I. Introduction

The purpose of this work instruction is to describe technical activities required for the extraction of RNA from tomato seeds. This protocol is used for confirmation of the presence of potentially relevant pospiviroidae (CEVd, CLVd, PCFVd, PSTVd, TASVd, TCDVd and TPMVd) in tomato seeds by isolation of RNA followed by RT TaqMan PCR. RNA from seed extract of tomato is isolated and purified with a Sbeadex Maxi Plant kit using Qiagen BioSprint 96 instrument and magnetic bead technology to automate nucleic acid purification. This work instruction follows the Naktuinbouw protocol SPN-V043e, v.2.1, 30-10-2017. Follow the section 5.2.2 in the Naktuinbouw protocol when using the KingFisher Flex 96 instrument for RNA isolation.

II. Related Work Instructions

WI-B-T-1-63 Detection of Pospiviroids in Tomato Seeds using Multiplex RT-qPCR on Quant7.
WI-B-T-1-64 Detection of Pospiviroids on Tomato Seeds using Conventional RT-PCR and Sequencing

III. Equipment, Materials and Reagents

A. Equipment

Natural programs.

a. MiniMix® 100 range (Interscience)
b. KingFisher Flex (ThermoFisher Scientific) or BioSprint 96 (Qiagen)
c. Biological Safety Cabinet, Class II, A2 (any vendor)
d. Fume Hood (any vendor)
e. Balance, capable of weighing 20-100 mg (any vendor)
f. Thermomixer (e.g. Eppendorf #5350), or water bath, capable of 65°C ±2°C (any vendor)
g. Microcentrifuge, bench-top, capable of at least 16,000 *g* (any vendor)
h. Vortex (any vendor)
i. Freezer, non-frost free, capable of -20 ±2°C (any vendor) or -80 ±2°C (any vendor)
j. Dedicated, annually-calibrated pipettors (P10, P50, P200, P1000, any vendor)
k. Automatic pipette controller for 25 ml pipettes (any vendor)

B. Materials

a. Grinding bags 100 ml (Interscience BagPage)
b. KingFisher 96 tip comb for DW magnets, (ThermoFisher Scientific, Cat. No. 97002534)
c. KingFisher 96 standard plate, 200µL (ThermoFisher Scientific, Cat. No. 97002540)
d. KingFisher deepwell 96 plate, (ThermoFisher Scientific, Cat. No. 95040450)
e. Sbeadex maxi plant kit, 960 samples (LCG genomics, Cat. No. 41620)
f. Sterile filter (barrier) pipette tips (P10, P50, P200, P1000, any vendor)
g. Gloves, non-powder (any vendor)
h. Weigh boats (any vendor)
i. Microcentrifuge tubes, 1.5-1.7 ml (certified DNase & RNase free, any vendor)
j. Paper mat or towels, absorbent (any vendor)
k. Disposable, absorbent bench underpads (any vendor)
l. Ice and ice bucket (any vendor)
m. Resealable plastic bags, 1 quart (any vendor)
n. Microcentrifuge tube openers (any vendor)
o. Round glass media/storage bottles (multiple sizes, any vendor)
p. Test tube racks (any vendor)

q. 25 ml serological pipettes, plastic or glass, sterilized (any vendor)

C. *Reagents*

a. Ethylenediaminetetraacetic acid disodium salt dihydrate (Sigma ED2SS)
b. DL-Dithiothreitol (DTT) (Sigma D0632)
c. Guanidine hydrochloride (Sigma 5010)
d. Sodium Acetate (Sigma S2889)
e. Glacial acetic acid (Sigma 695084)
f. Polyvinylpyrrolidon, average mol wt 10,000 (Sigma PVP10)
g. RNase-free water (any vendor)
h. Confirmed negative seeds for negative internal control
i. Dahlia latent viroid (DLVd) for positive internal control

IV. General Information and Recommendations

An instruction guide is included with each kit or can be found at the LGC web site: https://www.lgcgroup.com/LGCGroup/media/PDFs/Products/Extraction/Kits%20inserts/sbeadex-maxi-plant.pdf . Before using kit the first time please carefully read the guide section titled *Safety information*.

Aliquot the necessary volumes of all buffers to single-day use tubes to avoid contamination of the original buffers.

Gloves **must** be worn during the extraction procedure and changed often, especially if there is any suspicion of contamination with sample extracts. More importantly, gloves also prevent the introduction of RNase from hands that will degrade RNA.

Equipment **must** be cleaned between processing each lot of seeds to include lab bench, balances, pipettes, MiniMix, and sample racks.

V. LIMS Information

1. Each lot of seeds must be logged in as a single Order ID. Each Order ID will have 3 samples with the 'Pospi qPCR' test group assigned. Make sure the matrix is 'RNA' prior to assigning the tests. The 'Pospi qPCR' test group contains the 4 multiplex qPCR tests. The final determination test and information from the PPQ Form 391/ARM generated form must be assigned to the 1st sample only in the Order ID.

2. Assign all the lots (Order IDs) into a single Prep Batch that will be prepared in a single period. In order to create a new Prep Batch, in the Sample Tracking module, select 'Sample Preparation'. Query the Order IDs to be assigned. Check the '+' column for the all samples to be added to the new Prep Batch. Click 'New' then click 'OK' on the pop up, finally click 'Add to Batch'.

3. Create a Prep Batch Form by double clicking the column 'Site'. If this form needs to be reopened, do NOT double click again in the LIMS this will overwrite any changes that were made. Instead, open the form from the server at: I:\CPHST1-BV\LIMS\Prep\Pospi and make any necessary changes.

4. The sample ID can be copy and pasted from the LIMS. Record the actual weight of each sample, the analyst who weighed the sample, and the lot number of the reagents. Use the barcode scanner of the tablets to record the station equipment information. DO NOT MOVE EQUIPMENT BETWEEN EACH ASSIGNED STATION. Record the

location of the sample in the final RNA extraction plate, the Prep Batch ID number, and the extraction analyst. Print the second page of the Prep Batch form that contains the final RNA extraction plate sample location and keep in designated location.

VI. Homogenization of tomato seed samples

Once you start this section work quickly to complete this one and the next. *Do not stop and leave your samples for any amount of time longer than prescribed in the protocol. Perform all steps of the extraction procedure at room temperature, including the centrifugation steps, which do not need to be cooled.*

1. Prepare GH+ buffer and 2 M DTT following the recipes in Appendix 1.
2. Prepare and label 1.5 ml tubes and grinding bags for the number of samples extracted.
3. If the original container is not resealable bag, transfer seed lot from original container to resealable bag. Seal bag and agitate to mix thoroughly.
4. Weigh 3 g (±10%, approx. 1000 seeds) subsamples of seed lots (3 subsamples per lot), and transfer each subsample to one side of the plastic mesh in a grinding bag. (Check that all seeds are at the bottom of the bag.) Place upright in a test tube rack or other container.
   Note: Include a confirmed negative tomato seed sample to be used as a healthy plant control.
   Note: Weigh 1.2 g (±10%, approx. 400 seeds) per subsample if instructed to assay 8 subsamples per lot.

5. For each subsample and control, aliquot 20 ml GH+ extraction buffer and 3 µl DLVd control into a round glass media/storage bottle. Mix well.
   Note: Aliquot 12 ml GH+ extraction buffer and 2 µl DLVd control per subsample for 8 subsample testing.
   Note: The DLVd control should be tested prior to use. Dilute the DLVd control until the Ct value is approximately 15.

6. Add 20 ml GH+ extraction buffer with DLVd spike to each grinding bag containing seeds.
   Note: 12 ml GH+ extraction buffer for 8 subsample testing.

7. Soak the seeds for a minimum 30 minutes at room temperature.
8. Preheat thermomixer or water bath to 65°C.
9. Check that paddles of the MiniMix are forward to maximum position, and that it is set to run for 1 minute 30 seconds at position 4.
10. Place one unprocessed grinding bag loaded with subsample or control into the MiniMix and close the door.
11. After homogenization, remove the processed bag from the MiniMix, and keep the bag upright by placing in a rack or other container. Repeat from step 11 until all bags have been processed.
12. For each processed bag, label a 1.5 ml tube and add 75 µl 2 M DTT to the tube (150 µl if 1 M DTT).
13. Transfer 1.0 ml of seed extract into the corresponding labeled 1.5 ml tube.
14. Close and vortex each tube immediately.
15. Incubate the subsamples in thermomixer for 15 minutes at 65°C and 850 rpm, or water bath at 65°C for 15 minutes with periodic inversion for mixing.
16. Centrifuge the tubes for 10 minutes at 16,000 *g*.
17. Proceed to RNA isolation.

VII. RNA isolation

Table 1: Labeling and composition of BioSprint 96 plates. Parentheticals indicate color of cap label.

Plate	Type of plate	Purpose	Composition
Plate 1	96 standard	Tips	New 96 deep well tip comb
Plate 2	96 deep well	Binding/ lysate	600 µl binding buffer PN (green)
			50 µl Sbeadex particle suspension (white)
Plate 3	96 deep well	Wash 1	600 µl Wash buffer PN1 (red)
Plate 4	96 deep well	Wash 2	600 µl Wash buffer PN1 (red)
Plate 5	96 deep well	Wash 3	600 µl Wash buffer PN2 (yellow)
Plate 6	96 standard	Elution	100 µl Elution buffer PN (black)

1. Label and fill in the plates as indicated in Table 1. Vortex Sbeadex particle suspension (white) in between loading every 2 wells.
2. Transfer 250 µl of centrifuged extract (avoid pellet!) from the subsamples and healthy seed control into the binding plate.
3. Store the remainder of the extract and controls at -20 °C until the test is completed.
4. Select "Naktuinbouw" program on the BioSprint 96 (Appendix B).
5. Press start and open the casing door.
6. Put plate 1 into correct position in the BioSprint 96 unit and press start. Repeat for plates 2 – 5.
7. Load plate 6 and close the casing door, then press start to begin the program.
8. The program will take one hour to complete all steps.
9. Upon completion, cover the elution plate with foil sticker and store on ice.
10. Proceed directly with molecular testing or store purified RNA at -20 °C (short term) or -80°C (long-term).

Appendix A

Buffers and Chemical Solutions preparation

2 M Dithiothreitol (DTT) solution

DTT is used to stabilize enzymes and other proteins which possess free sulfhydryl groups.

Materials

A. Reagents
- a. DL-Dithiothreitol ($C_4H_{10}O_2S_2$) (Molecular weight = 154.25)
- b. Deionized/Milli-Q water (any vendor)

B. Equipment and disposables
- a. Measuring cylinder (any vendor)
- b. Conical flask/beaker (any vendor)
- c. Magnetic stirrer with stir bar (any vendor)
- d. Disposable spatula

Preparation of 25 ml 2 M aqueous DTT solution

1. Weigh out 7.72 grams of DTT.
2. Transfer to 50 ml beaker/conical flask.
3. Add 16 ml deionized/Milli-Q water.
4. Mix until all DTT is dissolved completely.
5. Adjust volume to 25 ml with deionized/Milli-Q water.
6. *Optional*: Sterilize solution by passing through 0.22 micron filter.
7. Aliquot and store at -20°C.

Tips:

1. A magnetic stirrer makes the dissolving process easy and convenient, but manual swirling using a glass pipette can also bring the ingredients into solution.
2. Do not try dissolving the powder in 25 ml of deionized/Milli-Q water as the solution volume will increase beyond 25 ml.
3. Do not autoclave the solution. Autoclaving will destroy the DTT.
4. Avoid repeated handling and multiple freeze/thaw cycles.

GH+ extraction buffer

Table 2: Reagents for GH+ extraction buffer.

Reagent	For 1 liter
guanidine-hydrochloride	573.0 g
4 M sodium acetate buffer	50.0 ml
EDTA (di-natrium)	9.3 g
Polyvinylpyrrolidone (PVP-10)	25.0 g

Materials

A. Reagents
- a. Listed reagents on table 2.

b. Deionized/Milli-Q water (any vendor)

B. Equipment and disposables
   a. Measuring cylinder (any vendor)
   b. Conical flask/beaker (any vendor)
   c. Magnetic stirrer with stir bar (any vendor)
   d. Disposable spatula

Preparation of 1 L GH+ Extraction Buffer

1. Measure approx. 400 ml of deionized/Milli-Q water into a glass beaker.
2. Add ingredients in quantities listed in table 1.
3. Mix and bring volume to 1 liter using deionized/Milli-Q water.
4. Transfer to glass media/storage bottle with screw cap.
5. Label and date.
6. Store at room temperature.

Tips: Heat solution (dial position 3.5) to help dissolve PVP-10.

4M Sodium acetate (CH_3COONa) solution, pH 5.2

Overview: Sodium acetate is a sodium salt of acetic acid. It is water-soluble. Anhydrous sodium acetate is very hygroscopic; it readily converts to trihydrate due to its hygroscopic nature.

Materials

A. Reagents
   a. Sodium acetate, anhydrous (CH_3COONa) (Molecular weight = 82.0343) (any vendor)
   b. Glacial acetic acid (any vendor)
   c. Deionized/Milli-Q water (any vendor)

B. Equipment and disposables
   a. Measuring cylinder (any vendor)
   b. Conical flask/beaker (any vendor)
   c. Magnetic stirrer with stir bar (any vendor)
   d. Disposable spatula

Preparation of 500 ml of 4M Sodium acetate solution, pH 5.2

1. Add 300 ml Milli-Q water to a 1 liter beaker or conical flask.
2. Weigh out 164 g of CH_3COONa.
3. Mix into water until sodium acetate is completely dissolved.
4. Adjust pH to 5.2 with glacial acetic acid working in a chemical fume hood.
5. Bring volume to 500 ml using deionized / Milli-Q water.
6. Mix and transfer to media/storage bottle that is safe for the autoclave.
7. *Optional*: Autoclave for 20 minutes at 15 lb/sq.in. (psi) from 121-124°C on liquid cycle).
8. *Optional*: Sterilize through 0.2 micron filter. Neither autoclaving nor filtration inactivates RNase enzymes.
9. Store solution at room temperature.

Tips:

1. A magnetic stirrer makes the dissolving process easy and convenient, but manual swirling using a glass pipette can also bring the ingredients into solution.
2. Do not try dissolving the powder in 500 ml of deionized / Milli-Q water as the solution volume will increase beyond 500 ml.
3. pH is temperature dependent. Adjust pH at room temperature (25°C).

Appendix B

Details of "Naktuinbouw" protocol loaded into BioSprint 96 software.

Table 2. BioSprint 96 program "Naktuinbouw"

Step	Plate	Steps per plate
Tip 1	Tipholder	1. Remove tip comb from this plate (96 DW tip comb) 2. Release tip comb in plate 2 "Wash 1"
Binding	Plate 1 "Cell lysate"	1. Fast mixing, 10 min. 2. Collect beads, 3 x 10 sec.
Wash 1	Plate 2 "Wash 1"	1. Release beads, bottom mix, 20 sec. 2. Fast mixing, 10 min. 3. Collect beads, 3 x 10 sec.
Wash 2	Plate 3 "Wash 2"	1. Release beads, bottom mix, 20 sec. 2. Fast mixing, 10 min. 3. Collect beads, 3 x 10 sec.
Wash 3	Plate 4 "Wash 3"	1. Release beads, bottom mix, 20 sec. 2. Fast mixing, 10 min. 3. Collect beads, 3 x 10 sec.
Elution	Plate 5 "Elution"	1. Release beads, bottom mix, 1 min. 2. Preheat and fast mixing at 65°C, 10 min. 3. Collect beads, 5 x 10 sec.

References:

1) Naktuinbouw. 2012. Potato spindle tuber viroid: Detection of Potato spindle tuber viroid (PSTVd) and/or Tomato chlorotic dwarf viroid (TCDVd) in tomato seed with real-time RT-PCR (TaqMan RT-PCR). European and Mediterranean Plant Protection Organization (EPPO) Database on Diagnostic Expertise. Paris, EPPO. Available at http://dc.eppo.int/validationlist.php?action=filter&taxonomic=Virus&organism=&validationprocess=&method (last accessed 18 August 2014).

Document Revision History

Status	Document Revision Number	Effective Date	Description
Original	Original	11/30/2018	To baseline the work instruction
Revision	01	12/13/2018	Changed to 3 subsamples per lot (Naktuinbouw protocol validated on both 3 and 8 subsamples per lot). DLVd control changed from 2 uL to 3 uL to adjust for additional extraction buffer.
Revision	02	See electronic signature	Updated correct revision number in header. Section V. LIMS information updated to 3 samples instead of 8. Added DLVd control note in section VI.3 to ensure the control is tested prior to use and the control is diluted until Ct value is approximately 15.
These Revisions Require Staff Re-Training? Yes (No)			

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