \leq$ TET Ct ≤ 30 then the control is valid.

ii. If the Nad5 internal control of the Healthy plant RNA control has a TET > 30, the control is invalid and cannot be used. (To resolve, see section VI.3, Note 1.)

iii. If the Nad5 internal control of the Healthy plant RNA control has a TET < 17.00, dilute the RNA 1:10 (2 µl RNA in 18 µl MG water) and retest.

b. If the FAM Ct of the Healthy plant RNA control is FAM > 0.00, then the entire run is invalid. This result would indicate contamination with pathogen target, which could have occurred either during the RNA extraction or the qPCR run.

i. Retest this control using this Work Instruction. If the Healthy plant RNA control retest again produces a FAM Ct > 0.00, re-extract RNA (using WI-B-T-G-1) as it does not pass quality control. (See section VI.3, Note 1.)

- Using the new Healthy plant RNA retest the control and samples. If the TET Ct is between $17 \leq$ TET Ct ≤ 30 and the FAM Ct = 0 for the new Healthy plant RNA control then the rerun of the samples and controls is valid.

c. If the ROX Ct of the Healthy plant RNA control is ROX > 0.00, then the entire run is invalid. This result would indicate contamination with pathogen target, which could have occurred either during the RNA extraction or the qPCR run.

i. Retest this control using this Work Instruction. If the Healthy plant RNA control retest again produces a ROX Ct > 0.00, re-extract RNA (using WI-B-T-G-1) as it does not pass quality control. (See section VI.3, Note 1.)

- Using the new Healthy plant RNA retest the control and samples. If the TET Ct is between 17 ≤ TET Ct ≤ 30 and the ROX Ct = 0 for the new Healthy plant RNA control then the rerun of the samples and controls is valid.

3. **SPCSV-EA-positive plant RNA control**. This control is from an infected plant and should have a positive Ct value for both FAM and TET TaqMan labels.

a. Nad5 internal control reaction:

i. If the Nad5 internal control of the SPCSV-EA-positive plant RNA control is 16 ≤ TET Ct ≤ 30 then the control is valid.

ii. If the Nad5 internal control has a TET Ct > 30 the control is invalid and cannot be used. Failure to detect the plant internal control could mean that the one-step quantitative RT-PCR reaction failed or the RNA extraction failed. (To resolve, see section VI.3, Note 1.)

b. Target virus positive control reaction of the SPCSV-EA-positive plant:

i. If the SPCSV-EA plant RNA positive control has a FAM = 0.00, the SPCSV-EA positive control is invalid. Failure to detect the virus could mean the following:

1. The one-step quantitative RT-PCR reaction failed. The real-time PCR should be re-run for all the samples and controls using this work instruction.

OR 2. The virus may not be present in the plant and a new positive control plant should be found.

ii. To be a useful SPCSV-EA positive plant RNA positive control, it should produce a FAM Ct in the range of 16 ≤ FAM Ct ≤ 28.

1. If the SPCSV-EA plant RNA positive control had a FAM Ct < 16, this control RNA should be diluted 1:10 and retested.

2. If the SPCSV-EA-positive plant RNA control has a FAM Ct > 3 Cts greater than what is expected as the upper Ct limit then this control is not valid. This could indicate that the RNA has degraded or has too low a concentration to be used as a positive control and may need to be regenerated using WI-B-T-G-1 for a higher concentration of RNA.

4. **SPCSV-WA-positive plant RNA control**. This control is from an infected plant and should have a positive Ct value for both ROX and TET TaqMan labels.

a. Nad5 internal control reaction:

i. If the Nad5 internal control of the SPCSV-WA-positive plant RNA control is 16 ≤ TET Ct ≤ 30 then the control is valid.

ii. If the Nad5 internal control has a TET Ct > 30 the control is invalid and cannot be used. Failure to detect the plant internal control could mean that the one-step quantitative RT-PCR reaction failed or the RNA extraction failed. (To resolve, see section VI.3, Note 1.)

b. Target virus positive control reaction of the SPCSV-WA-positive plant:

i. If the SPCSV-WA plant RNA positive control has a ROX = 0.00, the SPCSV-WA positive control is invalid. Failure to detect the virus could mean the following:

1. The one-step quantitative RT-PCR reaction failed. The real-time PCR should be re-run for all the samples and controls using this work instruction.

OR 2. The virus may not be present in the plant and a new positive control plant should be found.

ii. To be a useful SPCSV-WA positive plant RNA positive control, it should produce a ROX Ct in the range of 16 ≤ ROX Ct ≤ 28.

1. If the SPCSV-WA plant RNA positive control had a ROX Ct < 16, this control RNA should be diluted 1:10 and retested.

2. If the SPCSV-WA-positive plant RNA control has a ROX Ct > 3 Cts greater than what is expected as the upper Ct limit then this control is not valid. This could indicate that the RNA has degraded or has too low a concentration to be used as a positive control and may need to be regenerated using WI-B-T-x-x for a higher concentration of RNA.

5. Test Sample Nad5 internal control

The Nad5 internal control of the test sample should be in the range of 16 ≤ TET Ct ≤ 30.

i. If the TET Ct > 30 the sample should be retested.

- If the sample retest again produces a TET Ct > 30, new RNA must be prepared (using WI-B-T-G-1) as it does not pass quality control and retested.

ii. If the TET Ct < 16, this sample RNA should be diluted 1:10 and retested

B. Test Sample SPCSV-EA FAM Reaction Assessment

Test Sample SPCSV-EA reactions can only be determined after the controls are all determined to be valid, including the test sample Nad5 internal control.

1. If a test sample RNA produces a FAM Ct = 0.00 then it is determined to test negative for SPCSV-EA.

2. If a test sample RNA produces a FAM Ct value in the range of 16 ≤ FAM Ct ≤ 36, the test sample RNA should be retested with this work instruction to confirm the first run result.

    • If the test sample RNA retest again produces a FAM Ct in the range 16≤ FAM Ct ≤ 36 then it is determined to be positive for SPCSV-EA (if all other PCR controls are valid).

Note: A sample that consistently produces a FAM Ct >36 may need to be re-sampled and extracted.

C. Test Sample SPCSV-WA ROX Reaction Assessment

Test Sample SPCSV-WA reactions can only be determined after the controls are all determined to be valid, including the test sample Nad5 internal control.

1. If a test sample RNA produces a ROX Ct = 0.00 then it is determined to test negative for SPCSV-WA.

2. If a test sample RNA produces a ROX Ct value in the range of 16 ≤ ROX Ct ≤ 36, the test sample RNA should be retested with this work instruction to confirm the first run result.

    • If the test sample RNA retest again produces a ROX Ct in the range 16 ≤ ROX Ct ≤ 36 then it is determined to be positive for SPCSV-WA (if all other PCR controls are valid).

Note: A sample that consistently produces a ROX Ct > 36 may need to be re-sampled and extracted.

A sample that produces a ROX Ct < 16 needs to be diluted and re-tested.

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Document Revision History

Status (Original/Revision/Cancelled)	Document Revision Number	Effective Date	Description
Original	Original	xx-xx-2011	To baseline the work instruction.

Approved By Renee M. DeVries, NPGBL Quality Manager
Approved By Laurene Levy, NPGBL Technical Manager

Appendix 7 – Work instructions for geminivirus PCR protocol of Li et al. (2004)

Detection of Geminiviruses Infecting Sweet Potato by Polymerase Chain Reaction

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Introduction

Geminviruses of the family *Geminiviridae* is plant viruses that have a circular single-stranded DNA genome encapsidated within twinned isometric particles (Fauquet et al., 2003). The viruses in the family are divided into seven genera based on insect vector, host range, and genome organization. Members of the genus *Begomovirus* are transmitted by whiteflies, have single or bipartite component genomes, and infect dicotyledonous plants. Occurrence of the geminiviruses in sweet potato is widespread (Clark et al., 2012), and they have been commonly found in imported germplasm by virus-indexing in quarantine (our unpublished data).

At least 13 different geminiviruses infect sweet potato, and they are of particular significance to quarantine programs because infected plants are essentially symptomless, and recombination or reassortment among species and strains could lead to occurrence of more virulent strains or species. To detect these viruses, vine segments from sweet potato are graft-inoculated onto an indicator host, *I. setosa*, which develops symptoms if the source material was infected (Lotrakul et al., 1998). Infected materials undergo meristem tip culture with or without therapeutic treatments for virus elimination, but the rate of eradication is low. To identify a virus-free clone, many *in vitro* plantlets have to be grown in the greenhouse, and then tested two times by the grafting assay.

Nucleic acid-based techniques, including polymerase chain reaction (PCR), offer the potential of great savings in time, greenhouse space, efficiency and cost. The genomic sequences of the geminiviruses that infect *Ipomoea* spp., and those for many other geminiviruses, are available and can be utilized for designing primers for detection of geminiviruses in sweet potato by PCR. We developed a PCR assay for the detection of SPLCV and several other geminiviruses. The assay was used to test *in vitro* plantlets generated from infected sweet potatoes, greenhouse-grown sweet potato plants, and grafted indicator plants, and the sensitivity of the PCR was compared with that of the grafting assay.

Materials and Methods

Plant materials and sample preparations

Plant leaves are collected from several positions of a plant, and used to obtain total nucleic acids by a CTAB extraction method (Li et al., 2008) (see the protocol provided).

**Change gloves and other items (razor blade etc.) between samples in both sample collection and extraction.*

Primers

SPG 1	Forward	912 bp	CCCCKGTGCGWRAATCCAT
SPG 2	Reverse		ATCCVAAYWTYCAGGGAGCTAA
MDHh*	Forward	390 bp	GCATCTGTGGTTCTTGCAGG
MDHc*	Reverse		CCTTTGAGTCCACAAGCCAA

** You may try other internal primers.*

Materials

Taq DNA polymerase from several sources (Invitrogen, New England Biolabs were tested and there was no significant difference among them) were tested. The protocol was originally optimized for the Invitrogen Taq DNA Polymerase, but switched to use GoTaq Green Master Mix (Promega). Reaction conditions should be optimized if reagents and conditions are changed.

Procedure

1. Thaw GoTaq Green Master Mix, primer solutions and the extracts on bench (fast) or on a ice basket (slow). Centrifuge briefly to collect liquid to the bottom soon after melting and keep them on ice.

2. Label a set of PCR strips or a PCR plate (if more than 60 samples).

3. Prepare the PCR master mix (19 µl per reaction*):

Sterile water 7 µl

GoTaq Green Master Mix 10 µl

5 µM Forward primer 1 µl

5 µM Reverse primer 1 µl

** Always add extra volume, depending on the numbers of reactions.*

4. Transfer 1 µl of the DNA extract to a labeled PCR stripe tube or a PCR plate well.

5. Add 19 µl of the PCR master mix to the PCR tubes, mix well and centrifuge briefly.

7. Run the PCR at the following conditions:

94°C/30, 60°C/45, 72°C/1 min, 35 cycles

72°C/5 min, 1 cycle

8. Load 8 µl of the PCR product in 1.2% agarose gel for electrophoresis.

Other strains such as gel red can be used to replace ethidium bromide in gel staining. Mix the dye solution well before adding to the gel.

Anticipated Results

A 912-bp PCR amplicon is produced if the source plant is infected. The internal control of approximately 390 bp is produced, especially in uninfected samples. The internal band could be weak, occasionally absent, when the sample is infected. Amplification of weak non-specific band(s) occasionally occurs.

Summary

A polymerase chain reaction (PCR) is used to detect SPLCV in sweet potato. This document presents basic procedures for preparation of total nucleic acids from plant tissues and subsequent amplification of the viral targets by PCR using a pair of virus-specific primers. The CTAB protocol presented here for template preparations is inexpensive, and can be used during spring and early summer. The primers also detected other viruses in Begomovirus as well as *Beet top curly virus*. We have used this PCR assay to detect the viruses with high genetic divergence in more than 10 years, and found it is fast, sensitive and reliable for the sweet potato geminivirus detection.

References

Fauquet CM, Bisaro DM, Briddon RW, Brown JK, Harrison BD, Rybicki EP, Stenger, DC, and Stanley J. 2003. Revision of taxonomic criteria for species demarcation in the Family *Geminiviridae*, and an updated list of begomovirus species. Arch. Virol. 148: 405-421.

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Appendix 8 – Work instructions for qPCR protocol for sweepovirus of Ling et al., 2010

Real Time PCR for Sweet Potato Begomoviruses (Swepoviruses)

SPLCV- DNA virus

Materials:
DNA samples (DNeasy or other preparation)
Eppendorf HotMasterMix (2.5X)
[20µM] primers: SPLCV-F2, SPLCV-R2
[10µM] probe: SPLCV-P2
Microtubes, Optical Caps, 1.5mL tube
Statagene Mx3000P

Improved primer and probe in Real-time PCR for SPLCV are:

KL07-40	SPLCV.F2	5'GAG ACA GCT ATC GTG CC
KL07-41	SPLCV.R2	5'GAA ACC GGG ACA TAG CTT CG
KL07-42	SPLCV.P2	5'FAM-TAC ACT GGG AAT GCT GTC CCA ATT GCT-TAMRA

Method:
1. Prepare mastermix in a 1.5mL tube

Reagents	Volume
2.5X reaction mix	12.5µL
Primer 1	0.5µL
Primer 2	0.5µL
Probe	0.5µL
Dilute reference dye	0.375µL
dH2O	9.625µL

2. Transfer 24µL of mastermix into each microtube
3. Add 1µL of DNA for each sample, mix and briefly spin down to get rid of any air bubbles
4. Replace caps of microtubes with a strip of optical caps
5. Load samples into Stratagene machine, set thermal profile and other conditions accordingly with the probe

Thermal Profile: 1.95°C 10 min
2. 95°C 30 sec
3. 55°C 1 min
4. 72°C 30 sec
5. Go to step 2, repeat 40 times

Reference:

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