Feb 18, 2026

Real-time RT-PCR for detection of Yellow Dwarf Viruses

DOI
https://dx.doi.org/10.17504/protocols.io.dm6gpmm1jgzp/v1

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Protocol Citation: Stephen Byrne, Jack Perry, Louise McNamara, Md Munir Mostafiz 2026. Real-time RT-PCR for detection of Yellow Dwarf Viruses. **protocols.io** https://dx.doi.org/10.17504/protocols.io.dm6gpmm1jgzp/v1

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

Created: October 26, 2025

Last Modified: February 18, 2026

Protocol Integer ID: 230813

Keywords: yellow dwarf virus, BYDV, RT-PCR, aphid, barley, surveillance of key yellow dwarf virus, key yellow dwarf virus, pcr for detection, nucleotide, serial dilutions of gene fragment, yellow dwarf, single nucleotide polymorphism, gene fragment, aphid, aphid internal control, following species, pcr, barley sample matrix, species, high throughput assay, temperature of the shorter probe

Funders Acknowledgements:
Marie Skłodowska-Curie Actions (MSCA)
Grant ID: 101106698

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Abstract

The purpose was to create a useful assay to identify and distinguish BYDV-PAV, BYDV-MAV, and BYDV-PAS in a single reaction together with an internal control (for both an aphid and a barley sample matrix). The goal was to have a high throughput assay that could be used in the surveillance of key yellow dwarf viruses. Given the challenge in distinguishing all three species in one reaction, this required the design of shorter probes to distinguish between two of the species that were only two nucleotides apart in the target region. This required the use of locked-nucleotides to increase the annealing temperature of the shorter probes. These were designed to flank each of the two single nucleotide polymorphisms. The linearity and specificity of each target was confirmed using serial dilutions of gene fragments. The aphid internal control has been tested on the following species and shown to function: *Rhopalosiphum padi, Sitobion avenae, Metopolophium dirhodum.*

Materials

>IRE-24601-MAV-gbloc
TCAAAACCGGTCTCTATTAATGGGAGGTACAGAATGGTTAGAAGGCCCGATAGCATAGGCAAAGACAGCACAACACTAC
T
GAGCATGCTCAACCAATCCGACGTCAAAAGTTACATGTCGGCTGTGGCTCAGTGTGGTTTGGTGCTCAACGCTGGAGT
AC
CCATACTTGAAAGCTTCTATAAATGCCTATATAGAAGTTCAGGGTACAAGAAAGTGAGTGAGGAATTCATCAAAAACGTC
ATTTCGTATGGAACAGATGAGAGACTACAAGGTAGACGTACCTTCSAAGACACACCTATC
>IRE-24601-PAS-gbloc
TCAAAACCCGTCAGAATAGATGGCAAATACAGAATGGTGAGGAGACCAGACTGTATTGGAAAAGACAGCTGCACACTTT
T
GAGCATGTTGAATGAGGCTGACGTYAAGAGTTACATGTCAGCTGTGGCTCAGTGTGGACTCGTTTTGAATGCGGGTGTG
C
CCATTTTGGAAAGCTTCTACCGCTGCCTGTACAGAAGCTCTGGGTACAAGAAAGTGAGTGAGGAATACATCAAAAACGT
C
ATATCATATGGAACAGATGAACGCCTTCAAGGTAGACGTACCTTCAAGGAGACACCTATC
>IRE-24601-PAV-gbloc
TCAAAACCGGTCTCTATTAATGGAAAGTATAGAATGGTCAGAAGGCCCGATAGCATAGGCAAAGACAGCACAACACTAT
T
GAGCATGCTCAATCAATCCGACGTCAAGAGTTACATGTCGGCTGTTGCTCAGTGTGGCTTGGTGCTTAACGCTGGAGTA
C
CCATACTTGAAAGTTTCTATAAATGCCTGTATAGAAGCTCGGGGTACAAGAAAGTGAGTGAGGAATTTATTAAAAACGTC
ATATCGTATGGAACAGATGAGAGACTTCAAGGTAGACGTACCTATAACGAAACACCTATC

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.

Primer and Probe Design

1 The primer and probes for YDV were designed using genomes assembled from HTS of symptomatic barley samples in Ireland (Byrne et al., 2024). The primers are designed to target a region of 120 bp in ORF3-5 and were ordered from IDT as follows:

	Primer name	Sequence
	IRE-24601-for	ARA GTT ACA TGT CRG CTG TKG C
	IRE-24601-rev	TCC TCA CTC ACT TTC TTG TAC CC
	IRE-24601-HV-for	GGT GAG AAG GAG GTT GCT GTG
	IRE-24601-HV-rev	TGA AAA CAC CGG TGG ACT CCA
	IRE-24601-SA-for	GCG AAG TTT CTG TTG ATG GAG AC
	IRE-24601-SA-rev	GCA CCA GCA GAT CCC CAT TGG

Primers for the IRE-24601 assay. This includes primers to target a region in ORF3-5 of the BYDV genome, and primers to target GAPDH in barley and aphids.

The 5′ nuclease probes for each of the three species *Luteovirus pavhordei*, *Luteovirus mavhordei*, and *Luteovirus pashordei* were ordered from IDT as described in the table below.

	Probe Name	Sequence (with modifications)	Reporter Dye	Modifications
	IRE-24601-PAS-P	/5TexRd-XN/TGT GGA CTC GTT TTG AAT GCG GGT GTG C/3IAbRQSp/	Texas red	5' Texas Red®-X (NHS Ester) 3' Iowa Black™ RQ-Sp
	IRE-24601-MAV-APP	/5HEX/TG+G +T+TT GGT GC+T +C+AA C/3IABkFQ/	Hex	Affinity Plus™ 5' HEX 3' Iowa Black™ FQ
	IRE-24601-PAV-APP	/5Cy5/TG+G +C+TT GGT GC+T +T+AA C/3IAbRQSp/	Cy5	Affinity Plus™ 5' Cy®5 3' Iowa Black™ RQ-Sp
	IRE-24601-HV-P	/56-FAM/AGA TTC CAT /ZEN/GGG CCG CTG CTG GT/3IABkFQ/	Fam	Int ZEN™ 5' 6-FAM 3' Iowa Black™ FQ

	Probe Name	Sequence (with modifications)	Reporter Dye	Modifications
	IRE-24601-SA-P	/56-FAM/TTC TCT GAA /ZEN/CGC GAC CCA AAG GCC A/3IABkFQ/	Fam	Int ZEN™ 5' 6-FAM 3' Iowa Black™ FQ

Probes for the IRE-24601 assay. + indicates locked nucleotides in the probes with suffix 'APP' (Affinity Plus from IDT); these surround the two nucleotides distinguishing *Luteovirus pavhordei* and *Luteovirus mavhordei*

Please note that during development , it was demonstrated that longer probes designed without locked nucleotides fail to distinguish between *Luteovirus pavhordei*, and *Luteovirus mavhordei.* The shorter Affintiy Plus probes are required.

2 The primers and probes were all prepared as stocks of [M] 100 micromolar (µM) and two **working primer mixes** and two **working probe mixes** were prepared as follows:

	Mix Name	Sample Matrix	Components
	IRE-24601-hvITC-primers	barley	IRE-24601-for; IRE-24601-rev; IRE-24601-HV-for; IRE-24601-HV-rev (each at 10 uM)
	IRE-24601-saITC-primers	aphids	IRE-24601-for; IRE-24601-rev; IRE-24601-SA-for; IRE-24601-SA-rev (each at 10 uM)
	IRE-24601-hvITC-AAPs	barley	IRE-24601-PAS-P; IRE-24601-PAV-APP; IRE-24601-MAV-APP; IRE-24601-HV-P (each at 10 uM)
	IRE-24601-saITC-AAPs	aphids	IRE-24601-PAS-P; IRE-24601-PAV-APP; IRE-24601-MAV-APP; IRE-24601-SA-P (each at 10 uM)

Primer and probe mixes prepared for use in the assay. The combination of primer/probe mixes will depend on the sample matrix - this ensures the correct internal control is utilised.

Gene Fragments

3 Gene fragments were synthesised (IDT gBlocks) for use as control material and to test specificity of probes during initial testing and design phase. A 300 bp fragment covering the target region was synthesised for all three species. The sequence of all three is included under the protocols materials section.

4 The gene fragments were prepared as [M] 10 ng/ul stocks and then serially diluted for use in testing linearity and efficiency of each target, testing specificity, and for making

positive control mixes.

5 A positive control mix was created for routine use when testing plates of samples - for use in addition to real positive (aphid or barley positive for virus) and negative (aphid or virus free barley). The gBlock control mixes were prepared by mixing together the three gBlocks for each species (at [M] 0.1 ng/ul of each gBlock).

Real-Time RT-PCR

6 The assay was developed and run on an LC96 (Roche) (although should work on other systems compatible with dyes used here). The assay was optimised using PrimeTime™ One-Step RT-qPCR Master Mix from Integrated DNA Technologies.

7 The reactions were set-up on ice according to the following recipe (this is for the case of testing a barley sample matrix):

	Component	Amount (ul)
	PrimeTime One-Step RT- qPCR Master Mix (2X)	10
	IRE-24601-hvITC-primers (10 uM)	1
	IRE-24601-hvITC-AAPs (10 uM)	0.5
	Molecular Grade Water	6.5
	Total	18

RT-PCR reaction recipe - the final reaction volume will be 20 ul after addition of 2 ul of template RNA.

The reactions were prepared in white opaque plates and sealed with optically clear film. The master mix was prepared and dispensed into each well prior to addition of 2 µL of template RNA. All reactions were prepared on ice and the plate was briefly spun down before loading into the LC96.

8 The cycling parameters are:

1. 50 °C for 00:15:00
2. 95 °C for 00:03:00
3. 95 °C for 00:00:15
4. 60 °C for 00:01:00

Steps 3-4 are repeated for 40-45 cycles.

Analysis

9 The results are analysed with the LightCycler 96 software as a qualitative analysis. The internal control is identified in the analysis and used as control to validate extraction, and reaction. Samples negative for internal control are disregarded if virus negative.

10 A Cq cut-off of 30 cycles was implemented and minimum endpoint fluorescence threshold (EPF) was identified and selected for each target.

Protocol references

S. Byrne, M. Schughart and V. Ballandras et al. The first survey using high-throughput sequencing of cereal and barley yellow dwarf viruses in Irish spring and winter barley crops. *Irish Journal of Agricultural and Food Research.* 2024. Vol. 63(1):1-16. DOI: 10.15212/ijafr-2023-0110