

One-enzyme multiplex RTX-PCR for detection of RNA and DNA viruses in cereals using total nucleic acid templates

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Abstract

Background: Detection of mixed infections involving viruses with different genome types requires a diagnostic workflow that can amplify RNA and DNA targets in a single reaction, thereby avoiding separate nucleic-acid-specific procedures.

Aims: This study aimed to develop a one-step PCR assay for the simultaneous detection of multiple viruses using a universal single template, total nucleic acid (TNA).

Methods: Wheat and barley field isolates were subjected to extraction of TNA using potassium ethyl xanthogenate (PEX) buffer. Wheat dwarf virus (WDV) and barley yellow dwarf virus (BYDV) were then detected using a one-enzyme RTX-PCR-based assay. Conventional PCR and two-step RT-PCR were used to validate the TNA-RTX-PCR assay, and absolute quantification by qPCR was performed to determine WDV and BYDV titres.

Results: TNA-RTX-PCR confirmed the simultaneous detection of WDV and BYDV, enabling the detection of viruses with different genome types in a single reaction. Conventional PCR and RT-PCR validated the presence of WDV and BYDV in the same isolates. Absolute quantification showed different WDV and BYDV titres range coherent with the PCR detection.

Conclusions: This one-step TNA-RTX-PCR assay is an efficient approach for detecting viruses with different genome types in a single reaction and eliminates the need for separate reverse transcription and DNA-based PCR workflows.

Keywords: barley yellow dwarf virus, qPCR, RTX-PCR, total nucleic acid, wheat dwarf virus.

Introduction

Viral diseases of cereals represent a major constraint on wheat and barley production in many agricultural regions worldwide, including the European Union. Among the most prevalent pathogens are wheat dwarf virus (WDV) and barley yellow dwarf virus (BYDV), which co-occur across European cereal-growing regions and cause substantial yield and grain-quality losses (Ilbağ and

Çıtır, 2024). WDV, a member of the family *Geminiviridae*, is a single-stranded DNA virus persistently transmitted by the leafhopper *Psammotettix alienus* (Dahlbom, 1850). Its occurrence in the Czech Republic was first reported by Vacke (1961), and the virus has subsequently been documented in many other regions (Pfrieme et al., 2023). Two major WDV strains have been described: WDV-W, the wheat strain, and WDV-B, the barley strain (Kundu et al., 2009). The wheat strain is generally considered more

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Abbreviations: BYDV - barley yellow dwarf virus; one-enzyme RTX-PCR - PCR using RTX enzyme for one enzyme RT-PCR; PEX - potassium ethyl xanthogenate; qPCR - quantitative polymerase chain reaction; RT-PCR - reverse transcription polymerase chain reaction; TNA - total nucleic acid; WDV - wheat dwarf virus.

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virulent and has a broader host range, whereas the barley strain predominantly affects barley and related species (Kundu et al., 2009).

BYDV, a member of the family *Luteoviridae*, is a positive-sense single-stranded RNA virus persistently transmitted by more than 25 aphid species, with *Rhopalosiphum padi* being a predominant vector in European cereal systems (D'Arcy and Burnett, 1995; Jones et al., 2024). The virus infects virtually all members of the *Poaceae*, making more than 150 grass species potential inoculum sources (Miller and Rasochová, 1997). Both viruses co-occur in many cereal production areas, and their overlapping host ranges and vector-mediated spread increase the risk of mixed infections (Armand et al., 2023). Because their detection traditionally relies on different nucleic acid extraction and amplification strategies, co-infected samples present a practical diagnostic challenge (Jarošová et al., 2018).

Detection of DNA viruses typically relies on direct PCR, whereas RNA virus detection requires a prior reverse transcription step to synthesise complementary DNA (cDNA). Consequently, when both genome types must be screened simultaneously, parallel and time-consuming workflows are often required. To overcome this limitation, total nucleic acid (TNA) can be used as a universal diagnostic template, enabling the simultaneous detection of viruses with divergent genome types from a single sample (Parida, 2008). Hoffmeisterová et al. (2022) reported the proof-reading activity of a novel one-enzyme xenopolymerase/reverse transcriptase, RTX enzyme. The RTX enzyme enhances direct amplification of DNA from an RNA template in a single-enzyme reverse-transcription polymerase chain reaction (single-enzyme RTX-PCR), as well as from a DNA template, improving the sensitivity and efficiency of the reaction. We use this specific activity of the RTX enzyme coupled with the TNA as universal template in this study to improve the diagnostic efficiency and reduce both time and resource requirements compared with parallel DNA- and RNA-specific workflows.

Several PEX-based protocols for TNA isolation have been described. Jhingan (1992) developed a protocol for plant genomic DNA extraction using 4 mL PEX buffer in a 15-mL tube. Williams and Ronald (1994) adapted this method into a microscale format incorporating a multi-step lysis process. Hataya (2021) later optimised lysis conditions among protocol variants and identified an 80°C/10 min SPEX-B incubation as effective. In the present study, because pre-ground leaf tissues were used, this lysis step was replaced with a single 56°C/20 min incubation to streamline the procedure and improve compatibility with both RNA and DNA templates. Coupling TNA extraction with a single-step reverse transcription-PCR assay represents a practical advance for plant virus diagnostics, particularly where mixed infections are frequent. Here, we describe a modified PEX-based TNA isolation protocol combined with a one-step RTX-PCR assay for the simultaneous detection of WDV and BYDV from the same cereal leaf sample.

Materials and methods

Plant material and virus source: Twenty-two field samples of wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*) naturally infected with WDV, BYDV, or both viruses were collected from four regions of the Czech Republic (refer table below). Samples F1 - F18 were symptomatic with the typical signs of virus infection (reduced plant height and yellowing of leaves) and H1 - H4 were asymptomatic and were used as tested further to use as internal negative control. Leaf tissue was ground under liquid nitrogen and stored at -80°C until nucleic acid extraction.

Sample ID	Region	Type
F1	Poličná	Wheat
F2	Poličná	Wheat
F3	Poličná	Wheat
F4	Poličná	Wheat
F5	Ruzyně	Wheat
F6	Ruzyně	Wheat
F7	Ruzyně	Wheat
F8	Ruzyně	Wheat
F9	Ruzyně	Wheat
H1	Ruzyně	Wheat
H2	Ruzyně	Wheat
H3	Ruzyně	Wheat
F10	Ruzyně	Wheat
F17	Hradec	Wheat
F18	Hradec	Wheat
H4	Hradec	Wheat
F11	Čechtice	Wheat
F12	Čechtice	Wheat
F13	Čechtice	Wheat
F14	Čechtice	Barley
F15	Čechtice	Barley
F16	Hřidelec	Barley

Total nucleic acid extraction: Total nucleic acids were extracted using a modified PEX-based protocol (Hataya, 2021), in which the 80°C/10 min SPEX-B lysis step was replaced with incubation at 56°C for 20 min to accommodate pre-ground tissue. The PEX extraction buffer consisted of 6.25 mM polyethylene glycol (*Thermo Fisher Scientific*, Waltham, WA, USA), 100 mM Tris-HCl (pH 7.5), 700 mM NaCl, and 10 mM EDTA (pH 8.0). Approximately 50 - 100 mg of frozen leaf tissue was mixed with 1 mL of PEX buffer and incubated at 56°C for 20 min with intermittent vortexing. Chloroform (0.5 mL) was added, the mixture was vortexed vigorously for 30 s, and phases were separated by centrifugation at 13 000 × g for 10 min. The aqueous phase was transferred to a new tube, and TNA was precipitated by adding 0.7 mL of isopropanol and incubating at 4°C for 10 min, followed by centrifugation at 13 000 × g for 10 min. The pellet was washed twice with 70% ethanol, air-dried, and resuspended in 50 µL of RNase-free water.

Genomic DNA and total RNA extraction: For comparative assays, genomic DNA was extracted from 50 - 100 mg of frozen leaf tissue using a modified CTAB protocol (Doyle and Doyle, 1987; Schenk *et al.*, 2023) and resuspended in 50 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Total RNA was extracted from 50 - 100 mg of leaf tissue using *TRIzol*TM reagent (Thermo Fisher Scientific, Waltham, WA, USA) according to the manufacturer's instructions and resuspended in 50 µL of DEPC-treated water. The purity of all nucleic acid preparations was assessed using a *NanoDrop 2000* spectrophotometer (Thermo Fisher Scientific, Waltham, WA, USA). Samples with A_{260}/A_{280} ratios of 1.8 - 2.0 and A_{260}/A_{230} ratios > 1.8 were considered suitable for downstream applications.

Virus detection: WDV and BYDV were detected simultaneously using a one-step multiplex RTX-PCR assay as described by Hoffmeisterová *et al.* (2022), with minor modifications. Each 20 µL reaction contained 2 µL of 10× Phusion buffer (Thermo Fisher Scientific, Waltham, WA, USA), 1.2 µL of 10 mM dNTP mix, 0.6 µL of each primer (see table below), 2 µL of 5 M betaine (final concentration 0.5 M; Thermo Fisher Scientific, Waltham, WA, USA), 0.12 µL of 5U/µL RTX polymerase (Hoffmeisterová *et al.*, 2022), 6 µL of TNA (~100 ng), and nuclease-free water to a final volume of 20 µL. Positive controls consisted of DNA extracted from WDV-W-infected plants in our greenhouse experiments (strain accession: GenBank FJ546188) and RNA extracted from BYDV-PAV-infected plants (strain accession: GenBank FJ645745). Negative controls included a no-template control containing nuclease-free water and nucleic acids extracted from healthy plants.

Amplification was performed in a thermocycler *C1000* (Bio-Rad, Hercules, CA, USA) using the following programme: initial reverse transcription at 68°C for 30 min; 33 cycles of denaturation at 98°C for 10 s, annealing at 58°C for 20 s, and extension at 72°C for 20 s; followed by a final extension at 72°C for 10 min.

For validation, conventional PCR-based detection of WDV and two-step RT-PCR detection of BYDV were performed in parallel using established protocols. The same primer pairs were used in qPCR detection as well for both WDV and BYDV detection. WDV detection was carried out using 2× *DreamTaq PCR Master Mix* (Thermo Fisher Scientific, Waltham, WA, USA) in 10 µL reactions containing 5 µL of master mix, 0.5 µL of each primer (UniWDVfw/UniWDVrv), 0.5 µL of genomic DNA, and 3.5 µL of nuclease-free water. For BYDV detection, cDNA was first synthesised from 1 µg of total

RNA using *RevertAid* reverse transcriptase (200 U/µL; Thermo Fisher Scientific, Waltham, WA, USA) and random hexamer primers, following a three-step programme of 25°C for 10 min, 42°C for 60 min, and 70°C for 10 min. PCR amplification was then performed using the same *DreamTaq* reaction setup with PVinterF/YanRa primers and 0.5 µL of cDNA as template.

Both conventional assays used identical thermocycling conditions: 95°C for 3 min; 33 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min; and a final extension at 72°C for 10 min. PCR products were resolved by electrophoresis on 2% agarose gels stained with *SYBR*TM Safe DNA Gel Stain (Thermo Fisher Scientific, Waltham, WA, USA) and visualised under UV illumination.

Virus quantification: Viral titres were determined by absolute quantification using *SYBR Green I*-based qPCR on a *LightCycler*® 480 system (Roche, Basel, Switzerland). Each 12 µL reaction contained 6 µL of 2× *SYBR Green Master Mix*, 0.6 µL of each primer (10 µM stock), 1 µL of template (genomic DNA, ~100 ng, for WDV; five-fold diluted cDNA for BYDV), and nuclease-free water to 12 µL.

Plasmid standards were prepared by cloning the WDV and BYDV target amplicons into *pGEM-T Easy Vector* (Promega, Madison, WI, USA). Inserts were verified by Sanger sequencing, and plasmid concentrations were determined spectrophotometrically. Standard curves were constructed from eight-point, ten-fold serial dilution series spanning 10^2 to 10^8 copies per reaction, each run in three technical replicates. Viral genome copy numbers were calculated according to Lee *et al.* (2006): copies = (amount of DNA [ng] × 6.022×10^{23}) / (plasmid length [bp] × $1 \times 10^9 \times 660$).

qPCR cycling conditions were as follows: 95°C for 10 min; 40 cycles of 95°C for 15 s and 65°C for 60 s; followed by melt-curve analysis at 95°C for 15 s, 60°C for 1 min, and a ramp to 95°C. Standard-curve efficiency and linearity (R^2) were assessed for each run to confirm assay performance. Ct values were determined using the second-derivative method and converted to absolute copy numbers by linear regression against the standard curve (Jarošová and Kundu, 2010).

Results

Of the 22 field samples collected from four regions of the Czech Republic, 18 were infected with WDV, BYDV, or both viruses. Four samples, H1 - H4, yielded no amplification in any assay and were confirmed as healthy or virus-negative controls (Fig. 1).

Description	Primer	Product length	Annealing temperature	Primer sequence (5' - 3')	Reference
WDV	UniWDVfw UniWDVrv	152 bp	65°C	TCCGCGCTAGGACAGTCACT AAGATTGGCTCAAGGATATGACTCC	Gadiou <i>et al.</i> (2012)
BYDV	PVinterF YanRa	294 bp	60°C	GTTGAGTTTAAAGTCACACGC TGTTGAGGAGTCTACCTATTG	Jarošová and Kundu (2010) Malmstrom and Shu (2004)

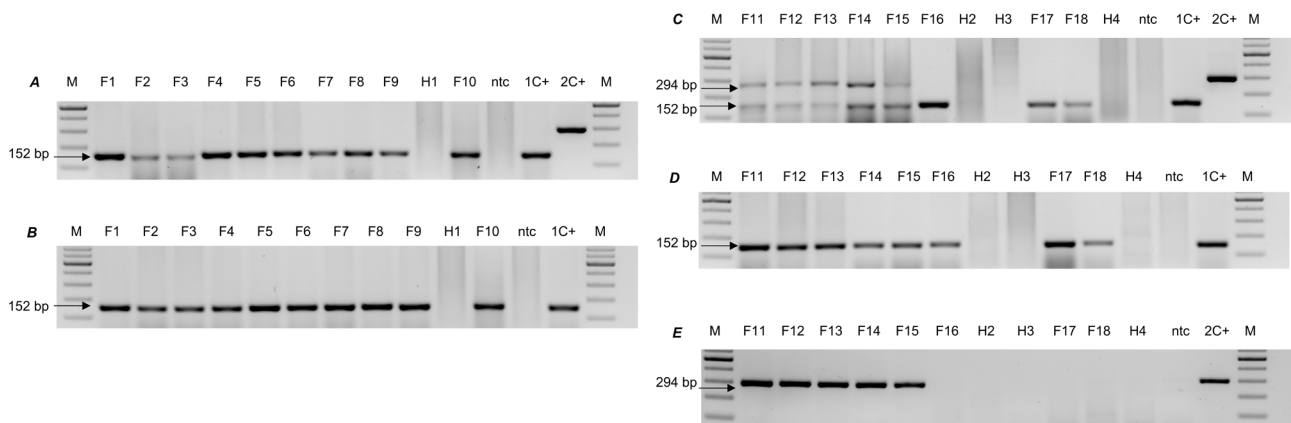


Fig. 1. Comparison of methods used to detect WDV and BYDV in wheat and barley field samples. (A) Detection of WDV in field samples using the TNA-RTX-PCR method. (B) Detection of WDV in field samples using the DreamTaq PCR method. (C) Detection of WDV and BYDV in mixed infections using the TNA-RTX-PCR method. (D) Detection of WDV in the same samples using the DreamTaq PCR method. (E) Detection of BYDV in the same samples using cDNA and the DreamTaq PCR method. M: *GeneRuler 100 bp Plus DNA Ladder* (Thermo Fisher Scientific, Waltham, WA, USA); lanes 1 - 18: WDV- and/or BYDV-infected field samples; H1 - H4: asymptomatic field samples; NTC: no-template control; 1C+: WDV positive control; 2C+: BYDV positive control.

Conventional PCR and two-step RT-PCR were used to confirm the presence of WDV and BYDV in the 22 field samples prior to evaluation of the multiplex RTX-PCR assay. WDV was detected by *DreamTaq* PCR using genomic DNA extracted with a modified CTAB protocol, yielding the expected 152 bp amplicon in 18 samples (F1 - F18) and no amplification in the four asymptomatic controls (H1 - H4) (Fig. 1B,D). BYDV was detected by two-step RT-PCR using total RNA extracted with *TRIzol*, in samples F11 - F15 (Fig. 1E). Corresponding to the conventional PCR workflows, the TNA template isolated by PEX-method, WDV was detected by one-step RTX-PCR in wheat field samples F1 - F10 (Fig. 1A), producing the expected 152 bp amplicon from TNA templates. This finding is consistent with the universal-template concept described by Parida (2008), in which TNA serves as a single-extraction solution for viruses with divergent genome types.

Simultaneous detection of both WDV (152 bp) and BYDV (294 bp) from a single TNA template was achieved in five co-infected samples, namely wheat samples F11 - F13 and barley samples F14 - F15, using the multiplex RTX-PCR assay (Fig. 1C). WDV was additionally detected as a single-virus infection in samples F16 - F18. The ability to distinguish single-virus infections from co-infections within the same reaction demonstrates the diagnostic utility of the multiplex format in field surveillance, particularly given the documented co-occurrence of both pathogens in European cereal systems (Armand et al., 2023). Table 1 summarizes the detection of different viruses by conventional PCR vs. TNA-RTX-PCR method.

RTX-PCR yielded detectable amplification, suggesting improved performance of the RTX-based assay in low-titre samples. However, this observation should be verified by formal analytical sensitivity testing. RTX polymerase combines reverse transcriptase and thermostable DNA polymerase activities, enabling

reverse transcription and PCR amplification in a single tube, whereas conventional *DreamTaq*-based detection of RNA targets requires a separate reverse transcription step (Hoffmeisterová et al., 2022). Assay reliability was further supported by consistent amplification of the WDV-W (GenBank FJ546188) and BYDV-PAV (GenBank FJ645745) positive controls and by the absence of amplification in negative controls.

Betaine is known to reduce secondary DNA structures and normalise the melting temperature of GC-rich regions (Lajin et al., 2013), which is particularly relevant in multiplex PCR. In the present assay, two primer pairs targeting sequences with different amplification properties had to perform consistently within the same reaction. The consistent amplification observed across positive samples, including those with relatively low viral titres, is consistent with the known benefits of betaine in stabilising multiplex amplification efficiency (Lajin et al., 2013).

Absolute quantification by qPCR confirmed WDV in all 18 infected samples (F1 - F18). Healthy controls (H1 - H4) yielded no detectable Ct values. WDV titres spanned more than four orders of magnitude, ranging from 0.84 to 2.46×10^5 copies per 100 ng DNA (Fig. 2B). This wide range indicates considerable variation in viral load across field samples and is consistent with previous reports of heterogeneous WDV accumulation in naturally infected cereals (Jarošová et al., 2018). The WDV standard curve had a slope of -3.388 , an amplification efficiency of 97.3%, and an R^2 value of 0.9887, confirming acceptable assay performance and linearity (Fig. 2A).

BYDV was detected exclusively in co-infected samples F11 - F15 (Fig. 2D). Titres ranged from 6.23×10^2 to 1.17×10^4 copies per 100 ng cDNA. The BYDV standard curve had a slope of -3.436 , an efficiency of 95.4%, and an R^2 value of 0.9793, confirming excellent linearity (Fig. 2C). Notably, WDV titres were consistently higher than BYDV titres across co-infected samples. The higher

Table 1. Detection by conventional PCR vs. TNA-RTX-PCR.

Sample ID	Symptoms	Detected virus	
		Conventional PCR	TNA-RTX-PCR
F1	Symptomatic	WDV	WDV
F2	Symptomatic	WDV	WDV
F3	Symptomatic	WDV	WDV
F4	Symptomatic	WDV	WDV
F5	Symptomatic	WDV	WDV
F6	Symptomatic	WDV	WDV
F7	Symptomatic	WDV	WDV
F8	Symptomatic	WDV	WDV
F9	Symptomatic	WDV	WDV
F10	Symptomatic	WDV	WDV
F11	Symptomatic	BYDV	WDV + BYDV
F12	Symptomatic	BYDV	WDV + BYDV
F13	Symptomatic	BYDV	WDV + BYDV
F14	Symptomatic	BYDV	WDV + BYDV
F15	Symptomatic	BYDV	WDV + BYDV
F16	Symptomatic	-	WDV
F17	Symptomatic	-	WDV
F18	Symptomatic	-	WDV
H1	Asymptomatic	Healthy (virus free)	
H2	Asymptomatic	Healthy (virus free)	
H3	Asymptomatic	Healthy (virus free)	
H4	Asymptomatic	Healthy (virus free)	

WDV titres observed in the co-infected samples may reflect differences in replication kinetics, tissue distribution, or the relative timing of infection. Alternatively, antagonistic interactions between the co-infecting viruses may have influenced their accumulation, such that infection by one virus could restrict the replication, movement, or systemic distribution of the other. Further experiments using controlled single and mixed infections, together with time-course analysis, would be required to distinguish among these possibilities (Kammann *et al.*, 1991; Miller and Rasochová, 1997). This observation is consistent with previous reports of asymmetric virus accumulation in co-infected cereals (Armand *et al.*, 2023), although it requires further targeted investigation. The WDV titres showed considerable variation among the field samples, spanning more than four orders of magnitude. The BYDV results are presented descriptively for the co-infected samples, and further evaluation would be required to determine the performance of the assay across a broader range of BYDV concentrations.

Discussion

Taken together, these results demonstrate that the TNA-RTX-PCR assay performs reliably across different infection types and viral loads. The co-occurrence of WDV and BYDV in cereal crops represents a diagnostic bottleneck because their divergent genomes, WDV as ssDNA and BYDV as positive-sense ssRNA, typically

require separate extraction and amplification workflows (Jarošová *et al.*, 2018). Recent advances in recombinant enzyme technology have generated interest in improving the specificity and fidelity of PCR-based assays. Such enzymes can enhance the accuracy of nucleic acid amplification in applications including pathogen detection and molecular diagnostics (Okano *et al.*, 2018). The present results show that the use of TNA as a universal template, combined with the bifunctional activity of RTX polymerase, can consolidate RNA- and DNA-virus detection into a single reaction.

The RTX enzyme provides reverse transcriptase and DNA polymerase activities in one enzyme system and has been reported to offer improved performance in reverse transcription and amplification workflows (Ellefson *et al.*, 2016). In this respect, the present assay addresses a key practical limitation associated with mixed-genome virus detection, namely the need to maintain separate workflows for RNA and DNA targets. Recent single-step multiplex assays have mainly focused on viruses with similar genome types, particularly positive-sense RNA viruses (Huang *et al.*, 2025), whereas the present approach enables simultaneous detection of DNA and RNA viruses from the same TNA preparation.

The method also offers practical advantages over TaqMan-based multiplex qPCR assays such as that described by Jarošová *et al.* (2018). Although probe-based qPCR is highly sensitive and quantitative, it requires fluorescent probes, real-time PCR instrumentation, and higher per-reaction costs. In contrast, the TNA-RTX-PCR

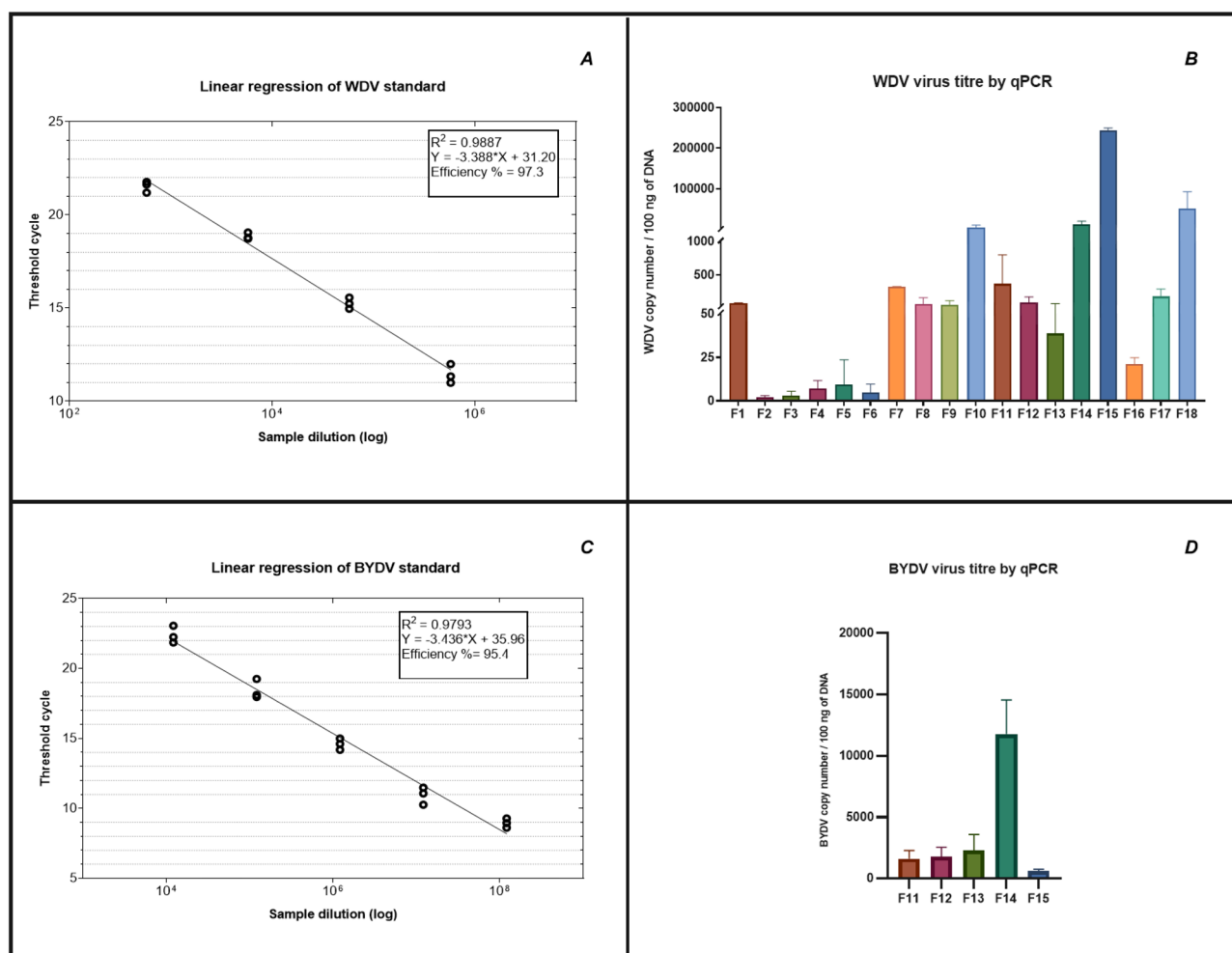


Fig. 2. Evaluation of WDV and BYDV copy numbers by qPCR. (A) Standard curve for *SYBR Green I*-based qPCR using UniWDVfw and UniWDVrv primers on ten-fold serial dilutions of WDV plasmid DNA (10^2 to 10^5 copies per reaction, in triplicate). (B) WDV copy number in each field sample per 100 ng DNA. Bars represent means $\pm$ standard errors from three technical replicates. (C) Standard curve for *SYBR Green I*-based qPCR using PVinterF and YanRa primers on ten-fold serial dilutions of BYDV plasmid DNA (10^2 to 10^6 copies per reaction, in triplicate). (D) BYDV copy number in each field sample per 100 ng cDNA. Bars represent means $\pm$ standard errors from three technical replicates.

assay uses a standard gel-based readout, making it more accessible for diagnostic laboratories without real-time PCR infrastructure and suitable for large-scale binary screening programmes in which reliable presence-or-absence detection is the main objective. Other studies have also achieved simultaneous detection of DNA and RNA plant viruses using multiplex RT-PCR approaches, including Zhang et al. (2017) for WDV and other cereal viruses and Periasamy et al. (2006) for rice tungro viruses. These methods primarily rely on total RNA as the template and can detect both genome types in a single reaction, offering simplicity and low cost. However, they are not explicitly designed around a universal nucleic acid template that treats DNA and RNA on equal footing from extraction through amplification. By consolidating RNA- and DNA-virus detection into a single reaction from a universal TNA preparation using a bifunctional RTX polymerase, the present assay provides a conceptually unified workflow that may be more robust when DNA and RNA virus titres

vary or when DNA virus loads are low, while retaining the option to apply qPCR as a confirmatory, quantitative step when needed.

The modified PEX-based TNA isolation protocol yielded nucleic acid preparations with A_{260}/A_{280} ratios of 1.8 - 2.0. Substitution of the 80°C/10 min SPEX-B lysis step with milder incubation at 56°C for 20 min was intended to reduce the risk of RNA degradation while preserving DNA template quality. The TNA-RTX-PCR assay can improve early and accessible diagnostics in official laboratories by consolidating WDV (ssDNA) and BYDV (ssRNA) detection into a single extraction and one-step multiplex reaction, reducing hands-on time, consumables, and infrastructure requirements compared with conventional separate DNA/RNA workflows (see table below). This is particularly relevant for field applications and large-scale surveillance, where rapid presence/absence decisions are prioritized; a two-tier strategy – qualitative TNA-RTX-PCR screening followed

Aspect	Conventional workflow (separate DNA/RNA)	Proposed TNA-RTX-PCR workflow
Nucleic acid extraction	Separate DNA extraction (<i>e.g.</i> , CTAB) and RNA extraction (<i>e.g.</i> , <i>TRIzol</i>)	Single TNA extraction (modified PEX buffer) from one leaf sample
Number of extraction steps per sample	≥ 2 independent extractions (DNA + RNA)	1 extraction (TNA)
Reverse transcription	Required as separate step for BYDV (two-step RT-PCR)	Integrated in one-step RTX-PCR (single enzyme, single tube)
Amplification reactions per sample	At least 2 separate reactions (WDV PCR + BYDV RT-PCR)	1 multiplex RTX-PCR reaction for both viruses
Instrumentation	Thermocycler; optionally real-time PCR if qPCR used	Standard thermocycler and gel electrophoresis; qPCR optional for quantification
Consumables	Separate kits/reagents for DNA and RNA; additional enzymes and tubes	Single extraction buffer; one enzyme mix; fewer tubes and reagents
Hands-on time	Higher (multiple extractions and setups)	Reduced (single extraction, single reaction setup)
Output	Separate results for WDV and BYDV; integration by user	Combined result for WDV and BYDV in one reaction
Suitability for high-throughput screening	More laborious and costly per sample	More scalable and cost-effective for binary screening

by selective qPCR confirmation – offers a cost-effective, scalable framework for epidemiological monitoring and can be extended to other mixed-genome virus complexes in cereals. The analytical detection limit of the multiplex TNA-RTX-PCR assay was not determined. The plasmid dilution series was used for qPCR quantification rather than assay validation. Future studies should assess the sensitivity and detection limit using serial dilutions of individual and mixed WDV and BYDV standards, analytical sensitivity, specificity, repeatability, and limit of detection of the assay under controlled conditions and assess its suitability for epidemiological surveys involving additional co-infecting cereal viruses.

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