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# Development and evaluation of one-step RT-qPCR TaqMan multiplex panels applied to six viruses occurring in lily and tulip bulbs

M.P.E. van Gent-Pelzer [*,1], A.M. Dullemans [*,1], M. Verbeek, P.J.M. Bonants, T.A.J. van der Lee

*Wageningen Plant Research, Wageningen University & Research, Droevendaalsesteeg 1, Wageningen 6700 AA, the Netherlands*

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ABSTRACT

One-step RT-qPCR TaqMan assays have been developed for six plant viruses with considerable economic impact in the growing of tulip and lily bulbs: lily mottle virus, lily symptomless virus, lily virus X, Plantago asiatica mosaic virus, tulip breaking virus and tulip virus X. To enhance efficacy and cost-efficiency these assays were combined into multiplex panels. Four different multiplex panels were designed, each consisting of three virus assays and an adapted assay for the housekeeping gene nad5 of lilies and tulips, that acts as an internal amplification control. To eliminate false negative results due to variation in the viral genome sequences, for each target virus two assays were developed on distinct conserved genomic regions. Specificity, PCR efficiency and compatibility of primers and probes were tested using gBlock constructions. Diagnostic samples were used to evaluate the strategy. High Throughput Sequencing of a set of the diagnostic samples, further verified the presence or absence of the viruses in the RNA samples and sequence variations in the target genes. This inter-changeable multiplex panel strategy may be a valuable tool for the detection of viruses in certification, surveys and virus diagnostics.

## 1. Introduction

Flower bulbs represent an important commodity crop for which the Netherlands is the largest exporting country (Niisato and Takeda, 2016). In the Netherlands, the total acreage of bulb flowers consisted in 2022 of 28045 ha (www.agrimatie.nl). Lilies and tulips are the most important bulb flower crops worldwide, and produced for bulb export but are also grown to produce cut flowers (Buschman, 2005).

Viral diseases and their associated symptoms have been documented extensively. A typical virus associated phenotype known as flower breaking in the flowers of tulip is a well-known phenomenon for hundreds of years and this phenotype was esthetically highly appreciated in the 17th century resulting in tremendous high prices to be paid for single bulbs. Subsequent observations demonstrated that flower breaking was a kind of degeneration and that it could be transmitted to other tulip plants (Cayley, 1928). Also viruses of lily were known already in the beginning of the 20th century (Ogilvie, 1928). The vegetative propagation of flower bulbs and the systemic infection of most viruses, propagules from the infected plants are likely to be infected as well (Sastry, 2013). Besides such vertical transmission from parent plants to the progeny, also horizontal transmission of viruses occurs, resulting in a potential build-up of viruses in the bulb stocks and mixed infections of different viruses are common (Moreno and López-Moya, 2020). The trade of flower bulbs is subject to certification programs, in the Netherlands carried out by the Bulb Flower Inspection Service (Bloembollenkeuringsdienst (BKD) Lisse, The Netherlands). In this certification program all flower bulb growers are officially registered and the quality of bulb stocks is monitored by field inspections and laboratory testing (Knippels, 2011). Laboratory testing was initially carried out with serological tests, since the nineteen eighties mostly by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) (Clark and Adams, 1977). It appeared to be critical to test the right material at the right moment, as the detectability could depend on host material and storage conditions (Derks et al., 1997). Later, several molecular tests were developed to detect plant viruses in bulb and leaf material with most of the time more sensitivity, including RT-PCR and real-time RT-PCR (Mackay et al., 2002; Miglino et al., 2007; Wei et al., 2012; Jin et al., 2017; Xu and Ming, 2022). The development of these molecular test is depending on the available sequence information and complicated by the sequence variation that can be found in these virus species.

* Corresponding authors.
*E-mail addresses:* marga.vangent@wur.nl (M.P.E. van Gent-Pelzer), annette.dullemans@wur.nl (A.M. Dullemans).
[1] These authors contributed equally to this work

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Six plant viruses which are known to regularly occur in lilies and tulips, were the targets of this study. Lily mottle virus (LMoV) and tulip breaking virus (TBV) are both members of the genus *Potyvirus* (family *Potyviridae*). Potyviruses are transmitted by aphids in a non-persistent manner (Fereres, 2016) and their long (approximately 700–900 nm) flexuous particles harbour a single stranded RNA molecule of app. 9.7 kb (Wylie et al., 2017). Lily symptomless virus (LSV) belongs to the genus *Carlavirus* (family *Betaflexiviridae*, subfamily *Quinvirinae*), which are also transmitted by aphids in a non-persistent manner. Carlaviruses have flexuous filamentous particles that are somewhat shorter than those of potyviruses (610–700 nm). These particles contain a ssRNA molecule of app. 7.4 – 7.7 kb (King et al., 2012). Lily virus X (LVX), tulip virus X (TVX) and Plantago asiatica mosaic virus (PlAMV) belong to the genus *Potexvirus* (family *Alphaflexiviridae*). Potexviruses are mainly transmitted by contact, but TVX is also transmitted by mites (Lommen et al., 2012). For PlAMV and TVX transmissions via weeds and soil are observed, but their transmission routes are not yet fully understood (Hammond, 2018). Potexvirus particles are flexuous and are 470–580 nm long, harbouring a ssRNA molecule of 5.9 – 7.0 kb (Kreuze et al., 2020).

As many different virus infections occur in bulbs, there is a need for simultaneous detection of viruses. Multiplex RT-PCR is more efficient and cost-effective than other PCR-based techniques (Kwon et al., 2013; Yu et al., 2013; Gambino, 2015; He et al., 2019). A probe based RT-qPCR is more specific and more sensitive and less laborious than conventional methods, therefore a probe based multiplex RT-qPCR is most suited molecular method for simultaneous identification and quantification of viruses in routine screening (Mortimer-Jones et al., 2009; Lin et al., 2013; Malandraki et al., 2017; Pallás et al., 2018). A one-tube reaction protocol (Osman et al., 2017) is even less laborious and further reduces the contamination risk.

In this study we aimed to rethink current assay designs to facilitate a more robust and more efficient multiplex strategy. We used synthetic double-stranded verified genomic DNA (gBlocks) for the testing of the RT-qPCR TaqMan assays and multiplex panels. This gBlocks contain two specific viral target genes and a universal target control (gBlock constructions). Quantification was determined with dilution series of gBlock constructions. With this approach we managed to overcome potential complexities associated with multiplex detection: difficult to assemble, modify and optimize as well as reduction of the sensitivity and availability of dyes with compatible excitation/emission spectra. Four different multiplex panels were designed, consisting of three virus assays and an adapted assay for the nad5 housekeeping gene of lilies and tulips that acts as an internal control. The auxiliary multiplex panel detect the same viruses using a second target gene and is interchangeable. These multiplex panels, facilitate the discovery of sequence variations in diagnostic samples from lily and tulip as was verified with High Throughput Sequencing (HTS).

## 2. Materials and methods

### 2.1. Primers and probes design

LMoV, LSV, LVX, PlAMV, TBV and TVX sequences available at the time this study started, were retrieved from public data bases (www.ncbi.nlm.nih.gov, 2013) and additional sequences (listed in Table S1) were generated in this study. Alignments were performed using the CLC Genomics Workbench (Qiagen Denmark_version 6.0.2) and conserved genomic regions were selected. For each virus two specific assays were developed for targets located in different conserved coding regions: coat protein (CP), helper component-protease (HC-Pro), RNA-dependent RNA polymerase domain (RdRp) and the first gene of the triple gene block (TGB1). All the primers and minor groove binding (MGB) TaqMan probes used in this study were designed to work efficiently at an annealing temperature of 60°C and synthesized by Applied Biosystems (Foster City, CA, USA). Target genes and primers and probes information is listed in Table 1.

#### 2.1.1. Internal positive control

An RNA specific Internal Positive Control (IPC) was developed based on the NADH dehydrogenase subunit 5 genome (nad5 gene) region of lily and tulip to monitor RNA extraction and RT-PCR and identify potential false-negative results due to technical failures in these processes or to PCR inhibition (Hoorfar et al., 2003; Noorani et al., 2013).

HTS reads obtained from diagnostic lily and tulip samples (lily: Dullemans et al., 2020; tulip: unpublished) were mapped (CLC Genomics Workbench version 6.5) to exon a and b of the apple nad5 gene (NCBI GenBank accession number D37958). Primers were developed flanking an intron and the probe was located at the exon-exon boundary according the strategy of Botermans et al., 2013.

The design of the internal nad5 lily and tulip (nad5-lt) control is shown in Fig. 1.

The obtained lily and tulip nad5 sequences were submitted to NCBI GenBank under accession numbers MT364895 and MT364896.

#### 2.1.2. Multiplex panel strategy

A multiplex panel was composed with four qPCR TaqMan assays based on the ability to simultaneously measure four different fluorophores in the Quantstudio 12 K Flex (Applied Biosystems (Foster City, CA, USA)). This real-time PCR machine offers maximum multiplexing and chemistry options, the fluorescence detection with enhanced OptiFlex system, allows up to six multiplexing targets. Due to its usefulness for epidemiological research the panels were grouped according to the transmission routes of the target viruses. Multiplex panel1 (MP1) and MP3 are targeting three viruses which are aphid-borne (LMoV and TBV, members of the genus *Potyvirus* and LSV, genus *Carlavirus*). MP2 and MP4 are targeting three viruses (LVX, PlAMV and TVX) belonging to the genus *Potexvirus* and transmitted by contact and mites (in the case of TVX). In every multiplex set up the detection of the IPC was included. MP1, MP2 and the auxiliary multiplex panels MP3, MP4 consist of hydrolysis probes labelled with four different fluorescent reporter dyes. Detailed information of the composition of the multiplex panels is shown in Table 1.

### 2.2. Design of qPCR TaqMan controls

For each virus a gBlock construction was designed (Carlson et al., 2013; Conte et al., 2018) containing two different target genes and in between the Universal Target Control (UTC) (Fig. 2). As both targets are present in a 1–1 ratio, this gBlock construction allows the direct comparison of the qPCR efficacy of the two qPCR TaqMan assays while the UTC serves as a reference that allows comparison of all other assays and could be used to check the performance and quality of the gBlock constructions as well. The sequences of the used gBlocks are provided in Table S2. The gBlock constructions were synthesized by Integrated DNA Technologies (Coralville, IA, USA).

#### 2.2.1. Assessment of gBlock constructions

The amount of the gBlock constructions was calculated to the number of copies using a converter made available by New England Biolabs, Ipswich, MA, USA (https://nebiocalculator.neb.com/#!/dsdnaamt). The gBlock constructions were serially diluted 10-fold in TE buffer from 1.0E+06–1 copy per µl.

In order to evaluate the quality of the synthesized gBlock construction, a highly efficient singleplex TaqMan assay described by Bonants et al. (2019) detects the UTC sequence. The composition of the singleplex reaction mixtures was as follows: 1× Premix Ex Taq (Takara Bio Europe SAS, Saint-Germain-en-Laye, France), 300 nM UTC forward and reverse primer and 100 nM, 0.125 µl ROX dye II (50x) and 1 µl of gBlock construction dilution series. PCR-grade water was added to a final volume of 25 µl. The reaction conditions: 2 min at 95°C followed by 40 cycles of 15 s at 95°C and 60 s at 60°C in the Quantstudio 12 K Flex (Applied Biosystems, Foster City, CA, USA). PCR-grade water was used as a negative amplification control.

**Table 1**
Primers and probes information for RT-qPCR TaqMan detection of the Universal Target Control (UTC), the Internal Positive Control (IPC) and the viruses LMoV, LSV, LVX, PlAMV, TBV and TVX. LNA modifications are indicated in the probe sequence by +. Target genes CP, HC-Pro, RdRp, TGB1 and Assay ID used in multiplex panels MP1, MP2, MP3 and MP4.

| Virus or control | Target gene | Primers Probes | Sequences(5'–3') | Reporter Dye | Quencher | Amplicon size (bp) | Assay ID | Multiplex Panel |
|---|---|---|---|---|---|---|---|---|
| LMoV | CP | Forw | TCGCTGAAGCTTATATTGAGAAGCA | | | 109 | 63193 | 1 |
| | | Rev | AAATCAAAAGCAAATCGTGCTAGATTGAA | | | | | |
| | | Probe | TCGCTGAAGGCCGTACCT | FAM | MGB NFQ | | | |
| | HC-Pro | Forw | ACCGACACGGATGAGAAGATGTATA | | | 98 | 63783 | 3 |
| | | Rev | TTTGCTTGACTTTCACGAACGTTT | | | | | |
| | | Probe | TAGCTCGAGAGGGCTACTGCTACATCA | ABY | QSY | | | |
| LSV | RdRp | Forw | GTCCAGTCGAGGAAGTGTTGAC | | | 152 | 63194 | 1 |
| | | Rev | AGTGCTTAGGCGCTCCTTAG | | | | | |
| | | Probe | CTGGCATGGAGAAGTTGAACC | VIC | MGB NFQ | | | |
| | CP | Forw | CAGTTCGGCGTTCCTTGAC | | | 98 | 63784 | 3 |
| | | Rev | GTCCAGCGTGCTTCTTCATG | | | | | |
| | | Probe | AATGGAGCGGTGCCCGTCGACTCC | JUN | QSY | | | |
| LVX | RdRp | Forw | CCAACCTCATTAACGGCAAAGAC | | | 75 | 63196 | 2 |
| | | Rev | GTGATTTCAGGAAGAGCGCTATCT | | | | | |
| | | Probe | CCAGTCCCCCGATTTT | FAM | MGB NFQ | | | |
| | CP | Forw | CAGCCCAGTCTGAGAGTGTAG | | | 70 | 63787 | 4 |
| | | Rev | CCAATCCCTTCCCACAGTGT | | | | | |
| | | Probe | CCGCTGAGGAGCTACAATCCATTGC | ABY | QSY | | | |
| PlAMV | RdRp | Forw | CAGCCCATCACTCTGGAGTTC | | | 104 | 63197 | 2 |
| | | Rev | TCCGGGTTGACAGGTTGTG | | | | | |
| | | Probe | AAGGAGGGTGTTGACTTT | VIC | MGB NFQ | | | |
| | CP | Forw | CGATCACACGAGGCTTAACAAC | | | 86 | 63789 | 4 |
| | | Rev | GTCGAAGCACGCGTTGA | | | | | |
| | | Probe | AAGCTCTGCTCAGCCACGCCCT | JUN | QSY | | | |
| TBV | HC-Pro | Forw | GGCAGTTGGCAAATTGATTGTTTC | | | 81 | 63785 | 1 |
| | | Rev | AGCATCTTCTGCACTGGTTCTC | | | | | |
| | | Probe | CATTCCCTGTCGAAAGGTCTCGAAATTTGT | ABY | QSY | | | |
| | CP | Forw | CACAAGTTGAATACCCGATAAGACCAA | | | 92 | 63311 | 3 |
| | | Rev | CGCCTCAGCTAGCGATGA | | | | | |
| | | Probe | CACTTTGCGCCAGATAATGGCACAC | FAM | MGB NFQ | | | |
| TVX | TGB1 | Forw | CCCCAACACCGCACTAAGG | | | 93 | 63790 | 2 |
| | | Rev | GGGCAGATGAGTTTGGAAATCAG | | | | | |
| | | Probe | ACTTCCTCAAGACCACCAGCCACAG | ABY | QSY | | | |
| | RdRp | Forw | AGTCCCTCCAACAGATCAAAGGA | | | 90 | 63202 | 4 |
| | | Rev | CCCACTGAGATTTGAGGAAGATGAG | | | | | |
| | | Probe | AAGGCGGCAGTCCCCA | FAM | MGB NFQ | | | |
| IPC | nad5-lt | Forw | TGGGAGCTAGTGCTTGCTATCT | | | 108 | 63786 | 1, 2, 3, 4 |
| | | Rev | CAATTAACATCACTACGGTCAGGCT | | | | | |
| | | Probe | TCGAACAAGAAGCCCCAAGAAGCAT | JUN or VIC | QSY | | | |
| UTC | Vkorc1 | Forw | ACGTTGGGCCTCTATCCTA | | | 234 | | |
| | | Rev | GCCAAGGCAAAGCAAGTTAG | | | | | |
| | | Probe | CAC+C+T+G+TGCCA | FAM | BHQ | | | |

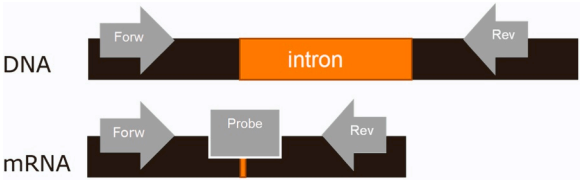



**Fig. 1.** Schematic design of the internal plant control nad5-lt.

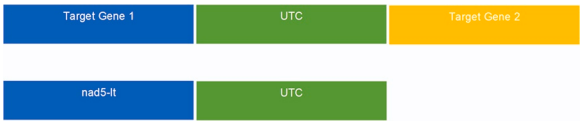



**Fig. 2.** Illustration of the gBlock constructions.

For each gBlock construction the amplification efficiency of the UTC was calculated using the Quantstudio 12 K Flex v 3.1 software. The real-time PCR software provides a standard curve and slope by measuring the quantification cycle as Ct value. The amplification efficiency of each test was determined based on the slope of the log-linear portion of the standard curve. The formula used for calculating PCR efficiency is E (Efficiency) = [10(-1/slope)]-1 as described in literature Kralik and Ricchi, 2017. The coefficient of determination ($R^2$) was calculated for each assay.

### 2.3. Singleplex qPCR TaqMan assay

The Quantstudio 12 K Flex was calibrated to measure the fluorescence upon degradation of the probes that were labelled with the dyes FAM, VIC, ABY, JUN and MUSTANG PURPLE as passive reference.

All the developed qPCR TaqMan assays for both target genes of each virus and also the IPC, were tested using the corresponding gBlock constructions with 1.0E+03 and 1.0E+04 copies. The composition of the singleplex reaction mixtures was as follows: TaqMan® Multiplex Master Mix (Applied Biosystems, Foster City, CA, USA), contains AmpliTaq® Fast DNA Polymerase Uracil-N Glycosylase (UNG), dNTPs with dUTP, MUSTANG PURPLE® dye (passive reference), and optimized buffer components (2x) 375 nM forward and reverse primer, 125 nM probe and 1 µl of gBlock construction dilution series. PCR-grade water was added to a final volume of 20 µl. The reaction conditions were: 20 s at 95°C followed by 40 cycles of 3 s at 95°C and 20 s at 60°C as fast modus in the Quantstudio 12 K Flex.

### 2.4. Multiplex qPCR TaqMan assay

Multiplex panels 1 and 2 were used for optimizing the multiplex qPCR TaqMan assay. An equal mixture (1.0E+04 copies) of the 3 MP corresponding virus gBlock constructions and the IPC gBlocks was used as a template. The multiplex reaction was conducted on a total volume of 20 µl, including 10 µl TaqMan® Multiplex Master Mix (2x), 375 nM for each primer, 125 nM for each probes and 1 µl of diluted gBlock constructions. The reaction conditions were similar to those described for the simplex assay in Section 2.3.

#### 2.4.1. Sensitivity and interchangeability of multiplex panels

Systematic comparisons were made between different concentrations of gBlock constructions to test the sensitivity, where IPC gBlocks and two virus gBlock constructions were in low copy number (1.0E+02 copies) while the third virus gBlock construction was added in variable amounts (1.0E+06–1 copy). For MP1 LMoV was added in variable amount, for MP2 both PlAMV and TVX were added in variable amount.

Interchangeability of the multiplex panel was tested by swapping the VIC and JUN probe dye (LSV and IPC) within MP1. In addition, we exchanged the two TaqMan assays from MP2 (PlAMV and IPC) with the auxiliary assay from MP4.

### 2.5. Multiplex one-step RT-qPCR TaqMan assay

To generate standard curves dilution series of gBlock constructions were used in combination with the TaqPath™ 1-Step Multiplex Master Mix (Applied Biosystems by Life Technologies) that includes thermostable MMLV reverse transcriptase, dNTPs, UNG, MUSTANG PURPLE dye (passive reference) and thermostable Fast DNA polymerase.

Multiplex one-step RT qPCR TaqMan was conducted in a total volume of 20 µl, including TaqPath™ 1-Step RT-qPCR Master Mix (4x), 375 nM for each primer, 125 nM for each probe, PCR-grade water and 1 µl of the corresponding gBlock constructions. The reaction conditions were as follows: UNG incubation 2 min at 25°C, RT incubation 15 min at 53°C, enzyme activation 2 min at 95°C followed by 40 cycles of 3 s at 95°C and 30 s at 60°C as fast modus in the Quantstudio 12 K Flex.

#### 2.5.1. Application of the multiplex one-step RT-qPCR TaqMan assay

To test the developed multiplex panels in practice. RNA from diagnostic samples was routinely extracted by the BKD. Eighty lily bulb samples and eighty tulip bulb samples with no, single or multiple virus infections were selected based on DAS-ELISA results. These samples were anonymous, no further metadata are known.

Multiplex one-step RT-qPCR TaqMan was conducted, by adding 2 µl RNA sample. The reaction conditions were similar to those described for the gBlock constructions, see Section 2.5.

### 2.6. Comparison of the multiplex panels

To compare the performance of the formulated panels the 160 selected diagnostic samples were screened using the multiplex panels MP1, MP2, MP3 and MP4.

Separately for each one-step RT-qPCR TaqMan assay the Ct was determined by a fixed threshold. TaqMan assays containing probes labelled with FAM, ABY and JUN dyes, had a threshold set on 0.2. The threshold for VIC labelled probe assay, was set at 0.1. The standard curves derived from dilution series of each gBlock construction, were used to calculate the amount of copy numbers of the IPC and viral targets in each sample. The measured Ct was computed into the target gene quantity as copy numbers per sample (2 µl) and a cut-off of 10 copies was set (Ruiz-Villalba et al., 2021).

Diagnostic samples were screened for intra-assay and inter-assay variability of the IPC. The gene copy numbers from MP1 were compared to the gene copy numbers of the auxiliary MP3. The same was done for MP2 in relation to the auxiliary MP4.

#### 2.6.1. HTS sequencing for confirmation of the multiplex panel results

Based on the multiplex panel results and the DAS-ELISA results which were obtained by the BKD, 18 lily and 15 tulip RNA samples of the 160 diagnostic samples were selected for HTS to verify the one-step RT-qPCR TaqMan results.

Total RNA was ribosomal RNA depleted using the Ribo-Zero Plant kit (Illumina Inc, San Diego CA, USA). Two dual unique indexed libraries were produced using the TruSeq stranded total RNA library prep kit (Illumina) as per the manufacturer's instruction. The resulting libraries were equimolar pooled and sequenced on an Illumina HiSeq using a 500 cycle V2 kit at Wageningen University and Research. Reads were split per sample by corresponding MIDs using CASAVA 1.8 software (Illumina) using stringent settings that allow no mismatch in the MID region. Data analysis was performed using the CLC Genomics Workbench (Qiagen, version 12.0.3). After quality trimming (settings: quality limit 0.05 %; short reads <100 nt and broken pairs were discarded), reads were used for read mapping to reference genomes and *de novo* assembly as described hereafter.

The trimmed reads were mapped with low stringency (Length fraction = 0.6, Similarity fraction = 0.6) to the reference genomes annotated with primer and probe regions (LMoV: NC_005288, LSV: NC_005138, LVX: NC_007192, PlAMV: KF471012 and KF601695, TBV: KF442403 and KF442402, TVX: NC_004322). Primer and probe sites were checked for polymorphism in the diagnostic samples. For each target region the coverage was determined at the 5' end of the probe. For the internal control region high stringent settings were used (Length fraction = 1.0, Similarity fraction = 1.0) to discriminate between reads originating from RNA and the potentially present contaminating DNA template.

Before the *de novo* assembly overlapping trimmed paired reads were merged. Contigs with a minimum length of 500 nt were analysed using NCBI blastn with the RefSeq viruses representative genomes downloaded from NCBI GenBank (Dec 9, 2019). All obtained near full length LMoV, LSV, LVX, TBV and TVX virus contigs were annotated and submitted to NCBI GenBank.

## 3. Results and discussion

### 3.1. Assessment of gBlock constructions

To check the constructed gBlocks an Universal Target Control was built in, using the corresponding TaqMan to qualify the synthetic targets. The performance of the seven gBlock constructions were analysed using the amplification efficiency of the UTC assay. The gBlock containing only the UTC sequence, gave a linear curve for 1.0E+06–10 copies per 1 µl, with correlation coefficient $R^2$ =0.999, slope = −3.29 and efficiency of amplification E =101 %. The UTC in the seven constructed gBlocks generated similar linear standard curves based on serial 10-fold dilutions (Figure S3) and met the requirements. PCR efficiency should be in the range of 0–1 (0–100 %); when E = 1 this means that the number of newly formed DNA amplicons is doubled in each cycle. This is not obviously to achieve repeatedly over time. In practice, this parameter is likely to be in the range 90–105 % (Johnson et al., 2013). This parameter can be estimated from the slope of the calibration curve. The dynamic range is defined by the MIQE guidelines as the range over which a reaction is linear (Bustin et al., 2009).The obtained results had a standard deviation of 0.0727 for all slopes and measured an amplification efficiency (E) between 97.3 % and 106.3 % The seven different gBlock constructions in our study proved to be well suited synthesized targets, the UTC assays resulted in a parallel slope and these results showed that all synthetic targets were qualified for reliable quantifications. This gBlock control strategy was already used by Bonants et al., 2019 and Van der Wolf et al. 2022.

### 3.2. Singleplex qPCR TaqMan assay

All fourteen TaqMan assays were verified for performance using two quantities (1.0E+03 and 1.0E+04) of corresponding gBlocks, the use of

gBlocks is ideal for research since the primers and probes fit perfectly and make target quantification possible. Amplification curves were evaluated using Quantstudio 12 K Flex software v 1.3, The optimal threshold for the baseline was chosen automatically. Cq Confidence provides a metric on the quality of the Cq determination and Cq Confidence value above 0.9 were very reliable (Thermo Fisher Scientific, Applied Biosystems, Quantstudio 12 K Flex user guide). The computed confidence score (Cq conf) of the amplification curves from 1.0E+04 copies were between 0.933 and 0.978. As expected, the ΔRn fluorescent signals differed per reporter dye (0.76–2.39), where VIC produces the lowest signal and FAM the highest. (Table 2). Between the two gBlock concentrations, the fourteen assay IDs show a Ct difference (Δ Ct) between the 10-fold dilution ranging from 2.29 to 3.45. Ideally PCR efficiency will demonstrate a change of 3.32 cycles for such 10 fold dilutions of template. To promote a user-friendly method, we aimed to keep all primers and probe concentrations in all assays the same. Most likely this resulted in TaqMan assays showing a difference in the measured delta Ct in the singleplex qPCR TaqMan assays. More important is that the two assays detecting the same virus, perform comparably by showing similar Δ Ct and negative template controls always test negative (Ct>40). The designed assays including nad5-lt performed well under the same conditions. As the commonly used nad5 assay by Botermans et al., 2013, is not based on sequence data from lilies and tulips, a specific qPCR TaqMan assay was developed as internal positive control on their nad5 gene (nad5-lt).

The newly developed lily and tulip nad5-lt qPCR assays appeared not only specific for lily and tulip but also more applicable for detection of monocotyledon plants in general, like grains, maize and grass (data not shown). Which was confirmed by NCBI blastn analysis of the nad5-lt primers and probe sequence. We recommend using an IPC that is specific for the host plant in the research.

### 3.3. Multiplex qPCR TaqMan assay

For the multiplex qPCR TaqMan assay, the primer and probe concentrations (375 nM and 125 nM respectively) as described previously, were used. Under these standardized qPCR conditions, multiplex panels MP1 and MP2 were studied, using automatic threshold settings. The designed multiplex panels maximize the available fluorophore options in the Quantstudio 12 K Flex providing three slots for virus detection and one for the internal positive amplification control (nad5-lt) and the final fifth window for the passive reference. Subsequently, we selected the viruses in the panels based on the dominant mechanism of horizontal transmission which would allow targeted effective risk assessment. Detection of 1.0E+04 copies of a gBlock construction was not influenced by the presence of the other gBlock constructions (1.0E+04 copies) in multiplex qPCR TaqMan assay and similar Ct values were obtained which indicated no loss of sensitivity (data not shown). Also the combination of four fluorescent dyes (FAM, VIC, ABY, JUN) in the multiplex panels, show no interference in their interaction/combination. Small differences were seen in the endpoint fluorescence (ΔRn) from FAM and ABY amplification. While the lowest ΔRn amplification signal derived from reporter dye VIC was seen consistently in singleplex and multiplex qPCR TaqMan (Figure S4 and S5).

**Table 2**
Performance of the designed TaqMan assays in singleplex reaction. The amplification data of the assays obtained with 1.0E+04 copies gBlock construction presented in cycle threshold (Ct), confidence of the amplification curve (Cq conf), the end point signal (ΔRn) and the Ct difference (ΔCt) between 1.0E+04 and 1.0E+03 gBlock target.

| Target | Assay ID | Region | Reporter | Cq conf | ΔRn | Ct | ΔCt |
|---|---|---|---|---|---|---|---|
| LMoV | 63193 | CP | FAM | 0.973 | 2.23 | 24.56 | 3.16 |
| | 63783 | HC-Pro | ABY | 0.969 | 1.73 | 26.50 | 3.15 |
| LSV | 63194 | RdRp | VIC | 0.972 | 0.79 | 27.55 | 2.98 |
| | 63784 | CP | JUN | 0.962 | 2.09 | 25.25 | 3.05 |
| LVX | 63196 | RdRp | FAM | 0.968 | 2.29 | 26.10 | 2.83 |
| | 63787 | CP | ABY | 0.974 | 1.87 | 27.03 | 2.96 |
| PlAMV | 63197 | RdRp | VIC | 0.970 | 0.66 | 26.45 | 3.45 |
| | 63789 | CP | ABY | 0.933 | 1.97 | 25.88 | 3.24 |
| TBV | 63785 | HC-Pro | ABY | 0.969 | 1.37 | 26.41 | 2.65 |
| | 63311 | CP | FAM | 0.963 | 0.76 | 27.23 | 2.80 |
| TVX | 63790 | TGB1 | ABY | 0.950 | 1.92 | 24.28 | 2.90 |
| | 63202 | RdRp | FAM | 0.978 | 2.39 | 25.45 | 2.87 |
| IPC | 63786 | nad5-lt | VIC | 0.978 | 1.05 | 28.07 | 2.30 |
| | 63786 | nad5-lt | JUN | 0.954 | 1.36 | 27.92 | 2.29 |

#### 3.3.1. Sensitivity and interchangeability of multiplex panels

To mimic the natural infections in which viruses can occur, in low concentrations and in mixed infections, gBlock constructions of two targets and IPC were tested at a fixed low concentrations (1.0E+02 copies) in combination with the third gBlock constructions in variable copy number (10-fold serial dilution (1.0E+06–1 copy)). The reaction sensitivity of the target with varying copy-numbers was not affected by other qPCR TaqMan assays and the multiplex panel also correctly identified all of its four target genes from the mixed template with different concentrations. Each multiplex panel has a large dynamic multiplex range of 1.0E+04 and no cross-signals were observed due to cross over of the fluorescent labels.

The sensitivity of qPCR TaqMan assays in multiplex panels was evaluated with the corresponding gBlock constructions, the lowest concentration that consistently could be amplified was 10 copies/µl. Each set of virus assays was mirrored by alternative target regions of the same virus. This maximizes both flexibility and robustness in the use of the multiplex panels. If one assay is no longer considered fit, it can be substituted by the alternative assay in the other panel that has the same specifications and the same alternative fluorophore. If the virus to be detect needs to be replaced by another virus to detect both assays have to be changed, example shown in Figure S6A. The same design principles are applied to all assays which are rigorously tested as individual components before inclusion into the multiplex sets. The assays can therefore be used in multiple combinations by replacing components by plug and play, examples with additional information are shown in Figure S6B. Confirmation of the quality of assays can be performed among the panels using the absolute quantification of the two alternative assays. Specification of new assays can easily be quantified by comparison with the IPC, after which they can be integrated in the panels. In this study the multiplex panels were grouped according the transmission routes of the target viruses, the panel grouping can be adjusted based on the research question.

### 3.4. Multiplex one-step RT-qPCR TaqMan

To overcome changes that occur due to buffer components or run profile, also TaqPath™ 1-Step Multiplex Master Mix in combination with a reverse transcriptase step for 15 min at 53°C, was used to obtain standard curves from the double-stranded gBlock constructions. From the 10-fold dilution series of the gBlock constructions, each one-step RT-qPCR TaqMan (Assay ID) generated the standard curve formula that was used for quantification of the diagnostic samples (Table 3). Remarkably both one-step RT-qPCR TaqMan assays for LSV resulted in inexplicable high amplification efficiency of target gene RdRp and CP (respectively 119.6 % and 112.4 %).

#### 3.4.1. Diagnostic samples

The multiplex qPCR TaqMan assays were applied to diagnostic samples obtained from lily and tulip bulbs. One hundred and sixty diagnostic RNA samples from lily and tulip were tested by the four multiplex panels.

By means of the IPC we were able to monitor the intra-assay variation of the 80 lily and 80 tulip diagnostic samples. Nad5-lt was measured by Assay ID 63786 in all samples, the quantity of nad5-lt gene was calculated in copies per sample. The IPC measured in lily samples was in

**Table 3**
The standard curve formula derived from 10-fold dilution series of the gBlock constructions containing the target gene sequence in multiplex one-step RT-qPCR TaqMan assay for each multiplex panel.

| MP1 | Target1 | Assay ID | Dye | Formula | $R^2$ | Eff% |
|---|---|---|---|---|---|---|
| LMoV | CP | 63193 | FAM | y = −1.426ln(x) + 40.131 | 0.983 | 101.6 |
| LSV | RdRp | 63194 | VIC | y = −1.271ln(x) + 42.167 | 0.993 | 119.6 |
| TBV | HC-Pro | 63785 | ABY | y = −1.414ln(x) + 40.708 | 0.992 | 102.9 |
| IPC | nad5-lt | 63786 | JUN | y = −1.430ln(x) + 41.857 | 0.999 | 101.2 |
| **MP2** | **Target1** | **Assay ID** | **Dye** | **Formula** | **$R^2$** | **Eff%** |
| LVX | RdRp | 63196 | FAM | y = −1.405ln(x) + 39.395 | 0.999 | 103.7 |
| PlAMV | RdRp | 63197 | VIC | y = −1.438ln(x) + 42.383 | 0.999 | 100.5 |
| TVX | TGB1 | 63790 | ABY | y = −1.415ln(x) + 39.412 | 1.00 | 102.8 |
| IPC | nad5-lt | 63786 | JUN | y = −1.430ln(x) + 41.857 | 0.999 | 101.2 |
| **MP3** | **Target2** | **Assay ID** | **Dye** | **Formula** | **$R^2$** | **Eff%** |
| LMoV | HC-Pro | 63783 | ABY | y = −1.387ln(x) + 40.360 | 0.999 | 105.6 |
| LSV | CP | 63784 | JUN | y = −1.327ln(x) + 38.874 | 0.996 | 112.4 |
| TBV | CP | 63311 | FAM | y = −1.430ln(x) + 42.216 | 0.998 | 101.2 |
| IPC | nad5-lt | 63786 | VIC | y = −1.346ln(x) + 40.327 | 0.998 | 110.2 |
| **MP4** | **Target2** | **Assay ID** | **Dye** | **Formula** | **$R^2$** | **Eff%** |
| LVX | CP | 63787 | ABY | y = −1.379ln(x) + 41.075 | 0.999 | 106.5 |
| PlAMV | CP | 63789 | JUN | y = −1.501ln(x) + 40.610 | 0.994 | 94.7 |
| TVX | RdRp | 63202 | FAM | y = −1.523ln(x) + 39.092 | 0.992 | 92.8 |
| IPC | nad5-lt | 63786 | VIC | y = −1.346ln(x) + 40.327 | 0.998 | 110.2 |

the range of 1.0E+05–1.0E+06 copies and tulip samples showed a broader range between 1.0E+04–1.0E+06 copies. The nad5-gene expression is known to be dependent on host, physiological state and other circumstances (Chan et al., 2014; Song et al., 2021).

The correlation ($R^2$-value) of the nad5-lt quantity was computed between the multiplex panels in all possible combinations (Fig. 3). In general, the IPC results of the multiplex panels show high correlation in quantity, regardless the probe is labelled with reporter dye JUN or VIC. This $R^2$-value can be used to validate new multiplex panel combinations in future efficiently.

Clearly, the reverse transcriptase and amplification was not negatively influenced by potentially co-extracted inhibitors since all diagnostic samples were positive for the IPC (nad5-lt). The amplification plot results showed that only the intended target gene was amplified from its target templates, and no signal was detected in all non-target pools.

Per multiplex panel different target genes were identified from samples with mixed infection and no cross-amplification was observed, suggesting that the panel was highly specific for detection of the target virus.

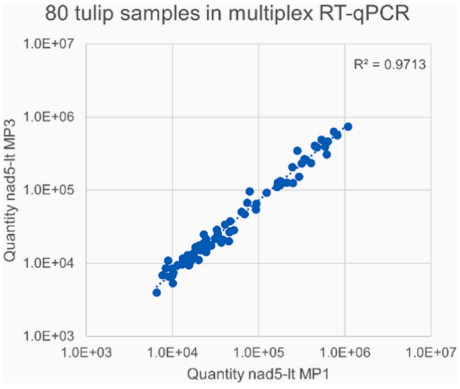
The quantity of the virus infections of the diagnostic samples revealed by the multiplex panels were also calculated in copies of the target gene per sample. In Fig. 4 these calculated values for both targets genes per virus were compared. In general the results from the two assays targeting the same virus were in accordance and there was a good correlation (between x and y) between quantity results of both target genes per virus. However, for some samples only one of the assays showed a positive result. For instance LMoV CP target gene did not show positive results for samples that were positive for LMoV according to target gene HC-Pro see Fig. 4 B1. The presence of multiple viruses in the diagnostic samples could be confirmed. In lily bulb samples we detected PlAMV, LSV and LVX and in tulip bulb samples LMoV, TBV and TVX, indeed the two target genes were not measured in the same amount in all samples. To clarify the different RT-PCR results for two assays of a specific virus, HTS was performed on a selection of diagnostic samples.

### 3.4.2. *HTS confirmation*

RNA samples whose PCR results for both assays did not match were checked with HTS.

Additional read mapping of data sets of the selected lily (18) and tulip (15) RNA samples provided additional sequence information in the primer probe regions. These for HTS selected samples are marked orange in Fig. 4.

A




B

| Correlation | Lily $R^2$-value | Tulip $R^2$-value |
|---|---|---|
| MP1:MP2 | 0.893 | 0.983 |
| MP1:MP3 | 0.945 | 0.972 |
| MP1:MP4 | 0.930 | 0.976 |
| MP2:MP3 | 0.947 | 0.963 |
| MP2:MP4 | 0.972 | 0.982 |
| MP3:MP4 | 0.967 | 0.970 |

**Fig. 3.** Internal Positive Control measured in one-step RT-qPCR TaqMan multiplex panels. A: Plot show the nad5-lt quantities (copies per assay) of two multiplex panels in 80 diagnostic tulip samples. B: $R^2$-value for nad5-lt calculated from 80 lily and 80 tulip samples for all multiplex panel combinations.

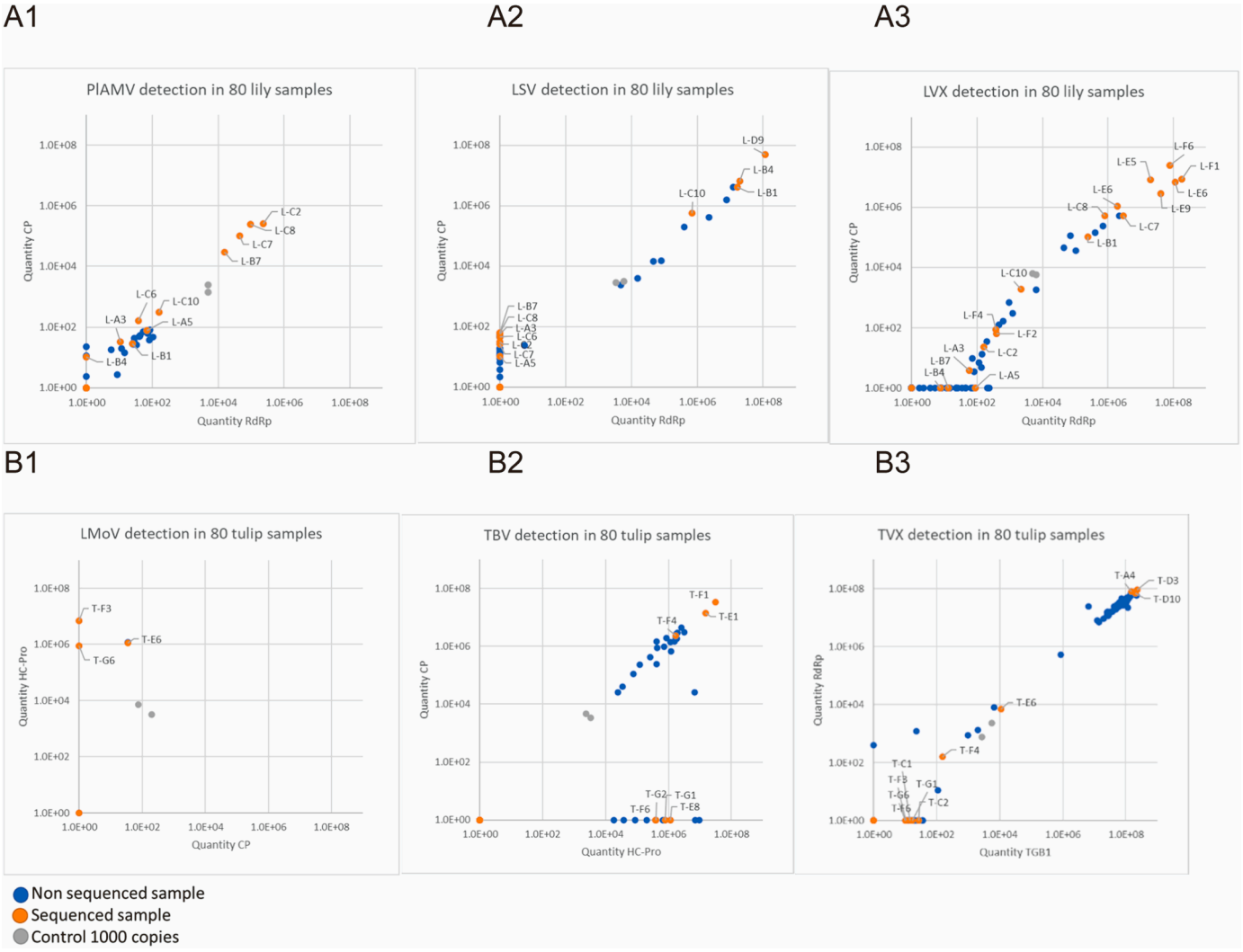



**Fig. 4.** Virus detection with one-step RT-qPCR TaqMan multiplex panels in 80 diagnostic lily and tulip samples, respectively plots A and B. Plots show the measured virus gene quantities, derived from two assay IDs for PlAMV (L-A1), LSV (L-A2), LVX (L-A3), LMoV (T-B1), TBV (T-B2), TVX (T-B3). In dots (only the positive samples are shown). The X-axis represents the target gene from multiplex panel 1 or 2, the Y-axis represents the target gene from the auxiliary multiplex panel 3 or 4. Non sequenced samples are indicated in blue dots. Samples which were selected for HTS are indicated in orange and labelled. The gBlock constructions (1.0E+03 copies) are indicated in grey dots.

The HTS data showed previously unknown sequence variation in the viral genomes. Some showed single nucleotide polymorphisms (SNPs) in the selected primer and or probe region, compared to the used viral genomes for primer and probe design (Table 1). SNPs may affect the results, with the risk of false negatives.

SNPs were found in the primer and probe binding sites of virus sequences in lily (LVX-CP, LSV-RdRp, LSV-CP, PlAMV-CP) and in tulip (LMoV-CP, LMoV-HC-Pro, TBV-HC-Pro, TBV-CP). For the complete information concerning the target coverage and number of SNPs in the primer- and probe binding sites, see Table S7.

In this discussion we highlight the results obtained for the lily LSV assays and the tulip TBV assays and the HTS results obtained from LSV and TBV which are used to explain the results obtained by one-step RT-qPCR TaqMan multiplex panels Table 4A and B respectively. Fig. 4 A2 shows the LSV detection (Assay ID 63194 versus 63784) in 18 lily samples (sequenced in orange). For four (L-B1, L-B4, L-C10 and L-D9) the results for both targets are in line. The primer and probe binding sites of both assays are confirmed by HTS. Sample L-C10 turned out to have one SNP in RdRp and the CP probe binding site and L-D9 turned out to have one SNP in CP forward primer binding site. These SNPs don't have a visible effect in the assays, as the calculated amount of virus target is for both assays in accordance. Seven lily samples (L-A3, L-A5, L-B7, L-C2, L-C6, L-C7 and L-C8) showed a very low detection of LSV (between 10 and 62 cp/assay) based on the CP assay only. A limited sequence reads were found on the LSV genome, but outside the primers and probe binding sites, so it was not possible to check the sequence of the binding sites. Low amount of reads and low amount of calculated target indicate a low amount of virus RNA in the lily RNA sample and not a loss of detection due to sequence polymorphisms (false negatives). These results are probably due to contamination of these samples, during routinely treatment of the bulbs in the field or the laboratory sampling process.

Fig. 4 B2 TBV detection (Assay ID 63755 versus 63311) show the 15 sequenced tulip samples in orange. The results for three samples are for both assays in line (T-F1, T-F4 and T-E1). T-F1 turned out to have one SNP in HC-Pro reverse primer binding site, T-F4 turned out to have one SNP in the CP forward and one in the CP reverse primer sequence. These SNPs had no visible effect in the assays, because the calculated amount of virus target is for both assays the same.

Four samples (T-E8, T-F6, T-G1 and T-G2) were clearly positive in HC-Pro assay and not in the CP assay. For all these four samples we found five SNPs in the CP forward primer and two SNPs at the CP probe

**Table 4**
Detailed information of a selection of diagnostic samples tested in multiplex RT-qPCR and HTS. Data for LSV (Table 4A) and TBV (Table 4B) for both target genes are shown. The target gene quantity is the calculated amount of copies per 2ul sample in the multiplex RT-qPCR reaction. The target coverage is the number of reads covering the 5′ nt of the probe at the reference genomes recalculated for 10 million trimmed reads. Read mapping only outside the primer probe region is indicated with an *. The single nucleotide polymorphism (SNP) in primer set (F+R) and probe region are indicated. Obtained near full length viral sequences LSV and TBV are submitted to NCBI GenBank.

4A

LSV

| Target gene | RdRp | | | | CP | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Sample | Target Quantity | Target Coverage | SNP Primers | SNP Probe | Target Quantity | Target Coverage | SNP Primers | SNP Probe | NCBI GenBank accession number |
| L-A3 | nd | 0* | | | 5.6E+01 | 0* | | | |
| L-A5 | nd | 0* | | | 1.0E+01 | 0* | | | |
| L-B1 | 1.7E+07 | 1.3E+04 | 0+0 | 0 | 4.1E+06 | 1.2E+04 | 0+0 | 0 | MT882679 |
| L-B4 | 2.0E+07 | 1.0E+04 | 0+0 | 0 | 6.6E+06 | 1.4E+04 | 0+0 | 0 | MT882680 |
| L-B7 | nd | 0* | | | 6.2E+01 | 0* | | | |
| L-C2 | nd | 0* | | | 3.1E+01 | 0* | | | |
| L-C6 | nd | 0* | | | 4.5E+01 | 0* | | | |
| L-C7 | nd | 0* | | | 2.6E+01 | 0* | | | |
| L-C8 | nd | 0* | | | 6.2E+01 | 0* | | | |
| L-C10 | 7.1E+05 | 2.8E+02 | 0+0 | 1 | 5.6E+05 | 7.0E+02 | 0+0 | 1 | MT882681 |
| L-D9 | 1.1E+08 | 2.9E+04 | 0+0 | 0 | 5.0E+07 | 4.7E+04 | 1+0 | 0 | MT882682 |
| L-E5 | nd | 0* | | | nd | 0* | | | |
| L-E6 | nd | 0 | | | nd | 0 | | | |
| L-E9 | nd | 0 | | | nd | 0 | | | |
| L-F1 | nd | 0 | | | nd | 0 | | | |
| L-F2 | nd | 0 | | | nd | 0 | | | |
| L-F4 | nd | 0 | | | nd | 0 | | | |
| L-F6 | nd | 0 | | | nd | 0 | | | |

4B

TBV

| Target gene | HC-Pro | | | | CP | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Sample | Target Quantity | Target Coverage | SNP Primers | SNP Probe | Target Quantity | Target Coverage | SNP Primers | SNP Probe | NCBI GenBank accession number |
| T-A4 | nd | 0 | | | nd | 0 | | | |
| T-C1 | nd | 0 | | | nd | 0 | | | |
| T-C2 | nd | 0 | | | nd | 0 | | | |
| T-D3 | nd | 0 | | | nd | 0 | | | |
| T-D10 | nd | 0 | | | nd | 0 | | | |
| T-E1 | 1.5E+07 | 5.8E+03 | 0+0 | 0 | 1.4E+07 | 4.7E+03 | 0+0 | 0 | MT895183 |
| T-E6 | nd | 0 | | | nd | 0 | | | |
| T-E8 | 1.1E+06 | 5.6E+02 | 0+2 | 1 | nd | 8.1E+02 | 5+0 | 2 | MT895184 |
| T-F1 | 3.1E+07 | 2.6E+03 | 0+1 | 0 | 3.3E+07 | 3.2E+03 | 0+0 | 0 | MT895185 |
| T-F3 | nd | 0 | | | nd | 0 | | | |
| T-F4 | 1.7E+06 | 4.7E+02 | 0+0 | 0 | 2.3E+06 | 4.7E+02 | 1+1 | 0 | MT895186 |
| T-F6 | 3.8E+05 | 1.3E+02 | 0+0 | 0 | nd | 2.6E+02 | 5+0 | 2 | MT895187 |
| T-G1 | 8.0E+05 | 8.3E+02 | 0+0 | 0 | nd | 1.4E+03 | 5+0 | 2 | MT895188 |
| T-G2 | 4.1E+05 | 1.2E+02 | 0+0 | 0 | nd | 1.5E+02 | 5+0 | 2 | MT895189 |
| T-G6 | nd | 0 | | | nd | 0 | | | |

binding sites. With the generated HTS data we now can conclude that due to limited available sequence data during design of the primers missing genome sequence information leads now to false negative results in the TBV CP TaqMan assay, which is luckily overcome by the second assay.

The negative PCR results of eight samples were confirmed by the HTS analysis. For these samples no TBV reads were found.

It appears that not every SNP in the primer or probe binding site has an effect on annealing and therefore has a decreasing effect in the amplification and the calculated quantity of the target gene. The observed genetic variability and the detection of mutations could lead to the modification of primers/probes, thereby providing methods that are applicable on the different occurring viruses and thus helping to cover the gaps in the current methods. In this study assembled genome sequences are submitted to NCBI GenBank (accession numbers are shown in Table 4 A+B and S7) to facilitate future assay development.

Although the multiplex RT-qPCR results of the selected diagnostic samples were confirmed or explained by the HTS data, this was not the case for all DAS-ELISA results from the provider (BKD). The highly sensitive multiplex panels detect very low concentrations of virus, these are most likely caused by smearing of samples with high virus loads.

Some LSV DAS-ELISA results were not in accordance with the RNA based analysis. Uneven spread of virus in the bulbs or mix up of the samples could explain these results. Since the RNA samples were isolated from the same bulbs but not from exactly the same sample as was used for the DAS-ELISA. RNA amplification based methods are more sensitive than protein based detection methods like DAS-ELISA and DAS-ELISA results are dependent on the quality and specificity of the used antiserum (Boonham et al., 2014).

The multiplex RT-qPCR results for LSV, LVX and PlAMV and TVX show samples with (very) low virus titres. In virus diagnostics it is always difficult to set thresholds to declare samples virus positive or not. Especially potexviruses, which can be present in high concentrations in plant material, are very sticky and persistent. This makes hygiene very important to avoid contamination during sample preparation. It is a warning for inspection services and research laboratories to be alert to contamination throughout the whole sample preparation and RNA isolation process.

At the meantime, these viruses can be present in low concentrations in bulb material, with negative results in serological tests but resulting in positive signals in RT-qPCR, although with rather high Cq values (unpublished results).

## 4. Conclusion

To exploit the PCR technology in a more efficient way and to meet the current needs in plant virus detection – i.e., effectiveness, reliability, transferability, and universal application, we developed one-step RT-qPCR TaqMan multiplex panel approach. With the proposed strategy we managed to mitigate potential difficulties associated with multiplex qPCR: such as difficulties to (i) assemble the test panels, (ii) modify and optimize assays in a multiplex system as well as (iii) the potential reduction of the sensitivity when multiple viruses are detected simultaneously. As final points, we considered two critical factors in virus detection in bulb flowers; the variability that is often observed in RNA viruses and the possibility of mixed infection with large differences in the virus load.

In conclusion, a universal strategy for high-coverage, highly inclusivity, and interchangeable, one-step RT-qPCR TaqMan multiplex panels was developed and validated in this study. It should facilitate the detection and differentiation of plant viruses under high throughput settings. Updating the input data from public databases is always essential for a thorough diagnosis. Subsequently sharing collected sequence data from diagnostic plant virus samples, will benefit the development of RT-qPCR TaqMan assays.

## 5. CRediT authorship contribution statement

**M.P.E. van Gent-Pelzer:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **A.M. Dullemans:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **M. Verbeek:** Writing – review & editing. **P.J.M. Bonants:** Writing – review & editing, Funding acquisition. **T.A.J. van der Lee:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jviromet.2024.114987.

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