Cucurbit Virus Diagnostic Assay QuickProtocol

Description: **Multiplex RT-PCR for Yellowing Viruses in Cucurbits**

Target virus(es): Cucurbit aphid-borne yellows virus (CABYV)
Cucurbit chlorotic yellows virus (CCYV)
Cucurbit yellow stunting disorder virus (CYSDV)
Squash vein yellowing virus (SqVYV)

Primers:

Target Virus	Primer Names	Sequence (5' → 3')	Amplicon Size (bp)
CABYV	CABYV_RdRp Forward[1]	AAGAGCGGCAGCTACAATAC	277
	CABYV_RdRp Reverse[1]	TGCCACATTCCGGTTCATAG	
CCYV	CCYV_RdRp Forward[2]	CTCCGAGTAGATCATCCCAAATC	953
	CCYV_RdRp Reverse[2]	TCACCAGAAACTCCACAATCTC	
CYSDV	CYSDV_RdRp Forward[3]	TTTCGGCTCCCAGAGTTAATG	492
	CYSDV_RdRp Reverse[3]	CGATCTCCGTGGTGTGATAAG	
SqVYV	SqVYV_Nib_RdRp Forward	TCTCTATCGCCCGTCCTATT	723
	SqVYV_Nib_RdRp Reverse	AGGCATGAGTGCTTCCATATC	

[1]Primer reference: Mondal et al. 2021. Plant Disease 105(11):3768.
[2]Primer reference: Kavalappara et al. 2021. Plant Disease 105(6):1862.
[3]Primer reference: Kavalappara et al. 2021. Viruses 13(6):988.

Protocol:

Reverse Transcription: Reverse transcription is carried out using the Promega M-MLV Reverse Transcriptase (Cat. No. M1701/M1705) as described below. Total sample reaction volume = 10 µL.

Step 1: Mix components below and incubate at 70°C for 5 min. Store on ice before adding components from Step 2.

4.75 µL nuclease-free water
0.5 µL random primers (10 µM)
1.5 µL sample RNA or total NA (concentration varies)

Step 2: Mix components below, increasing volumes as necessary for a master mix, and aliquot 3.25 µL of master mix into each completed reaction from Step 1. Incubate reactions at 25°C for 5 min, 37°C for 30 min, followed by 42°C for 25 min. Store completed reactions on ice or at -20°C until use in PCR.

2.0 µL M-MLV 5X Reaction Buffer
0.5 µL dNTPs (10 mM)
0.5 µL M-MLV (200 U/µL)
0.25 µL RNAse Inhibitor (40 U/µL)

Protocol (Continued):

PCR: PCR is carried out using the Applied Biosystems Platinum Multiplex Master Mix (Cat. No. 4464268) as described below. Mix components listed, increasing volumes as necessary for a master mix, and distribute 24 µL of master mix into each sample reaction tube. Add 1 µL of cDNA from Reverse Transcription Step 2 (above) for a total reaction volume of 25 µL. Incubate reactions at 95°C for 2 min; followed by 35 cycles of 95°C for 30 s; 57°C for 90 s; 72°C for 1 min; ending with72°C for 10 min. Store completed reactions on ice (temporary) or freeze at -20°C until analysis by agarose gel electrophoresis. Total sample reaction volume = 25 µL.

12.5 µL Platinum Multiplex Master Mix
0.25 µL each forward primer
0.25 µL each reverse primer
9.5 µL nuclease-free water

Notes:

- This is a two-step assay: one step for reverse transcription and one step for PCR.
- This assay was designed and optimized using Promega M-MLV Reverse Transcriptase and Applied Biosystems Platinum Multiplex Master Mix. It has been performed regularly and without issue. Assay performance with other polymerases may vary.
- This assay targets four RNA viruses that commonly occur in mixed infections in many cucurbit-producing regions of the United States. It does not include the DNA virus cucurbit leaf crumple virus (CuLCrV), which can also occur in mixed infections with one or more of the target viruses for this protocol. Additional or alternative diagnostic assays may be needed to determine the presence of CuLCrV in a sample, if CuLCrV is known to occur in your region.

Protocol Reference:

Mondal, S., Jenkins Hladky, L., and Wintermantel, W. M. 2023. Differential seasonal prevalence of yellowing viruses infecting melon crops in southern California and Arizona determined by multiplex RT-PCR and RT-qPCR. Plant Disease 107(9):2653-2664.

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