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YDV Multiplex PCR

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Protocol status: Working
We use this protocol and it's working

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Disclaimer

Abstract

This protocol describes the process of carrying out a multiplex PCR assay followed by capillary electrophoresis to detect C/BYDV and determine species (BYDV-MAV, BYDV-PAS, BYDV-PAV, CYDV (RPS or RPV)). The starting point for this is cDNA that has been synthesized from nucleic acid extracted from single aphids or leaf material - an internal control targeting GAPDH is included and primers can be selected depending on the sample matrix (aphid or barley). The BYDV primers were taken from:

Sõmera, M., Massart, S., Tamisier, L., Sooväli, P., Sathees, K. and Kvarnheden, A., 2021. A survey using high-throughput sequencing suggests that the diversity of cereal and barley yellow dwarf viruses is underestimated. *Frontiers in Microbiology*, *12*, p.673218.

In-house sequence data was used to modify primers where necessary, add a primer for CYDV and an internal control for both aphid and barley samples. The CYDV primers captures both RPS and RPV (high resolution melt analysis may be required to distinguish these if required).

Materials

Equipment

NAME	BRAND	SKU
MiniAmp Plus Thermal Cycler	Applied Biosystems	A37835

Equipment

NAME	TYPE	BRAND	SKU
5200 Fragment Analyzer System	Capillary Electrophoresis	Agilent Technologies	M5310AA

- Nuclease-free water **Merck MilliporeSigma (Sigma-Aldrich)**
- 2X QIAGEN Multiplex PCR Master Mix **Qiagen Catalog #**206143
- dsDNA reagent kit (1-500 bp) **Agilent Technologies Catalog #**DNF-905

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Troubleshooting

Safety warnings

Please read SDS associated with various consumables and kits used in this protocol and wear appropriate PPE. A site specific procedural risk assessment should be carried out prior to introducing this protocol to the lab.

Before start

This protocol assumes that you have already isolated Total RNA from plant or insect material, and converted to cDNA using random hexamers. The protocol uses capillary electrophoresis for separation of PCR products; however it is also possible to separate and visualise using a high quality agarose gel electrophoresis method.

PCR protocol

2h 45m

1 Identify samples for analysis, including positive (virus infected aphid/barley) and negative controls (virus free aphid/barley), a positive control consisting of a mix of gBlocks with sequences for all targets in the multiplex PCR, and a no-template control. Details on the gBlocks can be found here:

gBlocks-ordered-YDV-MULTIPLEX.... 2KB

2 Prepare the primer mix for the multiplex PCR (*YDVmixHV* when sample matrix is barley, or *YDVmixSA* when sample matrix is an aphid) using primers according to tables below. The primer mixes are prepared with each primer in the working solution at

[M] 10 micromolar (µM) .

YDVmixHV:

	A	B
	Primer Name	**Primer Sequence**
	RpvF	CTCGTGGGCTATCGCTATGG
	CydvR	TCATGGCGGAGCTCATGCAG
	GAPDH-HV-F	GGAGTCCACCGGTGTTTTCA
	GAPDH-HV-R	AGACAAACATGGGAGCGTCC
	GavF	GTTACAAGATCACAAACGTCAAG
	PasF	GAAGAGGGCCAAATTCTATACC
	PavF	CTTCACAATCAGCAGGAC
	Yan**R**24	TGTTGAGGRGTCTACCTATTTG

YDVmixSA:

	A	B
	Primer Name	**Primer Sequence**
	RpvF	CTCGTGGGCTATCGCTATGG
	CydvR	TCATGGCGGAGCTCATGCAG

	A	B
	GAPDH-SA-F	GGCGAAGTTTCTGTTGATGG
	GAPDH-SA-R	CAGCACCAGCAGATCCCC
	GavF	GTTACAAGATCACAAACGTCAAG
	PasF	GAAGAGGGCCAAATTCTATACC
	PavF	CTTCACAATCAGCAGGAC
	Yan**R**24	TGTTGAGGRGTCTACCTATTTG

3 Prepare a master-mix for the number of samples to be run so that each well will contain:

12.5 µL of 2X QIAGEN Multiplex PCR Master Mix **Qiagen Catalog #**206143

10.5 µL of molecular grade nuclease-free water

1 µL of working solution of appropriate primer mix prepared in step 2 above

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Note: use of the QIAGEN Multiplex PCR Master Mix (or equivalent)
is recommended - in the case of insect/plant/crop co-infection,
each target is amplified with similar efficiencies (in contrast to
other polymerase options). Using different enzymes will likely
require some optimisation.
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4 Pipette 1 µL of cDNA (or control) into each well of a PCR plate and keep on ice.

5 Pipette 24 µL of master-mix from step 3 into each well, seal the plate carefully with foil lid, and centrifuge the PCR plate briefly to ensure no droplets adhere to sides of sample wells.

6 Place the PCR plate in a 2h 30m

Equipment	
MiniAmp Plus Thermal Cycler	NAME
Applied Biosystems	BRAND
A37835	SKU

and run the following PCR cycle:

- 95 °C 00:05:00

followed by *40 cycles* of

- 95 °C 00:00:30
- 60 °C 00:01:30
- 72 °C 00:00:30

and a final elongation step of

- 68 °C 00:10:00

Capillary Electrophoresis

2h

7 Bring all reagents from dsDNA reagent kit (1-500 bp; DNF-905) to room temperature by removing from the fridge and freezer at least 00:30:00 prior to setting up a run. 30m

8 Set up a run on the 5200 Fragment Analyser by adding 1 mL of 1X Inlet Buffer into row A of the 1.2 ml deep 96-well plate (to make 12 mL fresh add 2.4 mL of 5X Inlet buffer to 9.6 mL of molecular grade water. Inlet buffer should be changed daily. Into the same plate place 1 mL of Capillary Storage solution into row H (note: the storage buffer only needs to be changed on a weekly basis). This plate needs to go into drawer "B" of the instrument.

9 Add 30 µL of ready to use marker (1 and 500 bp) to row A of a compatible 96-well PCR plate and add a drop of miner oil (provided in kit) to each well and place plate into drawer "M" of the instrument. The marker is good for 30+ injections or 1 month.

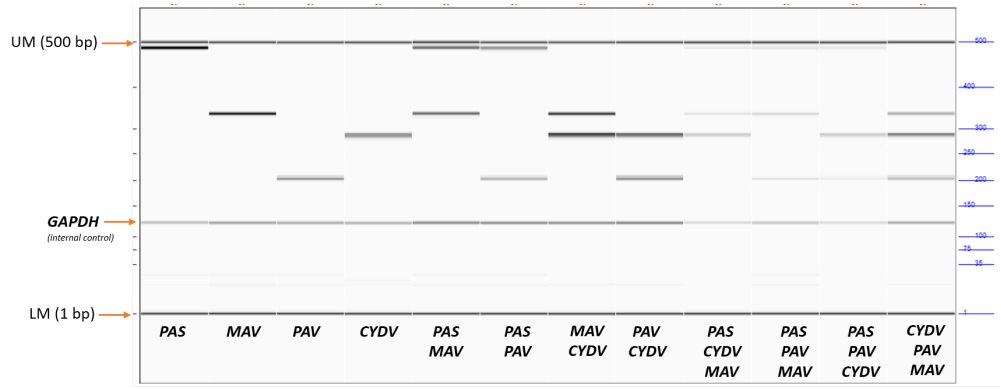
10 Prepare sufficient 1X conditioning solution for the number of samples you are going to run (e.g. 45 mL is sufficient for a single plate) and connect to correct fluid line.

11 Prepare sufficient gel for the number of samples you are going to run (e.g. 4.5 µL added to 45 mL of separation gel is sufficient for a single plate) and connect to correct fluid line.

12 The samples are prepared by adding 2 µL of PCR product to 22 µL of 1X TE (provided) in the well of a compatible PCR plate. Vortex the plate and centrifuge briefly to collect contents at bottom of the wells.

13 Update the solution levels on instrument and commence a run using method appropriate for the DNF-905-dsDNA (1 to 500 bp) kit.

14



An example of results from running PCR products from an amplification with YDVmixSA primer mix and various control template mixes (gBlocks) - all have internal GAPDH control, and then different combinations of BYDV-PAS, BYDV-MAV, BYDV-PAV, CYDV.

Protocol references

Sõmera, M., Massart, S., Tamisier, L., Sooväli, P., Sathees, K. and Kvarnheden, A., 2021. A survey using high-throughput sequencing suggests that the diversity of cereal and barley yellow dwarf viruses is underestimated. *Frontiers in Microbiology*, *12*, p.673218.