The purpose of this work instruction is to describe a one-step reverse transcription real-time PCR (RT-PCR) for simultaneous detection of pospiviroids (CEVd, CLVd, PCFVd, PSTVd, TASVd, TCDVd, and TPMVd) with internal controls.

I. Related Work Instructions

WI-B-T-2-16	Extraction of RNA from tomato seeds using Sbeadex Maxi Plant Kit
WI-B-T-1-64	Detection of Pospiviroids on Tomato Seeds using Conventional RT-PCR and Sequencing

II. Equipment, Materials and Reagents

A. Equipment
1. PCR clean station or set-up hood (any vendor)
2. ABI QuantStudio™ 7 Flex Real-Time PCR System
3. Labnet MPS 1000 Mini Plate Spinner
4. Bench-top micro centrifuge, i.e. Eppendorf MiniSpin or MiniSpin Plus.
5. Vortex (any vendor)
6. Dedicated, annually-calibrated pipettes (P10, P50, P200, multi-channel, any vendor)
7. Freezer, non-frost-free, capable of 20 °C ±2° C (any vendor)

B. Materials
1. 1.7 mL micro centrifuge tubes, clear and amber (pre-sterilized, certified DNase & RNase free, any vendor) and the tube openers.
2. ThermoFisher Scientific MicroAmp™ Optical 8-tube Strip, 0.2 mL (Cat #4316567); MicroAmp™ Optical 8-cap strips, 300/pc (Cat #4323032); OR MicroAmp™ Optical 96-Well Reaction Plate (Cat.#N8010560).
3. MicroAmp™ Cap Installing Tool (Cat #4330015);
4. MicroAmp™ Optical Adhesive Film (Cat. # 4311971); MicroAmp™ Adhesive Film Applicator (Cat. # 4333183); MicroAmp™ 96-Well Base (Cat. # N8010531)
5. 10 mL Bulk Reservoirs, Sterile (Vista Labs Cat #3054-1012)
6. Sterile filter (barrier) pipette tips for the corresponding pipettes (any vendor)
7. Gloves (any vendor)
8. Disposable bench tissue paper (any vendor)
9. Absorbent disposable bench under pads (any vendor)
10. Ice

C. Reagents
1. Molecular Grade (MG) water (any vendor)
2. Ultraplex 1-Step ToughMix Low ROX (4X) (VWR Cat. # 10804-954)
3. Primers and probes (Refer to Table 1)

III. Primer and Probe Reaction Mixture

Order primers and probes from Integrated DNA Technology (IDT) as described in Table 1.

Table 1. Primer and Probe Sequences

Primer Mix	Pathogens Detected (Targets)	Primer	Sequence
Pospi A Primer Mix (351a)	PSTVd (FAM), TCDVd (FAM), TPMVd (FAM), PCFVd (VIC), DLVd (ABY)	PSTV-231F1	GCCCCCTTTGCGCTGT
		PSTV-296R	AAGCGGTTCTCGGGAGCTT
		PCFVd-F	TCTTCTAAGGGTGCCTGTGG
		PCFVd-R	GCTTGCTTCCCCTTTCTTTT
		DaVd1-FT	GCTCCGCTCCTTGTAGCTTT
		DaVd1-RT	AGGAGGTGGAGACCTCTTGG
Pospi A probe mix (351b)		PSTV-251	6FAM-CAGTTGTTTCCACCGGGTAGTAGCCGA-QSY
		PCFVd-	VIC-CTCCCCCGAAGCCCGCTTAG-QSY
		DaVd1-P	ABY-CTGACTCGAGGACGCGACCG-QSY
Pospi B primer mix (352a)	CEVd (FAM), CLVd (FAM), DLVd (ABY)	CEVd-F2-	CTCCACATCCGRTCGTCGCTGA
		CEVd-R2	TGGGGTTGAAGCTTCAGTTGT
		CLVd-F	GGTTCACACCTGACCCTGCAG
		CLVd-F2	AAACTCGTGGTTCCTGTGGTT
		CLVd-R	CGCTCGGTCTGAGTTGCC
		DaVd1-FT	GCTCCGCTCCTTGTAGCTTT
		DaVd1-RT	AGGAGGTGGAGACCTCTTGG
Pospi B probe		CEVd-P2-	6FAM-CCCTCGCCCGGAGCTTCTCTCTG-QSY

mix (352b)		CLVd-P	6FAM-AGCGGTCTCAGGAGCCCCGG-QSY
		DaVd1-P	ABY-CTGACTCGAGGACGCGACCG-QSY or NED-CTGACTCGAGGACGCGACCG-NFQ-MGB
Pospi C primer mix (353a)	TPMVd (FAM), Nad5 (ABY)	TPMVd-F1	AAAAAAGAATTGCGGCCAAA
		TPMVd-R	GCGACTCCTTCGCCAGTTC
		Nad5 F	GATGCTTCTTGGGGCTTCTTGTT
		Nad5 R	CTCCAGTCACCAACATTGGCATAA
Pospi C probe mix (353b)		pUCCR2	6FAM-CCGGGGAAACCTGGA-NFQ-MGB
		Nad5-probe	ABY-AGGATCCGCATAGCCCTCGATTTATGTG-QSY or NED-AGGATCCGCATAGCCCTCGATTTATGTG-NFQ-MGB
Pospi TASVd-F2-200 (281a)	TASVd (FAM)	TASVd-F2	CKGGTTTCCWTCCTCTCGC
Pospi TASVd-R2-269 (281b)		TASVd-R2	CGGGTAGTCTCCAGAGAGAAG
Pospi TASVd-P2-228 (281c)		TASVd-P2	6FAM-TCTTCGGCCCTCGCCCGG-QSY

A. Primer and Probe Mix Preparation.

Note: Primer and probe 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 approved and validated positive and negative controls prior to testing samples. When a qRT-PCR assay is being conducted, the analyst should use a 'working solution' of primers and probes.

1. Tubes with lyophilized primer or probe (Table 1) are centrifuged for 60 seconds at minimum 10,000 rpm (6,708 x g) before opening to ensure that the lyophilized material is contained 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 the primers: primers are re-hydrated to

100 µM stock solution in molecular grade water and stored at -20 °C. The amount of water is determined by multiplying the nmoles provided by the vendor by 10 in µL. Store freezer stocks of probes in amber-colored micro centrifuge tubes.

a. Example: For 47.54 nmoles of primer, add 475.4 µL of MG water for a concentration of 100 µM.

3. Primers and probes for Pospi A, B and C should be prepared in a multiplex format. For a multiplex configuration, follow the tables below to prepare the primer mix and probe mix. The primer mix can be scaled up or down to accommodate the appropriate amount of samples. The Pospi T primer and probe mix is a simplex assay. The forward, reverse and probe can be diluted into separate 10 µM working solutions for use.

Table 2. Pospi A Primer Mix

Primer Name	Volume (µL)	Working Concentration
PSTV-231F1	100	10 µM
PSTV-296R	100	10 µM
PCFVd-F	100	10 µM
PCFVd-R	100	10 µM
DaVd1-FT	100	10 µM
DaVd1-RT	100	10 µM
MG Water	400	N/A
TOTAL	**1000**	**N/A**

Table 3. Pospi A Probe Mix

Primer Name	Volume (µL)	Working Concentration
PSTV-251T	20	10 µM
PCFVd-	20	10µM
DaVd1-P	20	10 µM
MG Water	140	N/A
TOTAL	**200**	**N/A**

Table 4. Pospi B Primer Mix

Primer Name	Volume (µL)	Working Concentration
CEVd-F2	100	10 µM
CEVd-R2	100	10 µM
CLVd-F	100	10 µM
CLVd-F2	100	10 µM
CLVd-R	100	10 µM
DaVd1-FT	100	10 µM

DaVd1-RT	100	10 µM
MG Water	300	N/A
TOTAL	**1000**	**N/A**

Table 5. Pospi B Probe Mix

Primer Name	Volume (µL)	Working Concentration
CEVd-P2-	20	10 µM
CLVd-P	20	10 µM
DaVd1-P	20	10 µM
MG Water	140	N/A
TOTAL	**200**	**N/A**

Table 6. Pospi C Primer Mix

Primer Name	Volume (µL)	Working Concentration
TPMVd-F1	100	10 µM
TPMVd-R	100	10µM
Nad5 F	100	10 µM
Nad5 R	100	10 µM
MG Water	600	N/A
TOTAL	**1000**	**N/A**

Table 7. Pospi C Probe Mix

Primer Name	Volume (µL)	Working Concentration
pUCCR2	20	10 µM
Nad5-probe	20	10 µM
MG Water	160	N/A
TOTAL	**200**	**N/A**

4. Vortex the primer/probe mix for 10 seconds at setting 7-10, then centrifuge briefly (10-20 seconds) at 10,000 rpm (6,708 x g).
5. Aliquot the primer/probe mixes and store at -20 °C for use in the future or on ice for same-day use. Do not use rehydrated primers or probes over one year old because of possible degradation.

IV. Real-Time PCR Instrument and Software Setup

1. In order to import sample numbers and test targets into the QuantStudio 7 software

follow JA-B-20 (latest version available at I:\CPHST1-BV\LIMS\Manuals).

2. Turn on the QuantStudio™ 7 Flex Real-Time PCR instrument, connect to the Real-Time System by clicking the serial number of the instrument under Home tab / Instrument Menu / Instrument Console. Click on manage instrument in order to check the instrument calibration status and calibration date for each dye.
3. Eject the instrument tray by clicking 'Open Door' to verify the correct plate adapter, then 'Close Door'.
4. Return to the home screen in the QuantStudio software. Select experiment setup.On the Experiment Properties screen:
   a) Name the experiment as the QCBatchID(s) if using LIMS
   b) Confirm the experiment settings:
      - Instrument type: QuantStudio™ 7 Flex System;
      - Block: 96-Well (0.2 mL)
      - Type of experiment: Standard Curve
      - Reagents: TaqMan® Reagents
      - Run properties for the instrument: Standard
5. From the Define screen, import the Sample Names following JA-B-20 or manually enter the sample names.
6. Import the target names from the DiagnosticTargetLibrary.txt file located on the desktop of the instrument's computer. Or manually enter the target names as the following:
   a) For Pospi A:
      - Target Name: PSTVd, TCDVd, TPMVd (Pospi A FAM) Reporter: FAM, Quencher NFQ-MGB
      - Target Name: PCFVd (Pospi A VIC) Reporter: VIC, Quencher NFQ-MGB
      - Target Name: DLVd-ABY (Pospi A ABY) Reporter: ABY, Quencher NFQ-MGB
   b) For Pospi B:
      - Target Name: CEVd, CLVd (Pospi B FAM) Reporter: FAM, Quencher NFQ-MGB
      - Target Name: DLVd-ABY (Pospi B ABY) Reporter: ABY, Quencher NFQ-MGB
   c) For Pospi C:
      - Target Name: TPMVd (Pospi C FAM); Reporter: FAM, Quencher NFQ-MGB

- Target Name: Nad5- ABY (Pospi C ABY); Reporter: ABY, Quencher NFQ-MGB

d) For Pospi T:

- Target Name: TASVd FAM (Pospi T FAM); Reporter: FAM, Quencher NFQ-MGB

7. Ensure the passive Reference is set to ROX.
8. From the Experiment Menu, select Assign. If the Sample Names were imported the wells will already be assigned with the appropriate sample name. Otherwise, select individual wells on the plate layout by highlighting the intended wells. Then, choose the targets and samples as defined above.
9. From the Experiment Menu, select Run Method confirm or enter the following information:

a) 25 (µL) in the Reaction Volume per Well

b) Thermal cycling conditions

i. Hold Stage

- Instrument automatic ramp 2.445 °C/s.
- Hold at 50.0 °C for 10 minutes with Data Collection OFF
- Hold at 95.0 °C for 3 minutes with Data Collection OFF

ii. PCR Stage

- Instrument automatic ramp 1.936 °C/s between cycling temperature
- 40 Cycles (AutoDelta disabled):
  - 95.0 °C for 10 seconds with Data Collection OFF
  - 60.0 °C for 1 minute with Data Collection ON

V. Real-time PCR Sample Set Up

1. In the LIMS, create a QCBatch and master mix form for each of the Pospi qRT-PCR assays. Record the location of the samples and quality controls in the PCR plate layout in the master mix form. The well number will automatically update as the sample numbers are pasted into their appropriate location.
2. Remove the primer/probe mix from the freezer and thaw at room temperature. As soon as the primer/probe mix is thawed, vortex at setting 7-10 for 10 sec. Spin for 5-10 sec at maximum speed on a bench-top micro centrifuge and return to ice. The Ultraplex 1-Step ToughMix Low ROX Master Mix should be kept on ice. To mix the solution, flick the tube several times, lightly vortex and spin briefly as described above.

3. If samples to be tested are frozen, remove the RNA samples from the -80°C freezer and thaw. Once samples are thawed, lightly vortex for 5 seconds at speed setting 7 and centrifuge for 30 seconds at 10,000 rpm (6,708 x g) to collect the sample at the bottom of the tube. Place samples on ice.

Steps 4 and 5 must be done in a decontaminated PCR hood/enclosure:

4. Prepare the Master Mix described in Table 8 (or Table 9 for Pospi T) for the desired total number of samples and quality controls. In order to ensure enough master mix is prepared at a given time, multiple all reagent total amounts in the master mix by 1.1. Lightly vortex the reaction mix and follow by a quick spin down. If a bulk reservoir is used, pour the mix into the reservoir after homogenization and pipette any additional reaction mix from the initial mix tube.

Table 8. RT-PCR Master Mix Composition (Pospi A, B, C)

Reagent	1 Reaction (µL)	Final Concentration (µM)
MG Water	11.5	---
Ultraplex 1-Step ToughMix Low ROX (4X)	6.25	1X
10 µM primer mix (forward and reverse)	0.75	0.3
10 µM probe mix	0.5	0.2
Total Master Mix	**19.0**	---
Sample	6.0	---
Total Reaction	**25.0**	---

Table 9. RT-PCR Master Mix Composition (Pospi T)

Reagent	1 Reaction (µL)	Final Concentration (µM)
MG Water	10.75	---
Ultraplex 1-Step ToughMix Low ROX (4X)	6.25	1X
10 µM Pospi TASVd-F2-200	0.75	0.3
10 µM Pospi TASVd-R2-269	0.75	0.3
10 µM Pospi TASVd-P2-228	0.5	0.2
Total Master Mix	**19.0**	---
Sample	6.0	---
Total Reaction	**25.0**	---

5. Put a 96-Well Reaction Plate or 8-tube strips onto a 96-well base or a tube rack, aliquot 19.0 µL of the composed Master Mix into each well/tube.
6. Take the PCR plate/tube strips containing the Master Mix to an assigned lab bench and place all items on a new disposable lab mat.
7. Add **6 µL** of RNA samples and controls to the corresponding well or tube. The total reaction volume is 25 µL.

a) After sample extraction, the samples are often stored in a 96-well plate format sealed with an aluminum foil cover. Do **NOT** remove the aluminum foil cover. This could cause significant contamination issues. To add the sample to the qRT-PCR reaction plate, pierce the aluminum foil with the pipette tip and withdraw the sample to be added to the qRT-PCR reaction.

8. The following lists the quality controls to be used for each test:

a) Pospi A

- PSTVd, TCDVd or TPMVd total RNA or synthetic dsDNA positive control
- PCFVd synthetic dsDNA positive control
- Healthy Seed Control (from the extraction)
- NTC

Note: The synthetic PSTVd control is known to cross react and produce a PCFVd VIC Ct value.

b) Pospi B

- CEVd or CLVd total RNA or synthetic dsDNA positive control
- Healthy Seed Control (from the extraction)
- NTC

c) Pospi C

- TPMVd total RNA or synthetic dsDNA positive control
- Healthy Seed Control (from the extraction)
- NTC

d) Pospi T

- TASVd total RNA or synthetic dsDNA positive control
- Healthy Seed Control (from the extraction)
- NTC

Note: It is recommended to add NTC to PCR plate first, followed by the healthy plant controls, then the samples to be tested and lastly, the positive controls. This helps to avoid contamination of samples by the positive controls.

9. Follow the instructions below based on the if a plate or tubes are used:

a) Plate:

i. Remove one Optical Adhesive Film from the box, hold only the tab outside the dashed line to avoid optical contamination of the film and to ensure transparency on the 96 wells.

ii. Carefully, peel back the white protective backing from the center sealing surface. With the adhesive side facing the plate, align and lower the film onto the reaction plate to cover all 96-wells.

iii. Applying firm pressure, gradually move the Adhesive Film Applicator across the film horizontally and vertically several times to ensure a good final seal for

all of the 96 wells.

iv. Hold the films short-edge in place with the applicator edge, then grasp one end of the tab and sharply pull away. Repeat to remove the other tab.

v. Finally, run the applicator edge along all four of the outer borders of the film.

vi. Place the film-sealed plate in a centrifuge balance plate (film-top to face centrally) into Labnet MPS 1000 Mini Plate Spinner, spin for 1 minute.

b) Tube Strip:

i. Remove 1 strip of Optical 8-cap strips to be used with the tube strip

ii. Line up the caps on top of the tubes.

iii. Using the MicroAmp™ Cap Installing Tool, press firmly down on the caps, rocking slowly back and forth to ensure a complete seal of the caps.

iv. Visually inspect the caps to ensure a complete seal.

v. For strip tubes, place the tubes in a bench top centrifuge with the 8-tube strip adapter and spin for 1 minute.

10. Take out the plate or tube strips and visually check each PCR mixture is at the bottom of a well.

11. Load the PCR reaction plate/tubes into QuantStudio™ 7 Flex Real-Time PCR instrument using the appropriate plate or tube adapter,

12. Start the PCR by clicking the START RUN button from Experiment Menu / Run group.

VI. Results Analyses

A. Acquire the real-time qRT-PCR results

1. Once the run is complete, in the QuantStudio software, select the Analysis group and highlight all the 96 wells on the Plate Layout:

   a. Click the Analysis Settings button in the top left corner, open the Analysis Settings verify:

      - Data Step Selection is on Stage 2, Step 2
      - Algorithm Settings is on Baseline Threshold

   b. Verify Amplification Plot is on Δ Rn vs Cycle; Plot Settings is on Log or Linear

   c. On the Options / Target drop-down menu, select one target at a time and verify the Auto threshold is selected.

2. Obtain the PCR results in Excel table format by clicking the Export button on the Experiment Menu. Select only the Results tab and check the All Fields box in the select content. Click Start Export on the bottom of the screen. Save the file at I:\CPHST1-BV\LIMS\FilesToBeParsed as the QCBatchID(s).

3. In order to import the Ct values into the LIMS, open the Quant7 parser located at I:\CPHST1-BV\LIMS\Parsers. Select the exported excel file from the above step, type in the QCBatchID, and start the task manager in the LIMS maintenance module. If more than one assay is ran on the same plate, not all of the quality controls Ct values will be imported when running the parser; manual entry will be required.

B. Quality Control PCR Reaction Assessment

All controls must be determined to be valid prior to test sample evaluation. If *any* control is determined to be invalid, all controls and samples must be retested. Repeat testing only once. If the controls fail on the retest, notify the Quality Manager.

1. The NTC must be 'Undetermined' in Pospi A, B and C. Pospi T frequently has non-specific amplification and in some cases can be accepted.
2. For Pospi A assay
   a) Pospi A positive control (See V.8.a) must produce FAM Ct values ≤32 with VIC and ABY as 'Undetermined'; otherwise the assay must be repeated.
   b) PCFVd synthetic dsDNA positive control produce a VIC and ABY Ct Values ≤32, and a FAM as 'Undetermined'; otherwise the assay must be repeated.
   c) Healthy Seed Control must produce an ABY Ct value ≤32, FAM and VIC as 'Undetermined'; otherwise the assay must be repeated.

3. For Pospi B assay
   a) Pospi B positive control (See V.8.b) must produce a FAM Ct value ≤32 and ABY as 'Undetermined'; otherwise the assay must be repeated.
   b) Healthy Seed Control must produce an ABY Ct value ≤32 and FAM 'Undetermined'; otherwise the assay must be repeated.

4. For Pospi C Assay
   a) Pospi C positive control (V.8.c) must produce FAM and ABY Ct values ≤ 32, otherwise the assay must be repeated.
   b) Healthy Seed Control must produce an ABY Ct value ≤32 and FAM 'Undetermined'; otherwise the assay must be repeated.

5. For Pospi T Assay
   a) Pospi T positive control (See V.8.d) must produce a FAM Ct value ≤ 32, otherwise the assay must be repeated.
   b) Healthy Seed Control must be comparable to NTC result.

6. The DLVd spike control in all samples must have an ABY Ct value ≤ 32 in both Pospi A and Pospi B assays.

7. The Nad5 internal control in all samples must have an ABY Ct value ≤ 32 in Pospi C assay. If the Nad5 Ct is > 32, it can be accepted only if the DLVd spike control Ct value is ≤ 32 in both Pospi A and Pospi B assays.

C. Sample PCR Reaction Assessment:

1. A sample is negative for the tested viroids if it produces a FAM 'Undetermined' result in all assays AND a VIC 'Undetermined' result.

2. A sample is **inconclusive** if it produces a positive viroid result with a Nad5 ABY Ct > 32 in Pospi C assay AND DLVd FAM Ct value >32 in either the Pospi A and Pospi B assays.
   a) The sample must be retested in all assays. If the sample is inconclusive with the retest, test the sample using WI-B-T-1-64 Detection of Pospiviroids on Tomato Seeds using Conventional RT-PCR and Sequencing.

3. A sample is **positive** for a viroid if it produces a FAM Ct value ≤ 32 in any of the assays and/or VIC Ct value ≤ 32 in the Pospi A assay.
   a) If a sample is positive, it must be tested using WI-B-T-1-64 Detection of Pospiviroids on Tomato Seeds using Conventional RT-PCR and Sequencing

VII. References

1. Bakker, D., Bruinsma, M., Dekter, R.W., Toonen, M.A.J., Verhoeven, J. Th. J. and
2. Koenraadt, H.M.S. 2015. Detection of PSTVd and TCDVd in seeds of tomato using real-time RTPCR. EPPO Bulletin. Volume 45, Issue 1, pages 14–21,
3. Boonham, N., L. González-Pérez, ,M.S. Mendez, E. Lilia Peralta, A. Blockley, K. Walsh, I. Barker & R.A. Mumford (2004). Development of a real-time RT-PCR assay for the detection of Potato spindle tuber viroid. Journal of Virological Methods 116:139-146.
4. Botermans, M., van de Vossenberg, B. T. L. H., Verhoeven, J. Th. J.,Roenhorst, J. W., Hooftman, M., Dekter, R. & Meekes, E. T. M. (2013) Development and validation of a real-time RT-PCR assay for generic detection of pospiviroids. J. Virol. Methods 187, 43–50.
5. Koenraadt, H., Jodlowska, A., van Vliet, A. & Verhoeven, K. (2009) Detection of TCDVd and PSTVd in seeds of tomato. Phytopathology 99, S66.
6. Harrie Koenraadt, Agata Jodlowska, Maaike Bruinsma, Michel Ebskamp, Ko Verhoeven and Annelien Roenhorst. (2017) Biological relevance of the detection of pospiviroids on pepper and tomato seeds (poster at APS, San Antonio, Tx)
7. Menzel, W., Jelkmann, W., and Maiss, E. (2002) Detection of four apple viruses by multiplex RTPCR assays with coamplification of plant mRNA as internal control. J. Virol. Methods 99: 81-92.
8. Monger, W., Tomlinson, J., Boonham, N., Marn, M.V., Plesko, I.M., Molinero-Demilly,V., Tassus, X., Meekes, E., Toonen, M., Papayiannis, L., Perez-Egusquiza, Z., Mehle, N., Jansen, C., Nielsen, S.L., (2010) Development and inter-laboratory evaluation of real-time PCR assays for the detection of pospiviroids. J. Virol. Methods 169, 207–210.

9. Mumford, R.A., A.L. Skelton, N. Boonham, K.I. Posthuma, M.J. Kirby, & A.N. Adams (2001) The Improved Detection of Strawberry Crinkle Virus Using Real-Time RT-PCR (TaqMan®). Acta Horticulturae 656: 81-86.

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