So 6.1 Method for testing *Pospiviroids* (CLVd, PCFVd, PSTVd, TASVd, TCDVd and TPMVd) on tomato (*Solanum lycopersicum*) and pepper (*Capsicum annuum*) seeds using TaqMan RT-PCR

VERSION: 1.0	**DATE:** 06/2020
PATHOGEN: Pospiviroids (CLVd, PCFVd, PSTVd, TASVd, TCDVd and TPMVd)	
HOST: tomato (*Solanum lycopersicum*) and pepper (*Capsicum annuum*)	
COMMON NAME: Pospiviroids (CLVd, PCFVd, PSTVd, TASVd, TCDVd and TPMVd) - tomato - pepper	
METHOD: So 6.1 TaqMan RT-PCR Method, Ver 1.0 (National Seed Health System)	
METHOD CLASS: STANDARD (A)	
SAMPLE: subsamples of 500 or 1000 seeds for tomato / 500 seeds for pepper	
AUTHORS: Li-fang Chen[1], Tracy Bruns[2], Samantha Thomas[1] [1]Bayer Crop Science, Chesterfield, MO; [2]Iowa state University Seed Science Center, Ames, IA	

1. OBJECTIVE

To detect the presence or absence of Pospiviroids in tomato and pepper seed by isolation of total RNA followed by Reverse Transcriptase (RT) quantitative PCR using TaqMan assays.

2. PRINCIPLE

Total RNA extracted from tomato/pepper seed is isolated and purified using Qiagen PowerPlant kit or other equivalent methods. The possible presence of viroid RNA can be detected by the specific set of primers and labelled TaqMan probes in a duplex RT-qPCR assay with an internal control (IC). An internal control is designed to detect the mitochondrial NADH dehydrogenase 5 (*Nad5*) from seed or an external spike of an RNA virus-*Squash mosaic virus* (SqMV) to monitor the quality of RNA extraction and potential inhibitory effects.

3. MATERIALS AND EQUIPMENT

Geno/Grinder 2010
IKA Tube Mill 100 control
IKA Mills MT 40.100
50 ml conical shaped tubes
steel balls or zirconium beads

RNA extraction buffer
Thermal shaker or heat block
Vortex mixer
Centrifuges for 50 ml sample tubes and microcentrifuge tubes
Vacuum manifolds (Optional)
Positive RNA controls
RNA extraction kit-Qiagen RNeasy PowerPlant Kit, Qiagen RNeasy Plant Mini kit, LQC Sbeadex Plant Maxi Kit or MagNA Pure LC Total Nucleic Acid kit
TaqMan RT-PCR reagents, including primers and probes
Quanta qScript XLT One-Step RT-qPCR ToughMix, Low Rox (2X) or Ultraplex 1-Step ToughMix (4X)
MicroAmp™Fast Optical 96-Well Reaction Plate
MicroAmp™ Optical Adhesive Film
Real-time PCR system

4. METHOD

4. 1. Sample preparation

4.1.1. Grinding (the following two options are approved for use in this method; accreditees proposing to use other equipment must develop a validation plan in conjunction with the NSHS AU)

A. Option 1: Geno/Grinder

i. Weigh subsamples of 500 or 1000 seeds for tomato or 500 seeds for pepper and place into 50 ml tubes. Add an appropriate ball bearing(s).

ii. Freeze sample tubes containing seeds and ball bearings at -80 or -20 °C overnight. *Option: Seed subsamples can be quickly frozen by placing tubes in liquid nitrogen*

iii. For tomato seeds, grind seeds using Geno/Grinder at 1400 - 1700 rpm, 2 minutes.

iv. For pepper seeds, grind seeds using a Geno/Grinder at 1400-1700 rpm for 2-2 minute increments. Refreeze samples between grinding via freezer, dry ice or liquid N2.

B. Option 2: IKA Mill (grinder)

i. Weigh subsamples of 500 or 1000 seeds for tomato or 500 seeds for pepper and place into 40 ml mill tube.

ii. Set the speed at 25,000 rpm for 20 seconds and transfer ground seed flours to testing tubes for RNA isolation.

Figure 1. Demonstration of ground tomato (left) and pepper (right) seed flours comparing to unground seeds

4.1.2. If desired, prepare the internal control (IC) and add to subsamples prior to sampling for RNA extraction (see appendices).

4.1.3. Preparation for RNA isolation:

A. Option 1:

i. Add RNA extraction buffer to each ground subsample:

	500 seed	1000 seed
Tomato	6 ml	8 ml
Pepper	12 ml	--

ii. Mix samples with buffer vigorously by vortex or shaking and incubate samples at room temperature for 30-45 minutes.

iii. Centrifuge the sample tubes at 4000 rpm for 5 minutes, transfer 200µl of supernatant to a bead tube or a new 2.0 ml tube and follow appropriate steps according to preferred RNA extraction method (4.2.).

B. Option 2:

i. Weigh 70-80 mg per subsample from ground seed flours into a bead tube or a new 2.0ml tube and follow appropriate steps according to preferred RNA extraction method(4.2.).

4. 2. RNA isolation

Options: using Qiagen PowerPlant kit, Qiagen RNeasy Plant Mini kit, LQC Sbeadex Plant Maxi Kit or MagNA Pure LC Total Nucleic Acid kit for RNA extraction
Follow Manufacturer's guidance. Use an elution volume of 100 µl.

4. 3. TaqMan RT-PCR

Work on ice as much as possible and prevent prolonged exposure of probes to light. Wear clean lab coat and gloves to minimize the risk of cross-contamination.

4.3.1. Prepare the TaqMan RT-PCR mixes according to the tables below and use the PCR mixes: Quanta qScript XLT One-Step RT-qPCR ToughMix, Low Rox (2X) or Ultraplex 1-Step ToughMix (4X). Fluorophores and quenchers of the probes also may need to be adjusted depending on the thermocycler equipment applied. Verify test performance by thorough in-lab validation.

4.3.2. Ensure to add IC (*Nad5* or SqMV) in each PCR mix and calculate the required amount for reaction mixes

Internal control	Final Conc.	Target	Sequence 5'-3'
Nad5-F	100 nM	Nad5	GATGCTTCTTGGGGCTTCTTGTT
Nad5-R	100 nM		CTCCAGTCACCAACATTGGCATAA
Nad5-Pr	50 nM		VIC-AGGATCCGCATAGCCCTCGATTTATGTG-NFQ-MGB
SqMV-F	200 nM	SqMV	TAGGAATTTCTGGGCAGAGT
SqMV-R	200 nM		GGGCTGTACTTTCTAAGGG
SqMV-Pr	100 nM		Texas Red-CAGCAGCTTGGAACTTATAATCCAAT-BHQ1

4.3.3. Ensure to include positive amplification controls for each PCR assay

PCR Mix 1: PSTVd/TCDVd/TMVd and PCFVd

Reagent	Final Conc.	Target	Sequence 5'-3'
RNase-Free Water			
MasterMix	1x		
PSTVd-231F1	300 nM	PSTVd/ TCDVd/ TMVd	GCCCCCTTTGCGCTGT
PSTVd-296R	300 nM		AAGCGGTTCTCGGGAGCTT
PSTVd-251T	200 nM		6FAM-CAGTTGTTT/ZEN/CCACCGGGTAGTAGCCGA-3IABkRQ
PCFVd-F	300 nM	PCFVd	TCTTCTAAGGGTGCCTGTGG
PCFVd-R	300 nM		GCTTGCTTCCCCTTTCTTTT
PCFVd-Pr	200 nM		VIC-CTCCCCCGAAGCCCGCTTAG-BHQ1
IC Forward		Internal Control	
IC Reverse			
IC Probe			
RNA extract	6 µl		
Total	25 µl		

PCR Mix 2: CEVd (optional) and CLVd

Reagent	Final Conc.	Target	Sequence 5'-3'
RNase-Free Water			
MasterMix	1x		
CLVd-F	300 nM	CLVd	GGTTCACACCTGACCCTGCAG
CLVd-F2	300 nM		AAACTCGTGGTTCCTGTGGTT
CLVd-R	300 nM		CGCTCGGTCTGAGTTGCC
CLVd-Pr	200 nM		6FAM-AGCGGTCTCAGGAGCCCCGG-BHQ1
CEVd-F2	300 nM	CEVd	CTCCACATCCGRTCGTCGCTGA
CEVd-R2	300 nM		TGGGGTTGAAGCTTCAGTTGT
CEVd-Pr	200 nM		6FAM-CCCTCGCCCGGAGCTTCTCTCTG-BHQ1
IC Forward		Internal Control	
IC Reverse			
IC Probe			
RNA extract	6 µl		
Total	25 µl		

PCR Mix 3: TPMVd

Reagent	Final Conc.	Target	Sequence 5'-3'
RNase-Free Water			
MasterMix	1x		
TPMVd-F1	300 nM	TMVd	AAAAAAGAATTGCGGCCAAA
TPMVd-R	300 nM		GCGACTCCTTCGCCAGTTC
pUCCR2	200 nM		6FAM-CCGGGGAAACCTGGA-NFQ-MGB
IC Forward		Internal Control	
IC Reverse			
IC Probe			
RNA extract	6 µl		
Total	25 µl		

PCR Mix 4: TASVd

Reagent	Final Conc.	Target	Sequence 5'-3'
RNase-Free Water			
MasterMix	1x		
TASVd-F2	300 nM	TASVd	CKGGTTTCCWTCCTCTCGC
TASVd-R2	300 nM		CGGGTAGTCTCCAGAGAGAAG
TASVd-Pr2	200 nM		6FAM-TCTTCGGCCCTCGCCCGR-BHQ1
IC Forward		Internal Control	
IC Reverse			
IC Probe			
RNA extract	6 µl		
Total	25 µl		

4.3.4. Transfer 19 µL of PCR mix into a 96-well reaction plate. Add 6 µL of RNA sample into 19 µL of PCR mix. Cover the plate with adhesive film.

4.3.5. Include a positive RNA control and a no-template control in each run.

4.3.6. Run the assay using the following program:

	Temperature	Time
cDNA synthesis	48 °C	15 min
Denaturation	95 °C	1 min
PCR cycling (40 cycles)	95 °C	10 s
	60 °C	60 s

4. 4. Evaluation of test result

4.4.1. Threshold setting has to be validated depending on the use of mastermix and thermal cycler

4.4.2. Results are valid only if positive controls give a clear signal with a Ct < 30 and negative controls have a Ct of > 35. The amplification of an internal control should give a clear signal, preferably a Ct < 30.

4.4.3. Determine if any viroids was detected in each seed lot and a positive detection of viroids is a Ct < 32.

5. REFERENCES

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6. APPENDICES

RNA extraction buffer

	100 ml	**1000 ml**
DI Water	35 ml	350 ml
PVP-40 (3%)	3 g	30 g
Guanidine Isothiocynate (4M)	47.30 g	473 g
Sodium acetate (0.2M)	1.6 g	16 g
0.5M EDTA (25mM)	5 ml	50 ml
Sodium sulfite (1%)	1 g	10 g
Adjust pH to 5.0 with 37% HCl		
Sodium metabisulfite	1 g	10 g

Preparation of SqMV as an Internal control for monitoring the quality of RNA extraction.

1. Take 0.1 g of SqMV infected tissue, grind and add 50 ml of GenEx buffer.
2. From this 50 ml suspension, make 10-fold serial dilutions from 10-1 to 10-4.
3. Take 10 µl from each dilution (at least 3 replications/dilution) and spike in to RNA extraction buffer and proceed with extraction procedure.
4. Run qRT-PCR to determine Ct values and Ct values should be determined to 28.
5. After determining Ct value of 28, aliquot in to 2 ml tubes and freeze the tubes for future use.
6. Thaw the tubes and use 10 µl spike for each tube (sub-sample).
7. Discard after 1 or 2 times of freeze and thaw cycles.
8. Ct value of SqMV spike in unknown samples should be 28 ±3.
9. Prepare new SqMV control if Ct values deviate from above values and validate.