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Viruses of Dormant Bulbs

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1. Literature Review

The most important bulb genera in New Zealand include *Allium*, *Tulipa*, *Iris* and *Lilium.* Members of these genera are imported and exported in great numbers. In 2007 $28 million in bulbs were exported (FreshFacts, 2007), over 50% of these bulbs were *Lilium* and 25% were *Tulipa*. To ensure that imported and exported bulbs are free from regulated viruses, sensitive testing methods that can be used on dormant bulbs are required. The aim of this project is to develop real time reverse transcription polymerase chain reaction (RT-PCR) protocols for the detection of important viruses in dormant bulbs. Protocols will be developed for 5 of the following viruses; *Arabis mosaic virus* (ArMV), *Cucumber mosaic virus* (CMV), *Iris yellow spot virus* (IYSV), *Lily symptomless virus* (LSV), *Lily virus X* (LVX), *Tobacco rattle virus* (TRV), *Tobacco ringspot virus* (ToRSV), *Tulip breaking virus* (TBV) and *Tulip virus X* (TVX). Below is a review of the scientific literature relevant to this project with particular emphasis on extraction procedures from bulb tissue and diagnostic methods.

Real time RT-PCR assays already exist for some of the target viruses mentioned above. Mumford *et al.* (2000) developed and optimized a Taqman® real-time RT-PCR method for TRV and it was tested successfully on 21 isolates of the virus in leaves and tubers from around the UK. There are also reports of 2 different real time RT-PCR methods for IYSV, one using Taqman® (Mumford *et al.*, 2008) and the other using SYBR Green (Pappu *et al.*, 2008) The primer and Taqman sequences used in the above papers are listed in Appendix 1. A quantitative real time RT-PCR (SYBR Green) has also been used to determine the quantities of different RNA segments of the CMV genome (Feng *et al.*, 2005), however the test was used on purified virions so the primers may not be specific enough for diagnostic work. These methods require investigation to verify whether they detect viruses in dormant bulbs reliably.

Real-time PCR assays have been shown to be more sensitive than Enzyme-linked immunosorbent assay (ELISA) and conventional PCR. A TaqMan assay for the detection of PMTV from potato tubers was 10,000 times more sensitive than the PMTV ELISA while a TaqMan assay for the detection of TRV from potato tubers was 100 times more sensitive than the TRV RT-PCR (Mumford *et al.*, 2000). A SYBR Green real-time PCR for the detection of IYSV in onion bulbs was also shown to be $1:10^{-3}$ more sensitive than ELISA and conventional PCR (Pappu *et al.* 2008).

A major technical issue in this project will be the development of an extraction method that can be used to isolate viral nucleic acids from bulb tissue of suitable quality for RT-PCR. Although all plant tissues contain compounds which are somewhat inhibitory to PCR, bulb tissue is particularly difficult to work with. Polysaccharides, which are present in bulb tissue may be co-extracted with nucleic acids causing inhibition of the PCR reaction (Pandey *et al.*, 1996) or may interfere with the extraction process itself (Gibbs *et al.,* 2002). In diagnostic PCR, the reduced sensitivity caused by such inhibition can lead to false negative results. This is especially relevant when detecting virus in dormant bulbs as the titre may be very low. The ideal way to overcome the challenge of these inhibitors is to isolate the nucleic acids from the polysaccharides during the extraction process.

The most popular RNA extraction techniques is the RNeasy extraction kit (Qiagen) which has been used on garlic, gladiolus, tulip and lily bulbs. This method relies on the RNA binding to a silica column while a number of different reagents are used to remove contaminating proteins and polysaccharides. This extraction method has been used successfully to extract nucleic acid of viruses in bulbs of agapanthus, freesia and narcissus (Gibbs *et al.*, 2002), garlic (Meenakshi *et al.*, 2006), gladiolus and onion (Miglino *et al.*, 2007), tulip (Sato *et al.*, 2002) and lily (Sharma *et al.*, 2005). This method is fast and has been used widely to extract

RNA from other plant tissues in MAFBNZ's Plant Health and Environment Laboratory (PHEL).

There are also reports of non-commercial extraction methods which have been developed by research groups. Gibbs *et al.* (2002) tested extraction methods on samples consisting of bulb tissue spiked with known amounts of TRSV-infected leaf tissue. Extracts were tested with RT-PCR and the method they found to be the best involved an extraction/grinding buffer combining cetyl trimethyl ammonium bromide, guanidine thiocyanate and polyvinylpyrolidone (PVP). The method was tested using tissue from agapanthus, freesia, gladioli, narcissus, onion and tulip. The protocol is included in Appendix 2. Another extraction protocol, originally developed by Hooft van Huijsduijnen (1985), but used on gladioli bulb tissue by Vunsh *et al*. (1991) used a hot phenol extraction buffer consisting of phenol, lithium chloride and SDS (Appendix 3). This method when used alone produced extracts which inhibited the detection of BYMV in corm tissue. However, they found that by using a sephadex G-50 column as the final step in the extraction process produced extracts that did not inhibit the RT-PCR reaction and allowed amplification of the diagnostic product. Although the extraction protocol itself is time consuming the use of a sephadex G-50 column (which are available in micro spin columns) may prove useful in conjunction with another extraction method.

RT-PCR has also been done using crude bulb extracts. *Onion yellow dwarf virus* (OYDV) and *Leek yellow stripe virus* (LYSV) have been detected in crude extracts of garlic (Dovas *et al*., 2001) with a high level of sensitivity. The extracts were prepared by grinding small amounts of garlic bulb tissue (0.1g) in a simple extraction buffer (50 mM Tris-HCl [pH.8.3], 75 mM KCl, 2% PVP) and then a brief centrifugation step for 2 minutes at 10000 x *g*. Even when the original bulb extract was diluted with extracts from healthy garlic bulbs the viral RNA was still detected by RT-PCR. Crude extracts have also been used in real time RT-PCR detection of OYDV and LYSV in garlic bulbs (Lunello *et al*., 2004) using an extraction buffer developed for immunocapture-RT-PCR (500 mM Tris-HCI [pH 8.2] containing 2% PVP-40, 1 % PEG 6000, 140 mM NaCl, 0.05% Tween 20, and 3 mM NaN_3).

Streptavidin magnetic capture hybridisation is another extraction method which has been used to isolate viral RNA from bulbs. This method has been used with the commercial Kingfisher automated extraction system as well as a manual procedure (Appendix 4) (Miglino *et al*., 2006; Miglino *et al*., 2007). The basic procedure involves grinding the bulb tissue in a guanidinium isothiocyanate buffer to lyse the cells. Then there is a hybridisation step which utilizes a biotinylated nucleotide probe that binds to target RNA sequences as well as streptavidin-coated beads. Once the probe has bound the target nucleic acids, the beads along with the nucleic acids can be separated magnetically from the rest of the homogenate. This method has been used to detect TRV in dormant gladioli corms and in leaves (Miglino *et al*., 2007) and potexviruses in *Allium* (Miglino *et al*., 2006). In both of these papers the RNeasy method has been compared to magnetic capture RT-PCR. In the first case RNeasy was found to be less sensitive and efficient (Miglino *et al*., 2006) while in the other paper no difference was observed (Miglino *et al*., 2007).

Immunocapture RT-PCR (IC-RT-PCR) has also been compared to RT-PCR against extracts derived from the RNeasy extraction method (Sato *et al*., 2002) as well as against crude extracts (Dovas *et al*., 2001). In both cases the IC-RT-PCR was less sensitive; the IC-RT-PCR protocol is also more time consuming than standard extraction methods.

An understanding of the distribution of virus particles within the bulb will be critical in developing a sampling protocol; unfortunately limited work has been done on this subject.

The distribution of several viruses in lily bulbs and leaves was examined by Sharma *et al.* (2005) in 18 different cultivars. The study looked at the detection of CMV, *Lily mottle virus* (LMoV), LSV, *Strawberry latent ringspot virus* (SLRSV) and TBV with viral species-specific primers in the outer and inner scales of bulbs as well as in the leaves before and at flowering. In 4 of 16 plants, viruses were detected in the inner scales but not the outer scales of the bulbs and in 10 plants, viruses were detected in the leaves but not the bulbs. There is no mention in this paper of when the bulb was sampled nor can we be certain whether the inability to detect the viruses in various parts of the plant is due to the absence of virus from that particular sampling point due to uneven distribution, overall low virus titre or PCR inhibition (there is no mention of internal amplification controls).

Van der Vlugt *et al*. (1993) used immunoblotting to examine the distribution of *Iris severe mosaic virus* (ISMV) in gladiolus bulbs after lifting, as well as after different stress treatments (cutting and heat). When testing the bulbs directly after lifting the only detectable amount of virus was in the basal plate of the bulb and not the scales (Fig. 1). When the gladiolus bulbs were subjected to stress, either by cutting or heat treatment, virus titre throughout scales increased to detectable levels (even in scales cut from the basal plate). Both of these stress-inducing treatments could be used to aid detection of viruses in bulbs but because they can take from 16 days to several months they may not be practical for routine testing. The study suggests that it is most likely that virus is present subliminally throughout the bulb and multiplication is induced by the increased metabolism during stress response. The basal plate of the bulb is the most metabolically active site hence under normal conditions virus titre is highest there.

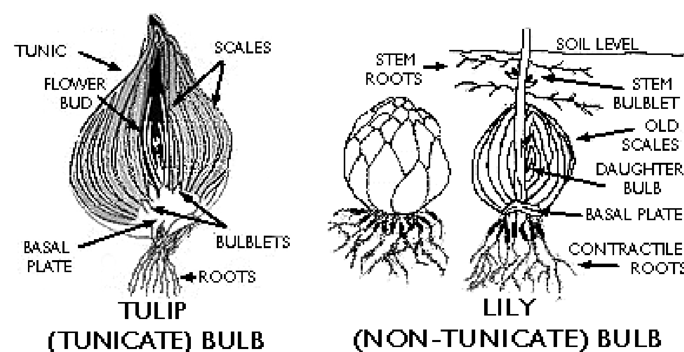


Figure 1.1. Basic anatomy of lily and tulip Bulbs (Hertogh & Le Nard, 1993)

The distribution of TRV in dormant tulip bulbs has been assessed by ELISA after 7-month incubations at temperatures ranging from 2 - 25ºC (van der Vlugt *et al.*, 1988). Each bulb was cut into 12 pieces and each piece tested for TRV. This study found there to be no evidence of a regular pattern of virus distribution. Furthermore, when samples from all parts of the bulb were pooled and tested the sensitivity of the test was not sufficient to detect the virus. Only in the sprouts could the virus be reliably detected.

2. Extraction of Nucleic Acid from Dormant Bulbs

2.1. MATERIALS AND METHODS

Nucleic acid of appropriate quality and quantity is essential for the molecular detection of pathogens in plants. Indeed, PCR reactions can be inhibited if plants compounds such as polysaccharides, polyphenolics are present in the nucleic acid extracts (Wei *et al.* 2008). Two extraction methods: (1) the standard Qiagen RNeasy plant mini kit; and (2) a modified Qiagen RNeasy plant mini kit (MacKenzie *et al.* 1997) were compared using two bulb species (*Lilium regale* and *Tulipa* 'Parade'). Extractions from two sampling sites (basal and transverse sections) of each bulb and from three bulbs of each species were investigated. The Qiagen RNeasy mini kit manufacturer's instructions were followed in the first method. In the second method the initial extraction step was modified as described by MacKenzie *et al.* (1997). Bulb tissue (0.1 g) was mixed with 1 mL RLT extraction buffer in both methods. The quality and quantity of extracted nucleic acid were measured by spectrophotometry using a Nanodrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, USA). Subsequent samples were then extracted using the best method.

One step RT-PCRs were done using three different plant internal amplification control (PIAC) primer pairs (Table 2.1), and two different *Taq* DNA polymerases with/without the addition of bovine serum albumin (BSA). BSA has been shown to enhance the efficiency or specificity of PCR (Wei *et al.* 2008). Two different *Taq* DNA polymerases (Invitrogen Platinum® *Taq* [Invitrogen, Carlasbad, USA] and Promega Go*Taq*® [Promega, Auckland, New Zealand]) were compared (Table 2.2) in one-step RT-PCR using PIAC primers designed to amplify a 181bp fragment from the *nad*5 gene (Menzel *et al.* 2002), with/without the addition of BSA (final concentration at 1 µg/µL). The PCR cycling conditions were as follows: one step of denaturation (94ºC, 3 min), 35 cycles of denaturation-annealing-elongation steps (94ºC, 20 sec; 55ºC, 30 sec; 72ºC, 1 min), and a final elongation step (72ºC, 7 min). All the RNA samples were tested initially using the *Taq* DNA polymerase selected during the previous experiment, and the *nad*5 gene PIAC primers. Subsequently at least two RNA samples from each bulb cultivar or species, especially those which had little or no amplification using the *nad*5 primers, were tested using PIAC primer pairs designed to amplify ~1065 bp and ~300-900 bp product from the *ndhB* gene (Graham and Olmstead, 2000) and the 5*s* gene (Kolchisky *et al.,* 1991), respectively. These PIAC primers were tested under the same amplification conditions and with/without the addition of BSA (final concentration at 1 µg/µL) as with the *nad*5 gene primers described previously.

Table 2.1 Information on PIAC primers

Targeted gene	Primer name and sequences (5´-3´)	size (bp)	References
Nad5 gene	Nad5-S (Forward): GATGCTTGTTGGGGCTTCTTGTT Nad5-AS (Reverse): CTCCAGTCACCAACATTGGCATAA	181 bp	Menzel et al., 2002
ndhB gene	9F (Forward): ATGGTTTCTCTTGGCTATATGG 13R (Reverse): GCATACGTTTCATGCTTGTTTGAG	~1065 bp	Graham and Olmstead, 2000
5s gene	Primer A (Forward): TTTAGTGCTGGTATGATCGC Primer B (Reverse): TGGGAAGTCCTCGTGTTGCA	~300-900 bp	Kolchisky et al., 1992

Table 2.2. Components of one step RT-PCR reaction

One step RT-PCR (10 µl total volume)			
Platinum® *Taq* DNA polymerase (Invitrogen)		**Go*Taq*® (Promega)**	
10 × PCR buffer	1.0 µl	2 × Go*Taq* Master Mix	5.0 µl
50mM $MgCl_2$	0.4 µl	5µM Forward primer	0.5 µl
10mM dNTP	0.2 µl	5µM Reverse primer	0.5 µl
5µM Forward primer	0.5 µl	RNA	1.0 µl
5µM Reverse primer	0.5 µl	0.1M DTT	0.5 µl
5U/ µl platinum *Taq* polymerase	0.1 µl	10 µg/µl BSA	1.0 µl
RNA	1.0 µl	RNasin® plus RNase inhibitor (Promega)	0.1 µl
0.1M DTT	0.5 µl	Superscript™II Reverse Transcriptase (20U/ µl, Invitrogen)	0.1 µl
10 µg/µl BSA	1.0 µl	Nuclease-free water	1.3 µl
RNasin® plus RNase inhibitor (Promega)	0.1 µl		
Superscript™ III Reverse Transcriptase (20U/ µl, Invitrogen)	0.1 µl		
Nuclease-free water	4.6 µl		

2.2. RESULTS

A total of 12 samples including basal and transverse tissues from three *Lilium regale* bulbs and three *Tulipa parade* bulbs were used for the RNA extraction using two different extraction methods. The quantity and quality of each RNA were evaluated by spectrophotometry using a Nanodrop® ND-1000 Spectrophotometer and one step RT-PCR using PIAC primers targeting the *nad*5 gene. The results showed that both quantity and quality of RNA extracted using the original Qiagen plant RNA extraction mini kit were slightly better than those extracted using the modified kit (Table 2.3 and Figure 2.1). In addition, a greater amount of nucleic acid was obtained if the tissue were ground in two times more volume of the Qiagen RNeasy plant mini kit RLT extraction buffer (0.1 g tissue in 2 mL RLT buffer). This modification was particularly important for bulb species yielding small quantities of RNA such as *Iris* sp. (Table 2.4).

The efficiency of nucleic acid extraction was further assessed using 148 RNA samples (extracted from 74 bulbs) representing 18 bulb species (including 5 in which 2 different cultivars were tested). With the exception of *Paeonia*, a greater quantity of RNA was extracted from basal than transverse sections (Table 2.5).

Using one step RT-PCR, Go*Taq*® DNA polymerase provided a greater sensitivity of *nad*5 amplification than Platinum® *Taq* (Figure 2.2). The addition of BSA inhibited *nad*5 amplification when used with Platinum® *Taq* but improved amplification with Go*Taq*® in some bulb species (Figure 2.2). The lower amplification efficiency of *Iris* bulb RNA might be due to the lower quantity of the RNA.

Three PIAC primer pairs using one step RT-PCR with Go*Taq*® DNA polymerase with/without BSA were compared (Figure 2.3, Table 2.5). The *ndhB* gene primers generally gave significantly better amplification efficiency using all RNA samples than the *nad*5 and 5*s* gene primers (Table 2.5). The RT-PCR amplification efficiency did not always correspond well with the RNA quantity (Table 2.5), suggesting that some extractions may contain inhibitors or that the primers do not amplify nucleic acid of all plant species equally efficiently.

Table 2.3. Comparison of the RNA quantity and quality extracted using the standard and modified Qiagen RNeasy plant mini kit. The RNA quantity and quality was measured using a Nanodrop® ND-1000 Spectrophotometer

No.	Bulb species	Sampling sites	RNA quantity (µg/µL)		RNA quality (260/280)	
			Standard kit	Modified kit	Standard kit	Modified kit
1	*Lilium regale* bulb 1	basal	617.84	234.78	2.10	2.09
2	*Lilium regale* bulb 1	transverse	43.58	23.22	1.84	1.61
3	*Lilium regale* bulb 2	basal	637.48	210.46	2.10	2.05
4	*Lilium regale* bulb 2	transverse	37.38	52.17	1.53	1.85
5	*Lilium regale* bulb 3	basal	948.18	350.71	2.05	2.00
6	*Lilium regale* bulb 3	transverse	132.14	101.37	1.88	1.53
7	*Tulipa* 'Parade' bulb 1	basal	69.85	55.93	2.03	2.04
8	*Tulipa* 'Parade' bulb 1	transverse	17.19	9.01	1.93	2.12
9	*Tulipa* 'Parade' bulb 2	basal	101.51	40.71	1.96	2.06
10	*Tulipa* 'Parade' bulb 2	transverse	23.34	12.56	1.94	2.16
11	*Tulipa* 'Parade' bulb 3	basal	180.89	211.98	2.01	0.87
12	*Tulipa* 'Parade' bulb 3	transverse	184.24	61.96	1.84	0.1

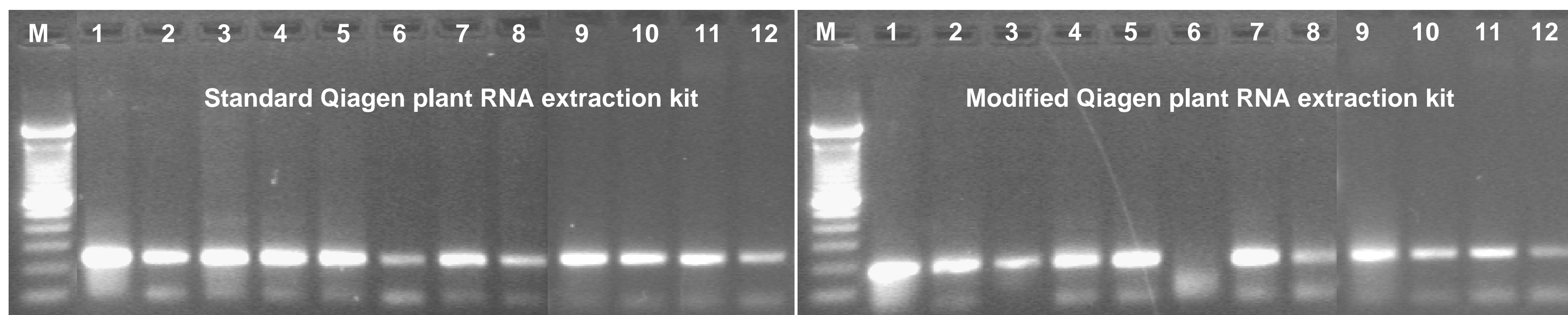
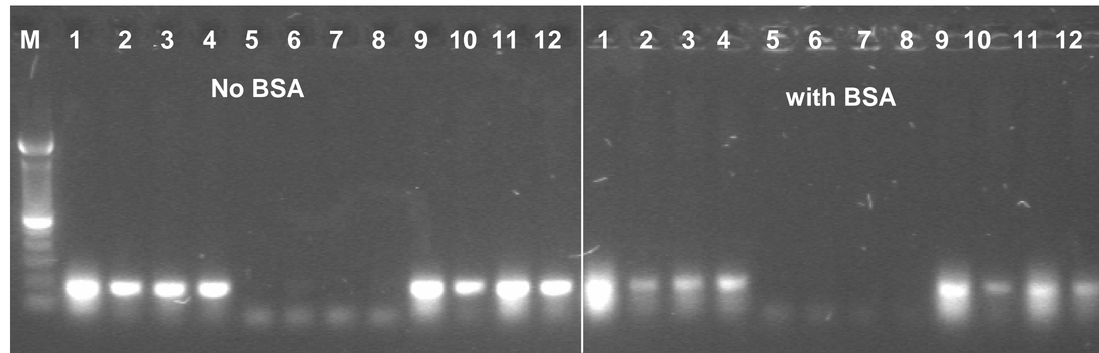


Figure 2.1. Comparison of the quantity and the quality of RNA extracted using the standard and modified Qiagen RNeasy plant mini kit. The RNA was amplified using the plant internal control amplification primer *nad*5 gene, Platinum *Taq* and with the addition of bovine serum albumin (BSA) in one step RT-PCR. The lane numbers correspond to the numbers listed in Table 2.3.

Table 2.4. Comparison of the RNA quantity and quality extracted from difficult bulbs using 1x and 2x RLT extraction buffer.

No.	Bulb species	Sampling sites	RNA quantity (µg/µL)		RNA quality (260/280)	
			1x RLT buffer	2x RLT buffer	1x RLT buffer	2x RLT buffer
1	*Allium sativum* bulb	basal	618.37	1261.88	2.11	2.18
2	*Allium sativum* bulb	transverse	513.5	693.84	2.10	2.16
3	*Iris* (bulbous) bulb	basal	55.35	634.81	1.96	2.14
4	*Iris* (bulbous) bulb	transverse	47.51	139.37	2.04	2.11
5	*Lilium regale* bulb	basal	617.84	852.39	2.10	2.11
6	*Lilium regale* bulb	transverse	43.58	226.25	1.84	2.09
7	*Tulipa* 'Parade' bulb	basal	69.85	427.63	2.03	2.08
8	*Tulipa* 'Parade' bulb	transverse	17.19	114.09	1.93	2.17

One step RT-PCR using Platinum *Taq*



One step RT-PCR using Go*Taq*®

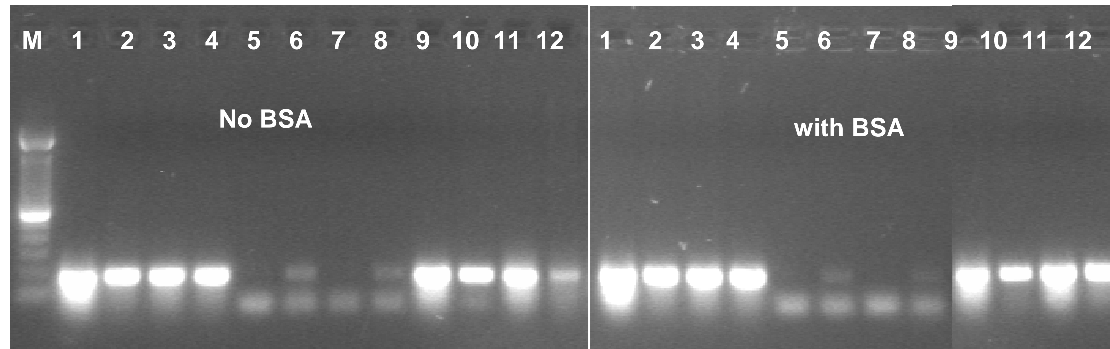


Figure 2.2. One step RT-PCR using two different Taq polymerases, with/without the addition of BSA. Lanes 1-12 represent RNA from Allium sativum bulb 1-basal (1768.1 ng/µL), Allium sativum bulb 1-transverse (493.4 ng/µL), Allium sativum bulb 2-basal (618.1 ng/µL), Allium sativum bulb 2-transverse (513.5 ng/µL), Iris bulb 1-basal (55.3 ng/µL), Iris bulb 1-transverse (47.5 ng/µL), Iris bulb 2-basal (39.5 ng/µL), Iris bulb 2-transverse (51.0 ng/µL), Allium cepa bulb 1-basal (1149.1 ng/µL), Allium cepa bulb 1-transverse (206.6 ng/µL), Allium cepa bulb 2-basal (971.4 ng/µL), and Allium cepa bulb 2-transverse (508.4 ng/µL), respectively. M: 100 bp DNA ladder (Invitrogen).

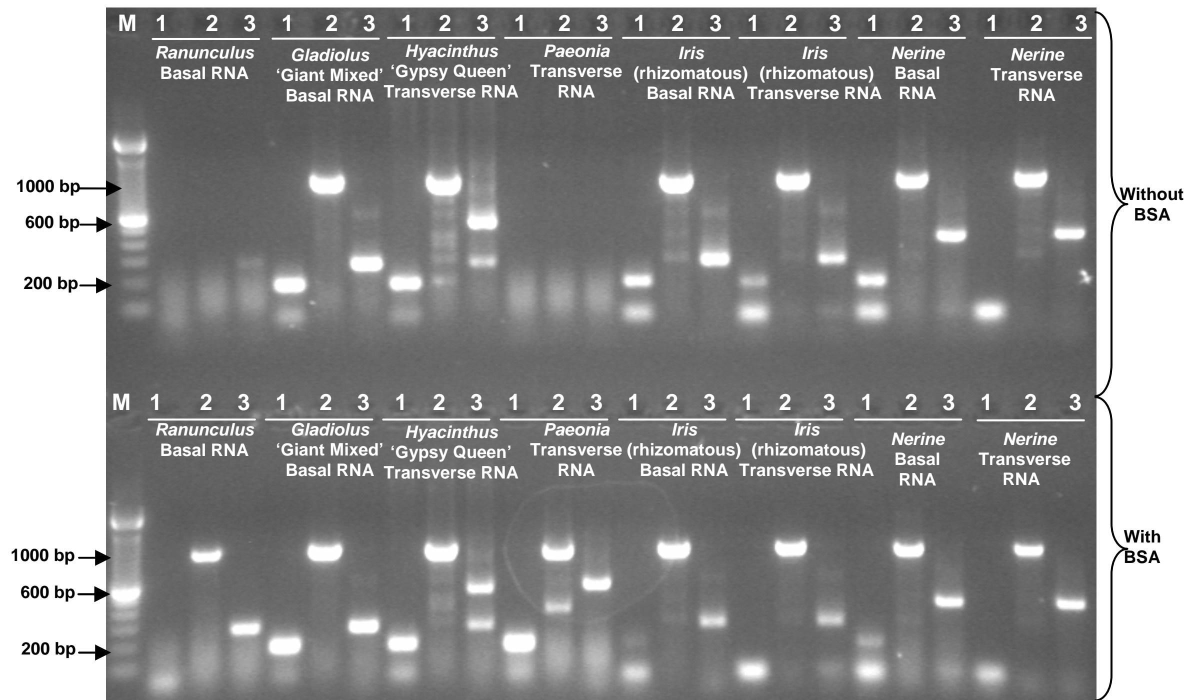


Figure 2.3. One step RT-PCR using GoTaq and three different PIAC primer pairs on different bulb samples, with/without the addition of BSA. Lanes 1-3 represent PIAC primers targeting the nad5 gene, ndhB gene and 5s gene, respectively. M: 100 bp DNA ladder (Invitrogen).

Table 2.5. Quantity and quality of nucleic acids extracted from different bulb samples measured using a Nanodrop® ND-1000 Spectrophotometer and compared with one step RT-PCR amplification using GoTaq, with/without the addition of BSA and three different primer pairs of internal amplification control.

No.	Bulb species (variety)	Quantity (ng/µL)	Quality (260/280)	*nad5* primers*		*ndh*B primers*		*5s* primers*	
				RT-PCR +BSA	RT-PCR no BSA	RT-PCR +BSA	RT-PCR no BSA	RT-PCR +BSA	RT-PCR no BSA
1	*Allium cepa*	Basal: 971.4	2.16	++	++	+++	++	++	+
		Transverse: 508.4	2.02	++	++	++++	+++	++	+
	Allium sativum	Basal: 1261.9	2.18	+++	+++	+++	+	++	+
		Transverse: 693.8	2.16	++++	++++	++++	+++	++	+
2	*Anemone*	Basal: 77.74	2.07	-	-	++++	+++	+++	++
		Transverse: 24.66	2.04	-	-	++++	+++	++	++
3	*Crocus* ('Pickwick)	Basal: 28.6	1.87	+++	+++	+++	++	+	+
		Transverse: 7.26	1.58	-	-	++	+	++	+
	Crocus ('Spring Blue')	Basal: 42.19	1.33	+	+	++++	++	++	++
		Transverse: 14.7	1.66	-	-	+++	++	+	+
4	*Freesia*	Basal: 97.1	1.98	+++	+++	++++	+++	++++	+++
		Transverse: 58.5	2.00	+	+	++++	+++	++	+
5	*Gladiolus* ('Mixed')	Basal: 10.7	2.42	+	+	++++	++++	+++	+++
		Transverse: 13.0	2.00	+	+	++++	++++	+++	+++
	Gladiolus ('Giant Mixed')	Basal: 196.3	2.08	+++	+++	++++	++++	++++	++++
		Transverse: 187.6	2.1	++	++	++++	+++	++	+
6	*Hyacinthus* ('Gypsy Queen')	Basal: 323.1	2.1	+++	+	++++	+++	++	+
		Transverse: 312.3	1.97	+++	++	++++	+++	+++	++
	Hyacinthus ('Super Mixed')	Basal: 310.1	2.06	++	++	++	++++	++	++
		Transverse: 262.6	2.08	++	+	++++	++	+	+
7	*Iris* (bulbous)	Basal: 94.81	2.11	++	+	++++	++++	+++	+
		Transverse: 181.38	2.04	++	+	++++	++++	++	+
	Iris (rhizomatous)	Basal: 61.68	2.02	+++	+++	++++	++++	++++	++
		Transverse: 11.55	1.97	+	+	++++	++++	++	+

1 Amplified products were recorded as very strong "++++" to weak"+" and no amplification was recorded as "-".

Table 2.5 (continued). Quantity and quality of nucleic acids extracted from different bulb samples measured using a Nanodrop® ND-1000 Spectrophotometer and compared with one step RT-PCR amplification using GoTaq, with/without the addition of BSA and three different primer pairs of internal amplification control.

No.	Bulb species (variety)	Quantity (ng/µL)	Quality (260/280)	*nad5* primers*		*ndh*B primers*		*5s* primers*	
				RT-PCR +BSA	RT-PCR no BSA	RT-PCR +BSA	RT-PCR no BSA	RT-PCR +BSA	RT-PCR no BSA
8	*Lachenalia*	Basal: 211.2	2.06	++++	++++	++++	++++	++++	++++
		Transverse: 122.8	1.98	+	+	++++	+++	+++	+++
9	*Lilium regale*	Basal: 335.61	2.08	+++	+++	++++	++++	+	-
		Transverse: 117.80	2.06	+++	+++	++++	++++	+	+
10	*Narcissus*	Basal: 472.5	2.07	+++	+++	++++	+++	+	+
		Transverse: 157.7	1.92	+++	+++	++++	++++	++	++
11	*Nerine*	Basal: 172.8	2.04	+	+	++++	+++	+++	++
		Transverse: 24.3	1.86	-	-	++++	+++	+++	+++
12	*Paeonia*	Basal: 7.55	1.15	++++	-	++++	-	+++	-
		Transverse: 403.57	1.75	++++	-	++++	-	++++	-
13	*Ranunculus*	Basal: 458.8	2.06	-	-	++++	-	++++	-
		Transverse: 62.5	1.79	-	-	+++	-	+++	-
14	*Sandersonia*	Basal: 99.8	2.03	++	+	++++	+++	++++	++
		Transverse: 37.6	1.97	++	+	++++	+++	++++	++
15	*Scilla*	Basal: 235.8	1.99	+++	+++	++++	+++	+++	+
		Transverse: 263.2	2.03	+++	+++	++++	+++	+++	+
16	*Sparaxis*	Basal: 153.7	2.02	+++	+++	++++	++++	+++	+++
		Transverse: 45.6	1.84	+	-	++++	+++	+++	++
17	*Tulipa* ('Parade')	Basal: 175.22	2.05	++	+	++++	+++	++	+
		Transverse:129.84	2.12	++	+	++++	++++	++	+
18	*Zantedeschia* ('Persuasion')	Basal: 196.7	2.06	++	++	++++	++++	+++	++
		Transverse: 24.3	1.97	-	-	++++	++++	+++	++
	Zantedeschia ('Treasure')	Basal: 216.7	2.07	+++	+++	++++	++++	+++	++
		Transverse: 10.4	1.84	+	+	++++	++++	++	+

1 Amplified products were recorded as very strong "++++" to weak"+" and no amplification was recorded as "-".

2.3. CONCLUSION

Nucleic acid of appropriate quality and quantity for PCR can be extracted from all 18 bulb species using the Qiagen RNeasy plant mini kit following the manufacturer's instructions. A greater amount of RNA was extracted if the volume of RLT extraction buffer was doubled.

Although nucleic acid of better quality and quantity was generally obtained from the basal sections, sufficient nucleic acid for reliable amplification was obtained from transverse sections. Future extractions will be done using transverse sections since removal of this tissue does not kill the bulb.

Further work on the distribution of the virus or the overall virus titre in bulbs would be useful for diagnostic purposes.

One step RT-PCR using Go*Taq*® DNA polymerase without the addition of BSA gave the most sensitive detection of the PIAC in most bulb samples, with the exception of RNA from *Paeonia* and *Ranunculus* for which the addition of BSA is crucial for successful amplification. However, Go*Taq*® DNA polymerase is only suitable for conventional PCR and a different *Taq* will be investigated for use in real-time PCR.

PIAC primers targeting the *ndhB* gene were more reliable than those targeting either the *nad*5 or 5*s* genes.

3. Development of real-time PCR methods

3.1. MATERIALS AND METHODS

3.1.1 Bulb samples and targeted viruses

A total of 165 bulbs representing 13 cultivars belonging to 9 different species (Table 3.1) were grown in a glasshouse at IDC-Tamaki. Leaves at around the flowering stage were collected and tested for the presence or absence of five viruses.

Five viruses were selected, all of which have been reported to infect ornamental plants in New Zealand (Pearson *et al.,* 2006; Ward *et al.,* 2008): *Arabis mosaic virus* (ArMV, *Nepovirus*), *Cucumber mosaic virus* (CMV, *Cucumovirus*), *Lily symptomless virus* (LSV, *Carlavirus*), *Tobacco rattle virus* (TRV, *Tobravirus*) and *Tulip virus X* (TVX, *Potexvirus*).

The positive and negative leaf samples and their corresponding bulbs were then used for the development of real-time PCR assays. If no positive samples were obtained for a particular virus, then commercial positive control virus-infected tissue was used in the assays.

Table 3.1. Bulb samples used for virus testing

No.	Species	Cultivar	No. of bulbs
1	*Freesia*	-	3
2	*Crocus*	'Pickwick'	3
		'Spring blue'	3
3	*Gladioli*	'Mixed'	1
4	*Iris x hybrida*	Bulbous	1
	Iris x hollandica	Rhizomatous	16
5	*Lilium regale*	-	11
6	*Paeonia lactiflora*	'Karl Rosenfield'	2
7	*Sparaxi*	'Granny's delight'	5
8	*Tulipa hybrida*	-	7
		'Parade'	71
		'Pink diamond'	38
9	*Vallota*	-	4
Total	**9**	**13**	**165**

3.1.2 Preliminary selection of positive and negative samples by serological method

Enzyme-linked immunosorbent assay (ELISA) test reagent sets for the detection of ArMV, CMV, LSV and TRV were obtained from Agdia (Elkhart, USA) and the reagent set for TVX was purchased from SEDIAG (Longvic, France). All the leaf samples were tested for the five targeted viruses by ELISA following the manufacturers' instructions to determine the positive and negative samples.

Samples were considered positive if the mean of the absorbance values were 2 times higher than the mean of the healthy controls.

3.1.3 Further selection of positive and negative samples using conventional RT-PCR

Total RNA of selected serological positive and negative samples for the five targeted viruses were extracted using the Qiagen RNeasy plant mini kit following the manufacturer's instructions.

Extracted RNA was tested using previously published primers for ArMV, CMV, LSV and TRV or newly designed primers for TVX (published primers were not available) in conventional one-step RT-PCR using SuperScript™ III reverse transcriptase (Invitrogen) and Go*Taq*® DNA polymerase (Promega) in a GeneAmp® PCR system 9700 (Applied Biosystem) following the protocol described in section 2.1. The primer information and PCR cycling conditions for each of the viruses are shown in Table 3.3 in result section 3.2.2. Obtained amplicons of the correct size were cut from the agarose gel and purified using Freeze 'N' Squeeze DNA Gel Extraction Spin Columns (Bio-Rad) and Illustra™ Microspin™ S-300 HR Columns (GE healthcare). Purified PCR products were sequenced and the sequences were compared with those available in the GenBank database using the Basic Local Alignment Search Tool (BLAST) program (Altschul *et al.*, 1997) to confirm the correct identity of the amplicons. The BLAST program searches nucleotide databases to find the identity of a nucleotide sequence.

To design TVX primers, a total of seven nucleotide sequences of TVX were collected from GenBank (www.ncbi.nlm.nih.gov) in November 2008 and aligned using BioEdit version 7.0.4 (Hall, 1999). A conserved sequence, the RNA-dependent RNA-polymerase region, was selected for primer design using Oligo Explorer 1.1.0 (Teemu Kuulasmaa, University of Kuopio, Finland [http://molbiol-tools.ca/molecular_biology_freeware.htm]). The optimal annealing and extension temperatures of the newly designed primers were optimized using a Palm-CyclerTM machine (Corbett Research, Sydney, Australia) before using for the RT-PCR detection.

3.1.4 Taqman probe and primer design

Nucleotide sequences of ArMV, CMV, LSV and TVX were collected from GenBank and the conserved regions were identified following alignment using BioEdit version 7.0.4. Primers and probes were then designed from the conserved sequence regions using Biosearch Technology online free software, RealTimeDesign™ (www.qPCRdesign.com). Probes had a FAM fluorescent dye and a BHQ quencher.

The detection of TRV was done using previously published primers (Mumford *et al*., 2000). The author demonstrated the robustness of the TRV protocol and the test was tested on a wide range of potato cultivars (Mumford *et al*., 2000). Primers and probe information are detailed in Table 3.4 in Results section 3.2.2.

All probes were obtained from Biosearch Technologies (www.biosearchtech.com) and primers were ordered from Invitrogen (www.invitrogen.co.nz).

To obtain the best amplification efficiency, a strategy of adding an extra 12-AT rich primer flap (AATAAATCATAA) at the 5'-end of each forward or reverse primer was introduced in this study. A primer flap is a nucleotide segment which is non-complementary to the target and is linked to the 5′ terminus of a primer. It has recently been recognized that a 5′ AT-rich non-complementary flap can improve significantly the efficiency of Real Time PCR (Afonina et al., 2007). Different combinations of the primer with/without flap were compared under the same reaction conditions.

3.1.5 Real-time RT-PCR

Real-time PCR reactions were carried out in a total volume of 10 µl using Superscript III one-step qRT-PCR system (Invitrogen) on a Rotor-Gene RG-3000 (Corbett Research, Sydney, Australia). Data were analyzed using the Rotor-Gene 6 software. Each 10 µL reaction contained 1 µL of relevant positive/negative RNA, 300 nM of each primer and probe (final concentration), 0.25 µL of 10 µg/µL bovine serum albumin (BSA, Sigama), 5 µL 2 × reaction mixture and 0.2 µL of SuperScript™ III reverse transcriptase and Platinum *Taq* mixture. The reactions were then conducted under generic conditions of 50°C for 30 min for cDNA synthesis, followed by amplification reactions at 95°C for 5 min, and 40 cycles of 95°C for 10 sec and 60°C for 45 sec.

The real-time RT-PCR products were used to run a 2% (w/v) agarose gel and amplicons with correct size were cut and purified using Freeze 'N Squeeze DNA Gel Extraction Spin Columns (Bio-Rad) and Illustra™ Microspin™ S-300 HR Columns (GE healthcare). Purified PCR products were sequenced and the primer and probe sequences were identified to confirm the correct identity of the amplicons.

3.2. RESULTS

3.2.1 ELISA results

Leaves were collected from all the 165 growing bulbous plants and tested using commercial ELISA kits for the five targeted viruses: ArMV, CMV, LSV, TRV and TVX. Results showed that a total of 130, 35, 44, 5 and 7 positive samples with different positive reaction strength were detected for ArMV, CMV, LSV, TRV and TVX, respectively. In most cases, multi-positive reactions were obtained from single plant (Table 3.2).

Table 3.2. ELISA results for Arabis mosaic virus (ArMV), Cucumber mosaic virus (CMV), Lily symptomless virus (LSV), Tobacco rattle virus (TRV) and Tulip virus X (TVX).

No.	Bulb species/cultivar	IDC Acession no.	ArMV	CMV	LSV	TRV	TVX
1	*Freesia* 1	09-2008-3419	+	+	+	-	-
2	*Freesia* 2	09-2008-3419	+	-	+	-	-
3	*Freesia* 3	09-2008-3419	+	-	-	-	-
4	*Crocus* 'Pickwick' 1	09-2008-3430	++	-	-	-	-
5	*Crocus* 'Pickwick' 2	09-2008-3430	+	-	-	-	-
6	*Crocus* 'Pickwick' 3	09-2008-3430	+	-	-	-	-
7	*Crocus* 'Spring Blue' 1	09-2008-3435	+	-	-	-	-
8	*Crocus* 'Spring Blue' 2	09-2008-3435	+	-	-	-	-
9	*Crocus* 'Spring Blue' 3	09-2008-3435	+	-	-	-	-
10	*Gladiolus* 'Mixed colours'	09-2008-3434	++	+	-	-	-
11	*Iris x hybrida* 1	09-2008-3525	-	-	-	-	+
12	*Iris x hollandica* 'Purple sensation' 1	09-2008-2812	-	-	-	-	-
13	*Iris x hollandica* 'Purple sensation' 2	09-2008-2812	-	-	-	-	-
14	*Iris x hollandica* 'Purple sensation' 3	09-2008-2812	-	-	-	-	-
15	*Iris x hollandica* 'Purple sensation' 4	09-2008-2812	-	-	-	-	-
16	*Iris x hollandica* 'Purple sensation' 6	09-2008-2812	-	-	-	-	-
17	*Iris x hollandica* 'Purple sensation' 7	09-2008-2812	-	-	-	-	-
18	*Iris x hollandica* 'Purple sensation' 8	09-2008-2812	-	-	-	-	-
19	*Iris x hollandica* 'Purple sensation' 28	09-2008-2812	-	-	-	-	-
20	*Iris x hollandica* 'Purple sensation' 31	09-2008-2812	-	+	-	-	-
21	*Iris x hollandica* 'Purple sensation' 37	09-2008-2812	-	+	-	-	-
22	*Iris x hollandica* 'Purple sensation' 41	09-2008-2812	-	-	-	-	-
23	*Iris x hollandica* 'Purple sensation' 49	09-2008-2812	-	-	-	-	-
24	*Iris x hollandica* 'Purple sensation' 51	09-2008-2812	-	-	-	-	-
25	*Iris x hollandica* 'Purple sensation' 61	09-2008-2812	-	-	-	-	-
26	*Iris x hollandica* 'Purple sensation' 64	09-2008-2812	-	-	-	-	-
27	*Iris x hollandica* 'Purple sensation' 66	09-2008-2812	-	-	-	-	-
28	*Lilium regale* 1	09-2008-2813	+	-	++++	-	-
29	*Lilium regale* 2	09-2008-2813	+	-	++++	-	-
30	*Lilium regale* 3	09-2008-2813	+	-	++++	-	-
31	*Lilium regale* 4	09-2008-2813	+	+	++++	-	-
32	*Lilium regale* 5	09-2008-2813	+	+	++++	-	+
33	*Lilium regale* 6	09-2008-2813	+	+	++++	-	+
34	*Lilium regale* 7	09-2008-2813	+	-	++++	-	-
35	*Lilium regale* 8	09-2008-2813	+	-	++++	-	-
36	*Lilium regale* 9	09-2008-2813	+	+	++++	-	-
37	*Lilium regale* 10	09-2008-2813	+	-	++++	-	-
38	*Lilium regale* 11	09-2008-2813	+	-	++++	-	-
39	*Paeonia* 'Karl Rosenfield' 1	09-2008-3436	+	-	-	-	-
40	*Paeonia* 'Karl Rosenfield' 2	09-2008-3436	+	+	-	+	-
41	*Sparaxis* 'Granny's delight' 1	09-2008-3428	+	-	-	-	-
42	*Sparaxis* 'Granny's delight' 2	09-2008-3428	-	-	-	-	-
43	*Sparaxis* 'Granny's delight' 3	09-2008-3428	-	-	-	-	-
44	*Sparaxis* 'Granny's delight' 4	09-2008-3428	-	-	-	-	-
No.	Bulb species/cultivar	IDC Acession no.	ArMV	CMV	LSV	TRV	TVX
45	*Sparaxis* 'Granny's delight' 5	09-2008-3428	-	-	-	-	-
46	*Tulipa x hybrida* 1	09-2008-2810	-	-	-	-	-
47	*Tulipa x hybrida* 2	09-2008-2810	++	+	++++	-	-
48	*Tulipa x hybrida* 3	09-2008-2810	+	-	++++	-	-

No.	Bulb species/cultivar	IDC Acession no.	ArMV	CMV	LSV	TRV	TVX
49	*Tulipa x hybrida* 4	09-2008-2810	++	-	+	-	-
50	*Tulipa x hybrida* 5	09-2008-2810	-	-	+++	-	-
51	*Tulipa x hybrida* 6	09-2008-2810	-	-	++	-	-
52	*Tulipa x hybrida* 7	09-2008-2810	-	-	-	-	-
53	*Tulipa x hybrida* 'Parade' 1	09-2008-2811	+	-	-	-	-
54	*Tulipa x hybrida* 'Parade' 2	09-2008-2811	+	+	+	+	-
55	*Tulipa x hybrida* 'Parade' 3	09-2008-2811	-	-	-	+	-
56	*Tulipa x hybrida* 'Parade' 4	09-2008-2811	++	+	+	-	-
57	*Tulipa x hybrida* 'Parade' 5	09-2008-2811	+	-	-	-	-
58	*Tulipa x hybrida* 'Parade' 6	09-2008-2811	+	+	+	+	-
59	*Tulipa x hybrida* 'Parade' 7	09-2008-2811	-	-	-	-	-
60	*Tulipa x hybrida* 'Parade' 8	09-2008-2811	+	+	+	+	-
61	*Tulipa x hybrida* 'Parade' 9	09-2008-2811	+	-	-	-	-
62	*Tulipa x hybrida* 'Parade' 10	09-2008-2811	+	-	-	-	-
63	*Tulipa x hybrida* 'Parade' 11	09-2008-2811	+	-	-	-	-
64	*Tulipa x hybrida* 'Parade' 12	09-2008-2811	+	-	-	-	-
65	*Tulipa x hybrida* 'Parade' 13	09-2008-2811	+	-	-	-	-
66	*Tulipa x hybrida* 'Parade' 14	09-2008-2811	+	-	-	-	-
67	*Tulipa x hybrida* 'Parade' 15	09-2008-2811	+	+	-	-	-
68	*Tulipa x hybrida* 'Parade' 16	09-2008-2811	+	-	-	-	-
69	*Tulipa x hybrida* 'Parade' 17	09-2008-2811	+	+	-	-	-
70	*Tulipa x hybrida* 'Parade' 18	09-2008-2811	-	+	-	-	-
71	*Tulipa x hybrida* 'Parade' 19	09-2008-2811	++	+	-	-	-
72	*Tulipa x hybrida* 'Parade' 20	09-2008-2811	+	+	-	-	-
73	*Tulipa x hybrida* 'Parade' 21	09-2008-2811	+	+	-	-	-
74	*Tulipa x hybrida* 'Parade' 22	09-2008-2811	++	+	-	-	-
75	*Tulipa x hybrida* 'Parade' 23	09-2008-2811	++	-	-	-	-
76	*Tulipa x hybrida* 'Parade' 24	09-2008-2811	+	-	-	-	-
77	*Tulipa x hybrida* 'Parade' 25	09-2008-2811	++	-	-	-	-
78	*Tulipa x hybrida* 'Parade' 26	09-2008-2811	-	-	-	-	-
79	*Tulipa x hybrida* 'Parade' 27	09-2008-2811	+	+	-	-	-
80	*Tulipa x hybrida* 'Parade' 28	09-2008-2811	+	-	-	-	-
81	*Tulipa x hybrida* 'Parade' 29	09-2008-2811	+	+	-	-	-
82	*Tulipa x hybrida* 'Parade' 30	09-2008-2811	-	-	-	-	-
83	*Tulipa x hybrida* 'Parade' 31	09-2008-2811	+	-	-	-	-
84	*Tulipa x hybrida* 'Parade' 32	09-2008-2811	+	-	-	-	-
85	*Tulipa x hybrida* 'Parade' 33	09-2008-2811	+	-	-	-	-
86	*Tulipa x hybrida* 'Parade' 34	09-2008-2811	+	-	-	-	-
87	*Tulipa x hybrida* 'Parade' 35	09-2008-2811	+	-	-	-	-
88	*Tulipa x hybrida* 'Parade' 36	09-2008-2811	+	-	-	-	-
89	*Tulipa x hybrida* 'Parade' 37	09-2008-2811	-	-	-	-	-
90	*Tulipa x hybrida* 'Parade' 38	09-2008-2811	+	-	-	-	-
91	*Tulipa x hybrida* 'Parade' 39	09-2008-2811	+	++	-	-	-
92	*Tulipa x hybrida* 'Parade' 40	09-2008-2811	+	-	-	-	-
93	*Tulipa x hybrida* 'Parade' 41	09-2008-2811	+	-	-	-	-
94	*Tulipa x hybrida* 'Parade' 42	09-2008-2811	+	-	-	-	-
95	*Tulipa x hybrida* 'Parade' 43	09-2008-2811	+	-	-	-	-
96	*Tulipa x hybrida* 'Parade' 44	09-2008-2811	+	-	-	-	-
97	*Tulipa x hybrida* 'Parade' 45	09-2008-2811	+	-	-	-	-
98	*Tulipa x hybrida* 'Parade' 46	09-2008-2811	+	-	-	-	-
99	*Tulipa x hybrida* 'Parade' 47	09-2008-2811	+	-	-	-	-
100	*Tulipa x hybrida* 'Parade' 48	09-2008-2811	+	-	-	-	-

No.	Bulb species/cultivar	IDC Acession no.	ArMV	CMV	LSV	TRV	TVX
101	*Tulipa x hybrida* 'Parade' 49	09-2008-2811	+	-	-	-	-
102	*Tulipa x hybrida* 'Parade' 50	09-2008-2811	+	-	-	-	-
103	*Tulipa x hybrida* 'Parade' 51	09-2008-2811	+	-	-	-	-
104	*Tulipa x hybrida* 'Parade' 52	09-2008-2811	+	-	-	-	-
105	*Tulipa x hybrida* 'Parade' 53	09-2008-2811	+	+	-	-	-
106	*Tulipa x hybrida* 'Parade' 54	09-2008-2811	++	-	-	-	-
107	*Tulipa x hybrida* 'Parade' 55	09-2008-2811	+	-	-	-	-
108	*Tulipa x hybrida* 'Parade' 56	09-2008-2811	+	-	-	-	-
109	*Tulipa x hybrida* 'Parade' 57	09-2008-2811	+	-	-	-	-
110	*Tulipa x hybrida* 'Parade' 58	09-2008-2811	+	-	-	-	-
111	*Tulipa x hybrida* 'Parade' 59	09-2008-2811	+	-	-	-	-
112	*Tulipa x hybrida* 'Parade' 60	09-2008-2811	+	+	+	-	-
113	*Tulipa x hybrida* 'Parade' 61	09-2008-2811	+	+	+	-	-
114	*Tulipa x hybrida* 'Parade' 62	09-2008-2811	+	-	-	-	-
115	*Tulipa x hybrida* 'Parade' 63	09-2008-2811	+	-	-	-	-
116	*Tulipa x hybrida* 'Parade' 64	09-2008-2811	+	-	-	-	-
117	*Tulipa x hybrida* 'Parade' 65	09-2008-2811	+	-	-	-	-
118	*Tulipa x hybrida* 'Parade' 66	09-2008-2811	+	-	-	-	-
119	*Tulipa x hybrida* 'Parade' 67	09-2008-2811	+	-	-	-	-
120	*Tulipa x hybrida* 'Parade' 68	09-2008-2811	+	-	-	-	-
121	*Tulipa x hybrida* 'Parade' 69	09-2008-2811	+	-	-	-	-
122	*Tulipa x hybrida* 'Parade' 70	09-2008-2811	+	-	-	-	-
123	*Tulipa x hybrida* 'Parade' 71	09-2008-2811	+	-	-	-	-
124	*Tulipa x hybrida* 'Pink diamond' 7	09-2008-2810	+	+	+	-	-
125	*Tulipa x hybrida* 'Pink diamond' 8	09-2008-2810	+	+	**+**	-	-
126	*Tulipa x hybrida* 'Pink diamond' 9	09-2008-2810	+	+	-	-	-
127	*Tulipa x hybrida* 'Pink diamond' 10	09-2008-2810	+	-	-	-	-
128	*Tulipa x hybrida* 'Pink diamond' 11	09-2008-2810	+	-	-	-	-
129	*Tulipa x hybrida* 'Pink diamond' 12	09-2008-2810	+	-	-	-	-
130	*Tulipa x hybrida* 'Pink diamond' 13	09-2008-2810	+	-	-	-	-
131	*Tulipa x hybrida* 'Pink diamond' 14	09-2008-2810	++	-	++++	-	+
132	*Tulipa x hybrida* 'Pink diamond' 15	09-2008-2810	+	-	-	-	-
133	*Tulipa x hybrida* 'Pink diamond' 16	09-2008-2810	+	-	-	-	+
134	*Tulipa x hybrida* 'Pink diamond' 17	09-2008-2810	+	-	++++	-	-
135	*Tulipa x hybrida* 'Pink diamond' 18	09-2008-2810	+	-	+	-	-
136	*Tulipa x hybrida* 'Pink diamond' 19	09-2008-2810	+	-	+	-	+++
137	*Tulipa x hybrida* 'Pink diamond' 20	09-2008-2810	+	-	+	-	-
138	*Tulipa x hybrida* 'Pink diamond' 21	09-2008-2810	+	-	+	-	-
139	*Tulipa x hybrida* 'Pink diamond' 23	09-2008-2810	+	-	++++	-	-
140	*Tulipa x hybrida* 'Pink diamond' 24	09-2008-2810	++	-	-	-	-
141	*Tulipa x hybrida* 'Pink diamond' 25	09-2008-2810	+	-	-	-	-
142	*Tulipa x hybrida* 'Pink diamond' 27	09-2008-2810	+	-	++++	-	-
143	*Tulipa x hybrida* 'Pink diamond' 28	09-2008-2810	+	-	-	-	-
144	*Tulipa x hybrida* 'Pink diamond' 29	09-2008-2810	+	-	-	-	-
145	*Tulipa x hybrida* 'Pink diamond' 30	09-2008-2810	++	-	++++	-	-
146	*Tulipa x hybrida* 'Pink diamond' 32	09-2008-2810	+	-	++++	-	-
147	*Tulipa x hybrida* 'Pink diamond' 33	09-2008-2810	+	-	-	-	-
148	*Tulipa x hybrida* 'Pink diamond' 34	09-2008-2810	+	+	-	-	-
149	*Tulipa x hybrida* 'Pink diamond' 35	09-2008-2810	+	+	-	-	-
150	*Tulipa x hybrida* 'Pink diamond' 37	09-2008-2810	+	-	+	-	-
151	*Tulipa x hybrida* 'Pink diamond' 38	09-2008-2810	+	-	+	-	-
152	*Tulipa x hybrida* 'Pink diamond' 39	09-2008-2810	+	-	+	-	-

153	*Tulipa x hybrida* 'Pink diamond' 40	09-2008-2810	+	-	-	-	-
154	*Tulipa x hybrida* 'Pink diamond' 42	09-2008-2810	+	-	++++	-	-
155	*Tulipa x hybrida* 'Pink diamond' 43	09-2008-2810	+	-	-	-	-
156	*Tulipa x hybrida* 'Pink diamond' 44	09-2008-2810	+	-	-	-	-
157	*Tulipa x hybrida* 'Pink diamond' 45	09-2008-2810	+	-	-	-	-
158	*Tulipa x hybrida* 'Pink diamond' 47	09-2008-2810	+	+	+	-	-
159	*Tulipa x hybrida* 'Pink diamond' 48	09-2008-2810	+	+	-	-	-
160	*Tulipa x hybrida* 'Pink diamond' 49	09-2008-2810	+	+	++++	-	++
161	*Tulipa x hybrida* 'Pink diamond' 50	09-2008-2810	+	-	-	-	-
162	*Vallota* sp. 1	09-2008-1330	-	-	-	-	-
163	*Vallota* sp. 2	09-2008-1330	-	-	+	-	-
164	*Vallota* sp. 3	09-2008-1330	-	-	+	-	-
165	*Vallota* sp. 4	09-2008-1330	-	-	+	-	-
	Total positive		**130**	**35**	**44**	**5**	**7**
	Total negative		**35**	**130**	**121**	**160**	**158**

*ELISA results were recorded as very strong "++++", to weak "+" and negative reactions were recorded as "–".

3.2.2 Taqman probes and primers

Newly designed real-time PCR primers and probes for ArMV, CMV, LSV and TVX were obtained from the coat protein (CP) gene. Information on new primers and probes and previously published primers and probe is given in Table 3.3 and 3.4.

Table 3.3. Conventional PCR primer information and PCR cycling conditions for *Arabis mosaic virus* (ArMV), *Cucumber mosaic virus* (CMV), *Lily symptomless virus* (LSV), *Tobacco rattle virus* (TRV) and *Tulip virus X* (TVX).

Virus	Primer name and sequences (5´-3´)	Product size (bp)	PCR cycling conditions	References
ArMV	ArMV-H428 (Forward): GCGGCGGATTGGGAGTT ArMV-C867 (Reverse): CGATGGTAGGGGGAGCGTATT	440 bp	94ºC, 5 min 94ºC, 30 sec, 54ºC, 35 sec, 72ºC, 1 min } 35cycles 72ºC, 5 min	Nassuth *et al.*, 2000
CMV	CMV1-F (Forward): GTAGACATCTGTGACGCGA CMV1-R (Reverse): GCGCGAAACAAGCTTCTTATC	540 bp	94ºC, 2 min 94ºC, 1 min, 54ºC, 1 min, 72ºC, 1 min } 35cycles 72ºC, 7 min	Blas *et al.*, 1994
LSV	LSV-F1 (Forward): GCAATCAAGACCAGCACAAG LSV-R1 (Reverse): TCCACACGATAGGGGCATAG	571 bp	94ºC, 2 min 94ºC, 45 sec, 58ºC, 30 sec, 72ºC, 2 min } 40cycles 72ºC, 5 min	Joe Tang, 2004, unpublished
TRV	TRV -261 (Forward): GACGTGTGTACTCAAGGGTT TRV-262 (Reverse): CAGTCTATACACAGAAACAGA	463 bp	94ºC, 2 min 94ºC, 45 sec, 58ºC, 30 sec, 72ºC, 2 min } 40cycles 72ºC, 5 min	Robinson, 1992
TVX	TVX-F (Forward): GATACATGCGGAGGTACAGG TVX-R (Reverse): TGAAGAACTTGGCTTTGAGG	201 bp	94ºC, 3 min 94ºC, 15 sec, 58ºC, 20 sec, 72ºC, 1 min } 40cycles 72ºC, 5 min	This paper

Table 3.4. Real-time RT-PCR primers and probes information

Virus	Primer/probe name	Primer/probe sequence (5'-3')	Location	Size	Reference
ArMV	ArMVcpF	TAGCCCTTGGAGACAATCCT	Coat protein	93bp	This paper
	ArMVcpR	CCTCCAAATCCCACATTAAC			
	ArMVcpProbe	TGCCCATATGATAGCTTGTCATGGAC			
CMV	CMVcpF	CCACACGGTAGAATCAATTTC	Coat protein	99bp	This paper
	CMVcpR	GGTCTTATTACGGTAAAGGTTG			
	CMVcpProbe	GCGCGAAACAAGCTTCTTATC			
LSV	LSVcpF	GGGCAGCATTGAGTTTGAGA	Coat protein	77bp	This paper
	LSVcpR	GCAGTCCAGCGTGCTTCTTC			
	LSVcpProbe	AGCGGTGCCCGTCGACTCCAT			
TVX	TVXcpF	CCAGATACATGCGGAGGTAC	Coat protein	117bp	This paper
	TVXcpR	GCTTGGTGAAGTCCCAATGG			
	TVXcpProbe	GATGCCCTCGGCCCCAACAACATC			
TRV	TRVcpF	CATGCTAACAAATTGCGAAAGC	Coat protein	87bp	Mumford *et al.*, 2000
	TRVcpR	TACAGACAAACCATCCACAATTATTTT			
	TRVcpProbe	ACGTGTGACACCAACCATGTCAGCAACT			

3.2.3 Selection of *Tulipa* samples for conventional RT-PCR testing

Based on ELISA results (Table 3.2), a total of 28 serological positive and negative *Tulipa* leaf samples were selected for RNA extraction (Table 3.5) and used for one-step RT-PCR targeting ArMV, CMV, LSV, TRV and TVX.

The newly designed TVX conventional PCR primers were tested in one-step RT-PCR using RNA from one ELISA TVX positive sample (*Tulipa x hybrida* 'Pink diamond' 50) (Table 3.5), The annealing temperatures (ranging from ~49°C to 62°C) and extension temperatures (ranging from ~62°C to 72°C) were first optimized. Results showed that an annealing temperature of ~53°C for 20 seconds provided the best amplification efficiency. Extension temperatures ranging from 61°C to 68°C for 1 minute showed similar amplification efficiency and were slightly better than a 72°C extension temperature (Fig. 3.1). The sequence of the obtained amplicon (Fig. 3.2) showed 100% nucleotide identity to New Zealand TVX isolate (Accession EU555190) (Fig. 3.3). Future TVX PCR tests will be done using an annealing temperature of 53°C for 20 second and an extension temperature of 68°C for 1 minute.

Correct size bands were obtained for LSV and TVX but none of the samples tested positive for ArMV, CMV and TRV (Fig. 3.4 and Table 3.6). All the ELISA positive samples for ArMV, CMV and TRV showed negative amplification in RT-PCR (Table 3.6). Variable results were obtained for LSV: sample numbers 1, 10, 13 and 14 showed weak positive/negative in ELISA but showed strong positive by RT-PCR, and sample numbers 5, 21, 22 and 27 showed strong positive in ELISA but weak positive/negative in RT-PCR (Table 3.6). Meanwhile, sample numbers 18 to 27 gave weak bands which were slightly smaller than the true size and were given question marks (3.6). Some ELISA negative or weak positive samples for TVX showed positive or strong positive amplification (Table 3.6).

The correctly size amplicon of LSV from one of the sample was sequenced (Fig. 3.4) and the obtained sequence showed 99% nucleotide identity with the Netherlands LSV isolate (accession X15343) (Fig. 3.5). This sample was then used for the development of the real-time PCR for LSV.

One clearly incorrectly sized band from sample number 24 for ArMV (Fig. 3.4) was sequenced and the result confirmed that this amplification is non-specific (data not shown).

Table 3.5. Samples tested by ELISA for *Arabis mosaic virus* (ArMV), *Cucumber mosaic virus* (CMV), *Lily symptomless virus* (LSV), *Tobacco rattle virus* (TRV) and *Tulip virus X* (TVX) which were selected for one-step RT-PCR tests.

No.	Bulb species/cultivar	ArMV	CMV	LSV	TRV	TVX
1	*Tulipa x hybrida* 1	-	-	-	-	-
2	*Tulipa x hybrida* 2	++	+	++++	-	-
3	*Tulipa x hybrida* 3	+	-	++++	-	-
4	*Tulipa x hybrida* 4	++	-	+	-	-
5	*Tulipa x hybrida* 5	-	-	+++	-	-
6	*Tulipa x hybrida* 6	-	-	++	-	-
7	*Tulipa x hybrida* 'Pink diamond' 12	+	-	-	-	-
8	*Tulipa x hybrida* 'Pink diamond' 13	+	-	-	-	-
9	*Tulipa x hybrida* 'Pink diamond' 14	++	-	++++	-	+
10	*Tulipa x hybrida* 'Pink diamond' 15	+	-	-	-	-
11	*Tulipa x hybrida* 'Pink diamond' 16	+	-	-	-	+
12	*Tulipa x hybrida* 'Pink diamond' 17	+	-	++++	-	-
13	*Tulipa x hybrida* 'Pink diamond' 18	+	-	+	-	-
14	*Tulipa x hybrida* 'Pink diamond' 19	+	-	+	-	+++
15	*Tulipa x hybrida* 'Pink diamond' 20	+	-	+	-	-
16	*Tulipa x hybrida* 'Pink diamond' 21	+	-	+	-	-
17	*Tulipa x hybrida* 'Pink diamond' 49	+	+	++++	-	++
18	*Tulipa x hybrida* 'Pink diamond' 50	+	-	-	-	-
19	*Tulipa x hybrida* 7	-	-	-	-	-
20	*Tulipa x hybrida* 'Parade' 1	+	-	-	-	-
21	*Tulipa x hybrida* 'Parade' 2	+	+	+	+	-
22	*Tulipa x hybrida* 'Parade' 3	-	-	-	+	-
23	*Tulipa x hybrida* 'Parade' 4	++	+	+	-	-
24	*Tulipa x hybrida* 'Parade' 5	+	-	-	-	-
25	*Tulipa x hybrida* 'Parade' 6	+	+	+	+	-
26	*Tulipa x hybrida* 'Parade' 7	-	-	-	-	-
27	*Tulipa x hybrida* 'Parade' 8	+	+	+	+	-
28	*Tulipa x hybrida* 'Parade' 9	+	-	-	-	-

*ELISA results were recorded as very strong "++++", to weak "+" and negative reactions were recorded as "–".

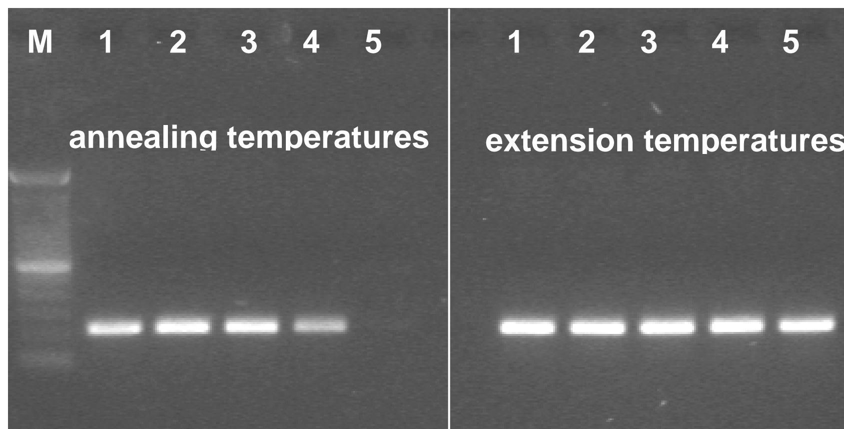


Figure 3.1. Optimization of annealing and extension temperatures for *Tulip virus X* (TVX) primers in one-step RT-PCR. Lanes 1 to 5 on the left part of the gel image represent annealing temperatures of 49.6ºC, 52.9ºC, 55.8ºC, 58.6ºC and 61.8ºC, respectively and lanes 1 to 5 on the right part of the gel image represent extension temperatures of 61.2ºC, 63.7ºC, 66.0ºC, 68.1ºC and 71.8ºC, respectively. M: 100 bp DNA ladder (Invitrogen). The TVX product size is 201 bp.

GATACATGCGGAGGTACAGGGATGCCCTCGGCCCCAACAACATCATGATTAAC
TGTGAGAGGACTCCCGCGGACCTGAGTCGCTGGGTTCGCAACCATTGGGACTTCA
CCAAGCCCTCCTACGCCAACGACTTCACCGCCTTCGACCAGTCACAAGATG
GAGCCATGCTGCAGTTCGAGAT**CCTCAAAGCCAAGTTCTTCA**

Figure 3.2. Sequence of *Tulip virus X* PCR amplicon. Letters in bold are primer binding sites.

```
>  gb|EU555190.1|  Tulip virus X from New Zealand replicase gene, partial cds

Length=704

 Score =  372 bits (201),  Expect = 4e-100
 Identities = 201/201 (100%), Gaps = 0/201 (0%)
 Strand=Plus/Plus

Query  1    GATACATGCGGAGGTACAGGGATGCCCTCGGCCCCAACAACATCATGATTAACTGTGAGA  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  401  GATACATGCGGAGGTACAGGGATGCCCTCGGCCCCAACAACATCATGATTAACTGTGAGA  460

Query  61   GGACTCCCGCGGACCTGAGTCGCTGGGTTCGCAACCATTGGGACTTCACCAAGCCCTCCT  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  461  GGACTCCCGCGGACCTGAGTCGCTGGGTTCGCAACCATTGGGACTTCACCAAGCCCTCCT  520

Query  121  ACGCCAACGACTTCACCGCCTTCGACCAGTCACAAGATGGAGCCATGCTGCAGTTCGAGA  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  521  ACGCCAACGACTTCACCGCCTTCGACCAGTCACAAGATGGAGCCATGCTGCAGTTCGAGA  580

Query  181  TCCTCAAAGCCAAGTTCTTCA  201
            |||||||||||||||||||||
Sbjct  581  TCCTCAAAGCCAAGTTCTTCA  601
```

Figure 3.3. BLAST result of the sequence of *Tulip virus X* PCR amplicon.

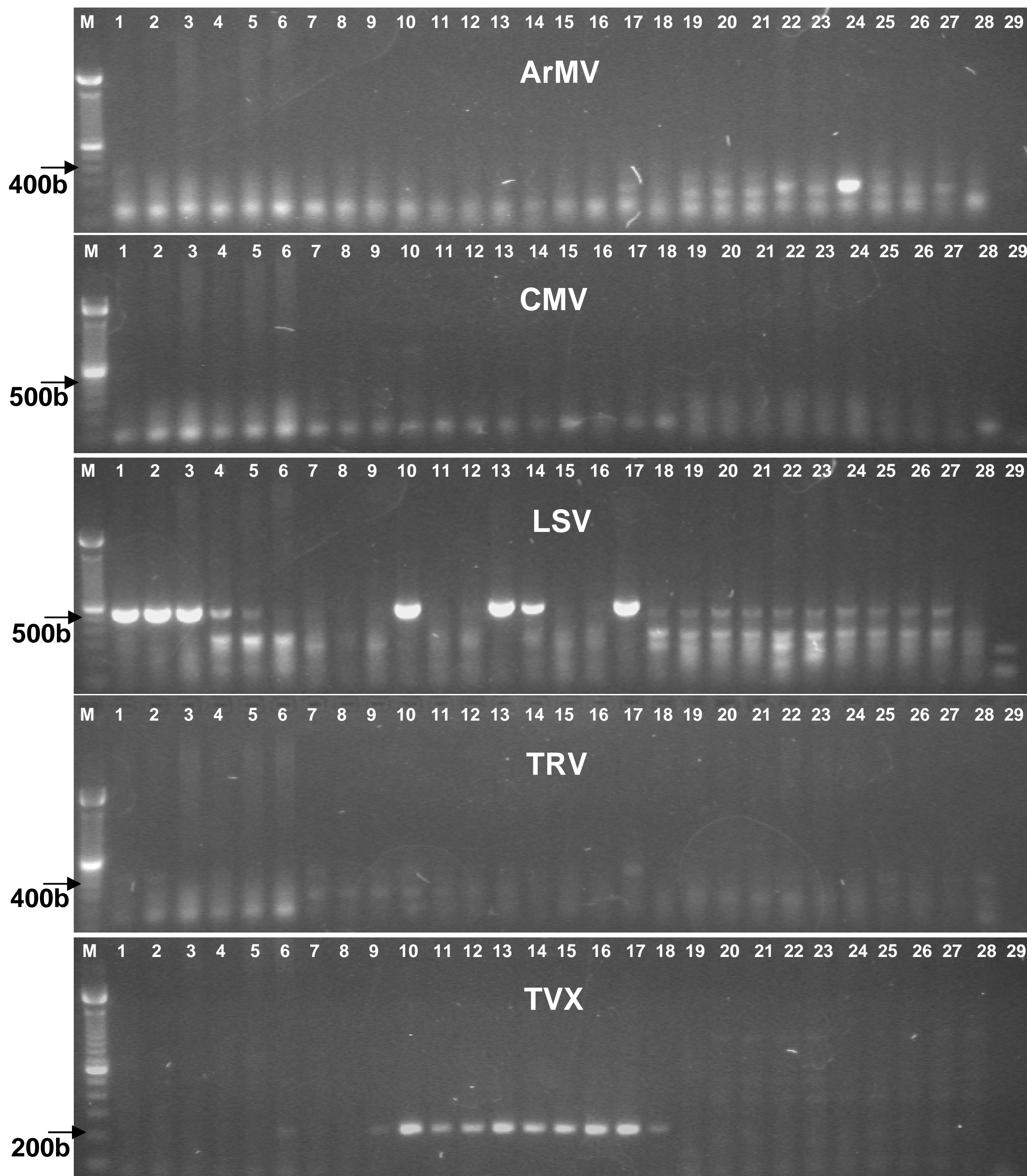


Figure 3.4. PCR results for the five targeted viruses. The correct size amplicons for *Arabis mosaic virus* (ArMV), *Cucumber mosaic virus* (CMV), *Lily symptomless virus* (LSV), *Tobacco rattle virus* (TRV) and *Tulip virus X* (TVX) were 440bp, 540bp, 571bp, 463bp, and 201bp, respectively. M: 100 bp DNA ladder (Invitrogen). Lane 29 is the negative control.

Table 3.6. Conventional one-step RT-PCR results for the 28 *Tulipa* RNA samples tested for *Arabis mosaic virus* (ArMV), *Cucumber mosaic virus* (CMV), *Lily symptomless virus* (LSV), *Tobacco rattle virus* (TRV) and *Tulip virus X* (TVX).

		RT-PCR results					ELISA results				
No.	Plant	ArMV	CMV	LSV	TRV	TVX	ArMV	CMV	LSV	TRV	TVX
1	*Tulipa x hybrida* 1	-	-	++++	-	-	-	-	-	-	-
2	*Tulipa x hybrida* 2	-	-	++++	-	-	++	+	++++	-	-
3	*Tulipa x hybrida* 3	-	-	++++	-	-	+	-	++++	-	-
4	*Tulipa x hybrida* 4	-	-	+	-	-	++	-	+	-	-
5	*Tulipa x hybrida* 5	-	-	+	-	-	-	-	+++	-	-
6	*Tulipa x hybrida* 6	-	-	-	-	+	-	-	++	-	-
7	*Tulipa x hybrida* 'Pink diamond' 12	-	-	-	-	-	+	-	-	-	-
8	*Tulipa x hybrida* 'Pink diamond' 13	-	-	-	-	-	+	-	-	-	-
9	*Tulipa x hybrida* 'Pink diamond' 14	-	-	++++	-	+	++	-	++++	-	+
10	*Tulipa x hybrida* 'Pink diamond' 15	-	-	-	-	++++	+	-	-	-	-
11	*Tulipa x hybrida* 'Pink diamond' 16	-	-	-	-	++++	+	-	-	-	+
12	*Tulipa x hybrida* 'Pink diamond' 17	-	-	++++	-	++++	+	-	++++	-	-
13	*Tulipa x hybrida* 'Pink diamond' 18	-	-	+++	-	++++	+	-	+	-	-
14	*Tulipa x hybrida* 'Pink diamond' 19	-	-	-	-	++++	+	-	+	-	+++
15	*Tulipa x hybrida* 'Pink diamond' 20	-	-	-	-	++++	+	-	+	-	-
16	*Tulipa x hybrida* 'Pink diamond' 21	-	-	-	-	++++	+	-	+	-	-
17	*Tulipa x hybrida* 'Pink diamond' 49	-	-	++++	-	++++	+	+	++++	-	++
18	*Tulipa x hybrida* 'Pink diamond' 50	-	-	?	-	+	+	-	-	-	-
19	*Tulipa x hybrida* 7	-	-	?	-	-	-	-	-	-	-
20	*Tulipa x hybrida* 'Parade' 1	-	-	?	-	-	+	-	-	-	-
21	*Tulipa x hybrida* 'Parade' 2	-	-	?	-	-	+	+	+	+	-
22	*Tulipa x hybrida* 'Parade' 3	-	-	?	-	-	-	-	-	+	-
23	*Tulipa x hybrida* 'Parade' 4	-	-	?	-	-	++	+	+	-	-
24	*Tulipa x hybrida* 'Parade' 5	-	-	?	-	-	+	-	-	-	-
25	*Tulipa x hybrida* 'Parade' 6	-	-	?	-	-	+	+	+	+	-
26	*Tulipa x hybrida* 'Parade' 7	-	-	?	-	-	-	-	-	-	-
27	*Tulipa x hybrida* 'Parade' 8	-	-	?	-	-	+	+	+	+	-
28	*Tulipa x hybrida* 'Parade' 9	-	-	-	-	-	+	-	-	-	-

*RT-PCR and ELISA results were recorded as very strong "++++", to weak "+" and negative reactions were recorded as "–", unsure results were marked as "?".

GCAATCAAGGCCAGCACAAGAATCCGGCTCTGCAAGCGAGACCCCTGCACGTG
GGAGGCCCACACCCAGTGATGCACCAAGAGACGAGCCCACCAATTACAATAATA
ACGCTGAGTCACTGTTAGAGCAACGACTAACCCGGCTGATCGAGAAGCTCAATGC
TGAAAAGCACAATTCCAATCTGCGAAATGTGGCTTTCGAGATCGGGAGGCCCTCG
CTGGAACCCACGAGTGCCATGCGAAGGAACCCTGCGAACCCCTACGGGAGATTC
TCAATTGACGAACTCTTCAAGATGAAGGTTGGCGTCGTATCTAACAACATGGCAA
CCACTGAACAAATGGCCAAGATAGCGTCAGACATCGCAGGGCTTGGGGTACCAA
CGGAGCACGTCGCATCAGTAATATTGCAAATGGTCATCATGTGTGCTTGCGTGAG
CAGTTCGGCGTTCCTTGACCCTGAGGGCAGCATTGAGTTTGAGAATGGTGCGGTG
CCCGTCGACTCCATCGCTGCGATCATGAAGAAGCACGCTGGACTGCGGAAAGTCT
GCCGGCT**CTATGCCCCTATCGTGTGGA**

Figure 3.5. Sequence of *Lily symptomless virus* PCR amplicon. Letters in bold were primer binding sites.

```
> emb|X15343.1|POLS3TER Lily symptomless virus RNA 3-terminal
region encoding 25kD, 12kD,7kD, 16kD proteins and 32kD coat protein
Length=2613, Score = 1035 bits (560), Identities = 568/572 (99%),
Gaps = 0/572 (0%)

Query  1     GCAATCAAGGCCAGCACAAGAATCCGGCTCTGCAAGCGAGACCCCTGCACGTGGGAGGCC  60
             ||||||||| |||||||||||||||||||||||||||||||||||||||||||||| |||||
Sbjct  1361  GCAATCAAGACCAGCACAAGAATCCGGCTCTGCAAGCGAGACCCCTGCACGTGGCAGGCC  1420

Query  61    CACACCCAGTGATGCACCAAGAGACGAGCCCACCAATTACAATAATAACGCTGAGTCACT  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1421  CACACCCAGTGATGCACCAAGAGACGAGCCCACCAATTACAATAATAACGCTGAGTCACT  1480

Query  121   GTTAGAGCAACGACTAACCCGGCTGATCGAGAAGCTCAATGCTGAAAAGCACAATTCCAA  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1481  GTTAGAGCAACGACTAACCCGGCTGATCGAGAAGCTCAATGCTGAAAAGCACAATTCCAA  1540

Query  181   TCTGCGAAATGTGGCTTTCGAGATCGGGAGGCCCTCGCTGGAACCCACGAGTGCCATGCG  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1541  TCTGCGAAATGTGGCTTTCGAGATCGGGAGGCCCTCGCTGGAACCCACGAGTGCCATGCG  1600

Query  241   AAGGAACCCTGCGAACCCCTACGGGAGATTCTCAATTGACGAACTCTTCAAGATGAAGGT  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1601  AAGGAACCCTGCGAACCCCTACGGGAGATTCTCAATTGACGAACTCTTCAAGATGAAGGT  1660

Query  301   TGGCGTCGTATCTAACAACATGGCAACCACTGAACAAATGGCCAAGATAGCGTCAGACAT  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1661  TGGCGTCGTATCTAACAACATGGCAACCACTGAACAAATGGCCAAGATAGCGTCAGACAT  1720

Query  361   CGCAGGGCTTGGGGTACCAACGGAGCACGTCGCATCAGTAATATTGCAAATGGTCATCAT  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1721  CGCAGGGCTTGGGGTACCAACGGAGCACGTCGCATCAGTAATATTGCAAATGGTCATCAT  1780

Query  421   GTGTGCTTGCGTGAGCAGTTCGGCGTTCCTTGACCCTGAGGGCAGCATTGAGTTTGAGAA  480
             ||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1781  GTGCGCTTGCGTGAGCAGTTCGGCGTTCCTTGACCCTGAGGGCAGCATTGAGTTTGAGAA  1840

Query  481   TGGTGCGGTGCCCGTCGACTCCATCGCTGCGATCATGAAGAAGCACGCTGGACTGCGGAA  540
             ||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1841  TGGAGCGGTGCCCGTCGACTCCATCGCTGCGATCATGAAGAAGCACGCTGGACTGCGGAA  1900

Query  541   AGTCTGCCGGCTCTATGCCCCTATCGTGTGGA  572
             ||||||||||||||||||||||||||||||||
Sbjct  1901  AGTCTGCCGGCTCTATGCCCCTATCGTGTGGA  1932
```

Figure 3.6. BLAST result of the sequence of *Lily symptomless virus* PCR amplicon.

3.2.4 Optimization of different primer combination for Real-time RT-PCR

Positive control RNA samples of LSV and TVX used were from *Tulipa* plants and were confirmed by sequencing in section 3.2.2 and 3.2.4. The positive samples for ArMV, CMV and TRV were obtained from infected *Chenopodium quinoa, Viola* and *Paeonia*, respectively which were collected in New Zealand and were confirmed by sequencing by Landcare Research, Tamaki, Auckland (data now shown).
Four different primer combinations: (1) both forward and reverse primers with flaps; (2) both forward and reverse primers without flaps; (3) forward primer with flap and reverse primer without flap; (4) reverse primer with flap and forward primer without flap, were investigated for obtaining the best amplification by real-time RT-PCR using ArMV, LSV, TRV and TVX.

For each primer combination, three different dilutions of each RNA sample (1:10, 1:100 and 1:1000) and a water negative control were included. Results showed that the best combination for all the four investigated viruses was both forward and reverse primers without flaps (Fig. 3.7). Consequently, CMV primers were also used without flap.

The real-time RT-PCR products for ArMV, CMV, LSV, TRV and TVX were purified and sequenced. The sequence results confirmed that all the primers and probes for ArMV, CMV, LSV and TVX bound to the correct sites while TRV primers had perfect-match but probes had variable sequences (Table 3.7).

In order to know if real-time RT-PCR for ArMV and TRV would work from *Tulipa* samples, artificial reactions were conducted. The serially diluted positive control RNA of ArMV and TRV were tested with and without the addition of negative control *Tulipa* RNA. Results showed that similar amplification efficiency were obtained from with and without the addition of negative control *Tulipa* RNA (Fig. 3.8).

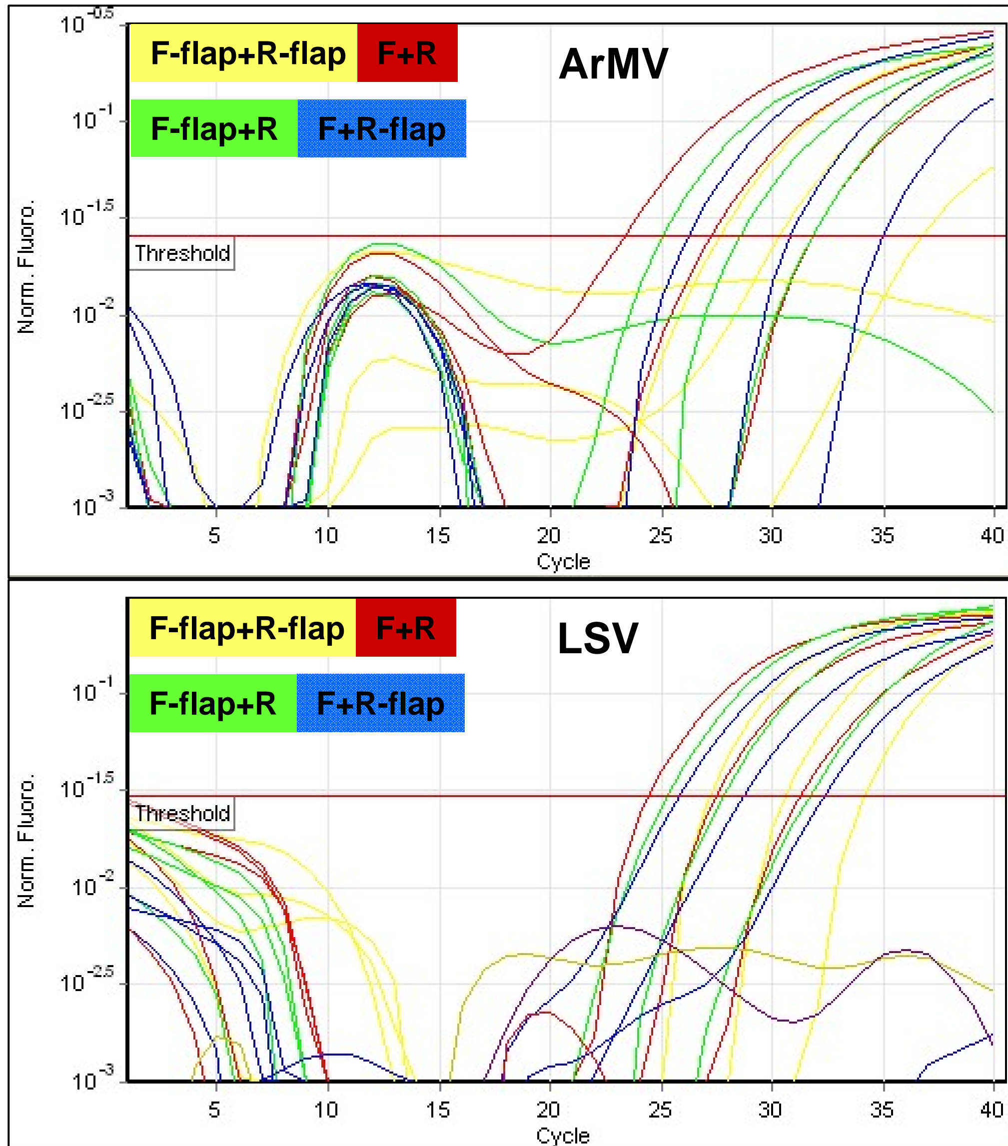


Figure 3.7. Optimization of primer combinations in real-time PCR for *Arabis mosaic virus* (ArMV), *Lily symptomless virus* (LSV), *Tobacco rattle virus* (TRV) and *Tulip virus X* (TVX). F=forward primer, R=reverse primer. Amplification curves in yellow, red, green and blue colour represent four different primer combinations of F-flap + R-flap, F + R, F-flap + R and F + R-flap, respectively. Each combination has four lines representing three different dilutions (1:10, 1:100 and 1:1000) plus a water negative control.

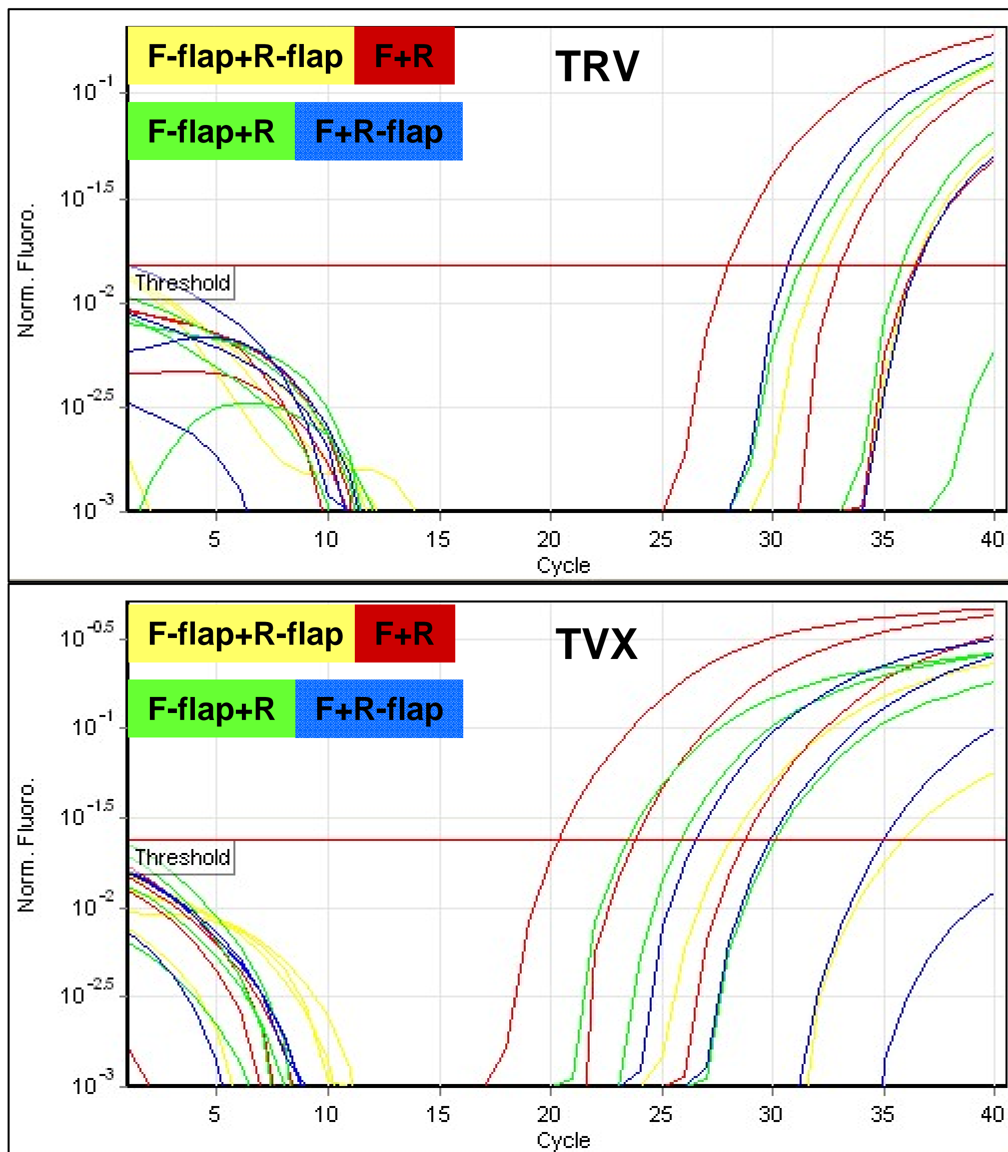


Figure 3.7 (continued). Optimization of primer combinations in real-time PCR for *Arabis mosaic virus* (ArMV), *Lily symptomless virus* (LSV), *Tobacco rattle virus* (TRV) and *Tulip virus X* (TVX). F=forward primer, R=reverse primer. Amplification curves in yellow, red, green and blue colour represent four different primer combinations of F-flap + R-flap, F + R, F-flap + R and F + R-flap, respectively. Each combination has four lines representing three different dilutions (1:10, 1:100 and 1:1000) plus a water negative control.

Table 3.7. Sequence results for real-time RT-PCR products of *Arabis mosaic virus* (ArMV), *Cucumber mosaic virus* (CMV), *Lily symptomless virus* (LSV), *Tobacco rattle virus* (TRV) and *Tulip virus X* (TVX).

Virus	Real-time PCR sequences*	Similarity
ArMV	**TAGCCCTTGGAGACAATCCT**TTTGCCCACATGAT AGCTTGTCATGGATTACATCATGGAATTCTTGATCT TAA**GTTAATGTGGGATTTGGAGG**	97% identity to German ArMV isolate (EU617327)
CMV	**CCACACGGTAGAATCA**G**ATTT**CGGCAAAGGATTAATT CTGATCTGAATGCGCGAAACAAGCTTCTTATCATAGTCC GTGACTGAATCTGGCAAAGA**CAACCTTTACCGTAATAAGACC**	95% identity to French CMV isolate (Y18138)
LSV	**GGCAGCATTGAGTTGAGA**ATGGT GCGGTGCCCGTCGACTGCATCGCTGCGAT CATGAA**GAAGCACGCTGGACTGC**	96% identity to Indian LSV isolate (AM422452)
TRV	**CATGCTAACAAATTGCGAAAGC**AAGTTGCTGACA TGGTCGGTGTCACACGTAGGTGT GCGG**AAAATAATTGTGGATGGTTTGTCTGTA**	98% identity to American TRV isolate (EU569291)
TVX	**CCAGATACATGCGGAGGTAC**AGGGATGCCCTCGGCCCC AACAACATCATGATTAACTGTGAGAGGACTCCCGCGGACC TGAGTCGCTGGGTTCGCAA**CCATTGGGACTTCACCAAGC**	100% identity to New Zealand TVX isolate (EU555190)

*Sequences in bold are primer binding sites and in red are probe binding sites.

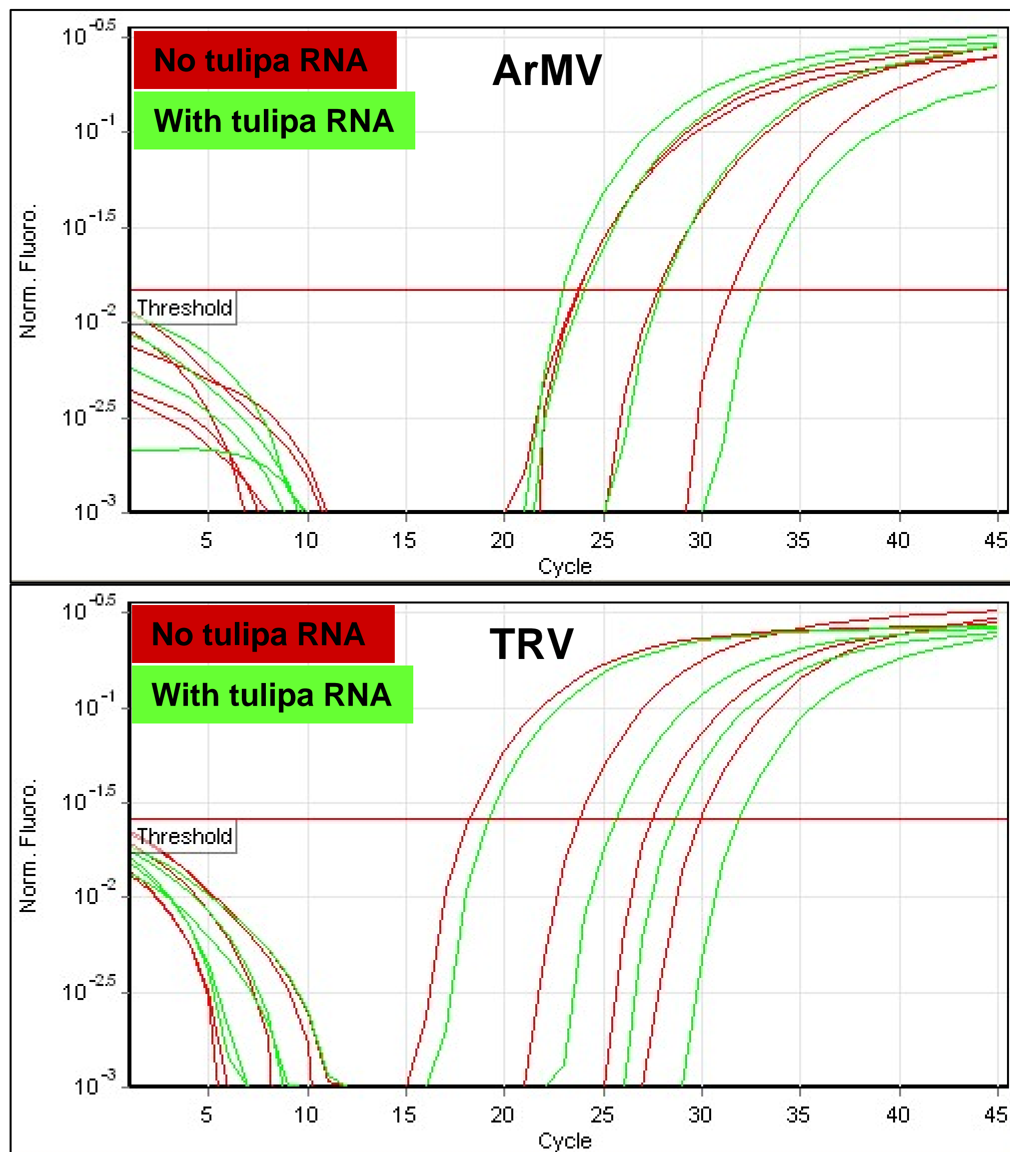


Figure 3.8. Comparison of the amplification efficiency of *Arabis mosaic virus* (ArMV), and *Tobacco rattle virus* (TRV) infecting non-*Tulipa* hosts with/without the addition of negative control *Tulipa* RNA. Amplification curves in red and green colour represent reaction mixture without and with the addition of negative *Tulipa* RNA respectively. Four different dilutions (1:10, 1:100, 1:1000 and 1:10000) plus a water negative control were used in each reaction.

3.2.5 Testing of Tulipa samples using the developed Real-time RT-PCR

The best primer combinations of forward and reverse primers without flap for each virus was used to test all the 28 RNA samples from Tulipa leaves by real-time RT-PCR. Results showed that all the samples were negative for ArMV, CMV and TRV which are consistent with the results obtained by conventional RT-PCR (Table 3.8). Higher amplification efficiency by real-time RT-PCR was observed for LSV and TVX when compared with conventional RT-PCR results (Table 3.8).

Table 3.8. Real-time one step RT-PCR results for the 28 *Tulipa* RNA samples tested for *Arabis mosaic virus* (ArMV), *Cucumber mosaic virus* (CMV), *Lily symptomless virus* (LSV), *Tobacco rattle virus* (TRV) and *Tulip virus X* (TVX).

		Real-time-PCR results					RT-PCR results				
No.	Plant	ArMV	CMV	LSV	TRV	TVX	ArMV	CMV	LSV	TRV	TVX
1	*Tulipa x hybrida* 1	-	-	++++	-	+	-	-	++++	-	-
2	*Tulipa x hybrida* 2	-	-	++++	-	-	-	-	++++	-	-
3	*Tulipa x hybrida* 3	-	-	++++	-	-	-	-	++++	-	-
4	*Tulipa x hybrida* 4	-	-	++++	-	-	-	-	+	-	-
5	*Tulipa x hybrida* 5	-	-	++++	-	-	-	-	+	-	-
6	*Tulipa x hybrida* 6	-	-	++++	-	+	-	-	-	-	+
7	*Tulipa x hybrida* 'Pink diamond' 12	-	-	-	-	-	-	-	-	-	-
8	*Tulipa x hybrida* 'Pink diamond' 13	-	-	-	-	-	-	-	-	-	-
9	*Tulipa x hybrida* 'Pink diamond' 14	-	-	++++	-	+	-	-	++++	-	+
10	*Tulipa x hybrida* 'Pink diamond' 15	-	-	-	-	++++	-	-	-	-	++++
11	*Tulipa x hybrida* 'Pink diamond' 16	-	-	-	-	+++	-	-	-	-	++++
12	*Tulipa x hybrida* 'Pink diamond' 17	-	-	++++	-	+++	-	-	++++	-	++++
13	*Tulipa x hybrida* 'Pink diamond' 18	-	-	++++	-	++++	-	-	+++	-	++++
14	*Tulipa x hybrida* 'Pink diamond' 19	-	-	-	-	+++	-	-	-	-	++++
15	*Tulipa x hybrida* 'Pink diamond' 20	-	-	-	-	+++	-	-	-	-	++++
16	*Tulipa x hybrida* 'Pink diamond' 21	-	-	+	-	++++	-	-	-	-	++++
17	*Tulipa x hybrida* 'Pink diamond' 49	-	-	++++	-	++++	-	-	++++	-	++++
18	*Tulipa x hybrida* 'Pink diamond' 50	-	-	-	-	+++	-	-	?	-	+
19	*Tulipa x hybrida* 7	-	-	-	-	-	-	-	?	-	-
20	*Tulipa x hybrida* 'Parade' 1	-	-	-	-	-	-	-	?	-	-
21	*Tulipa x hybrida* 'Parade' 2	-	-	-	-	-	-	-	?	-	-
22	*Tulipa x hybrida* 'Parade' 3	-	-	-	-	-	-	-	?	-	-
23	*Tulipa x hybrida* 'Parade' 4	-	-	-	-	-	-	-	?	-	-
24	*Tulipa x hybrida* 'Parade' 5	-	-	-	-	-	-	-	?	-	-
25	*Tulipa x hybrida* 'Parade' 6	-	-	-	-	-	-	-	?	-	-
26	*Tulipa x hybrida* 'Parade' 7	-	-	-	-	-	-	-	?	-	-
27	*Tulipa x hybrida* 'Parade' 8	-	-	-	-	-	-	-	?	-	-
28	*Tulipa x hybrida* 'Parade' 9	-	-	-	-	-	-	-	-	-	-

*RT-PCR results were recorded as very strong "++++", to weak "+" and negative reactions were recorded as "–", unsure results were marked as "?"

3.3. CONCLUSION

Taqman real-time one step RT-PCR assays for ArMV, CMV, LSV and TVX were successfully developed for the detection of these viruses from RNA extracted from Tulipa leaf samples. The published TRV Taqman real-time RT-PCR assay also worked well for the detection of Tulipa leaf samples.

It was reported that real-time PCR primers with the addition of an extra 12-AT rich primer flap at the 5'-end increase significantly the amplification efficiency (Afonina et al., 2007). However, no advantages of adding the flap to ArMV, LSV, TRV and TVX primers have been found in this study.

Twenty-eight Tulipa samples were selected for a comparison study between ELISA, conventional one step RT-PCR and real-time one step RT-PCR tests. The results showed that generally the same positive and negative samples were detected by real-time PCR and conventional RT-PCR but with higher sensitivity. ELISA provided lower sensitivity and specificity when compared with both real-time and conventional RT-PCR; false positive results were found for ArMV, CMV and TRV, false negative results were noted for TVX and variable results were obtained with LSV.

ELISA has been widely used in plant virology because the test is inexpensive and is easy to perform on large number of samples. However, the detection of viruses from dormant bulbs has been shown not be always reliable. For a reliable ELISA detection, dormancy of tubers may need to be broken (e.g. Potato virus Y and Potato virus A in potato tubers; Vetten et al., 1983) or dormant tubers may need to be stored at a specific temperature for up to three weeks prior to the testing (e.g. Iris severe mosaic virus in iris bulbs [ISMV; van der Vlugt, 1993]; Lily mottle virus (LMoV) in lily bulbs [Derks et al., 1997]; Tomato spotted wilt virus (TWSV) in dahlia bulbs [Schadewijk, 1996]).

4. Assessment of the reliability of the developed real-time RT-PCR on bulb samples

4.1. MATERIALS AND METHODS

4.1.1 Virus

Among the five targeted viruses, TVX was selected for verifying the specificity and sensitivity of the developed real-time RT-PCR method based on the results from previous study of which variable positive results were obtained between conventional and real-time RT-PCR (Table 3.8).

4.1.2 Bulb samples

A total of 24 *Tulipa x hybrida* bulbs were selected from the results obtained in section 3 where variable results between ELISA, conventional and real-time RT-PCR methods were obtained from the leaf samples (Tables 3.6 and 3.8). Total RNA was extracted from bulbs using the Qiagen RNeasy plant mini kit following the manufacturer's instructions.

4.1.3 Evaluation of the sensitivity and specificity of the real-time RT-PCR method

The specificity and sensitivity of the developed real-time RT-PCR for TVX was first evaluated by comparison with the conventional RT-PCR method on the selected 24 bulb samples.

Based on these results, a total of four bulbs with different positive strengths were selected for further evaluation of the sensitivity by serial dilution of the bulb saps / RNAs in ELISA, conventional RT-PCR and real-time RT-PCR.

TVX ELISA (SEDIAG, Longvic, France) was conducted following the manufacturer's instruction. Conventional RT-PCR for TVX was carried out as described in Table 2.2 and 3.3. The TVX Real-time RT-PCR was performed as described in sections 3.1.4 and 3.1.5.

4.2. RESULTS

4.2.1 Comparison of the conventional and real-time RT-PCR

Conventional and real-time RT-PCR results from the 24 bulb samples showed that in conventional RT-PCR, only four positive samples were found (Fig. 4.1, samples numbers 1, 3, 9 and 15) while six positive samples were found by real-time RT-PCR (Fig. 4.2, samples numbers 1, 2, 3, 9, 15 and 16). These results suggest that real-time RT-PCR showed higher sensitivity. Meanwhile, non-specific amplifications were observed from the conventional RT-PCR (Fig. 4.1).

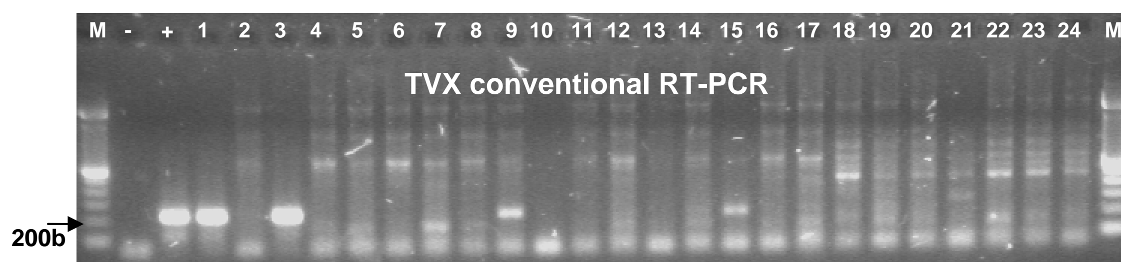


Fig. 4.1. Results of the conventional RT-PCR for the detection of *Tulip virus X* (TVX) on 24 bulb samples listed in Table 4.1.

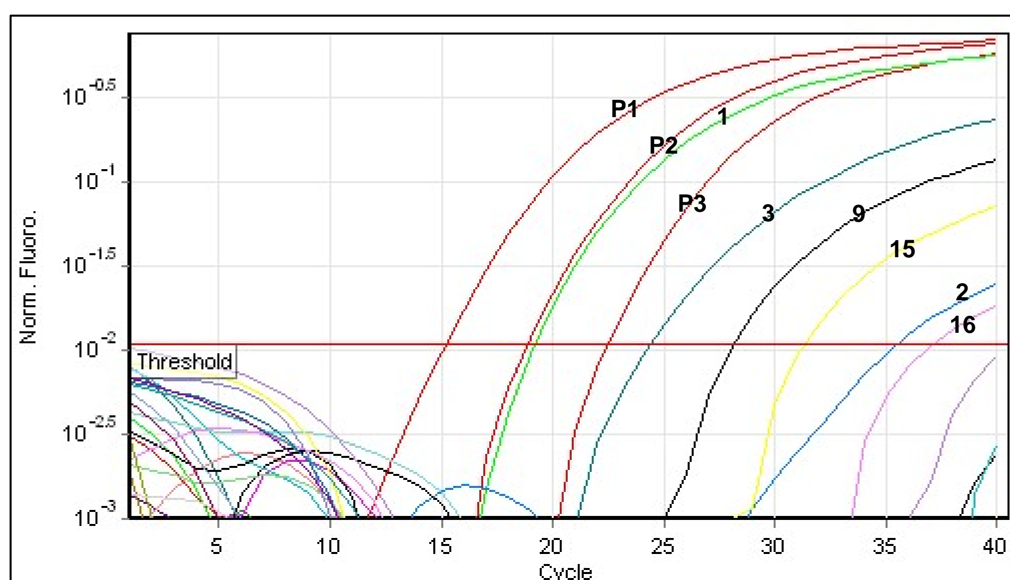


Fig. 4.2. Results of the real-time RT-PCR for the detection of *Tulip virus X* (TVX) on 24 bulb samples listed in Table 4.1. P1-3 represents a 10-fold serial dilution of the positive control. Samples with fluorescent signal above the threshold were positive samples. Numbers correspond to the bulb samples listed in Table 4.1.

4.2.2 4.2.2. Sensitivity of the real-time PCR

Four bulb samples: *Tulipa x hybrida* 1, *Tulipa x hybrida* 2, *Tulipa x hybrida* 'Pink diamond' 20 and *Tulipa x hybrida* 'Pink diamond' 49 (Table 4.1) with different positive strength based on the TVX conventional and real-time RT-PCR results (Fig. 4.1 and 4.2) were selected to further evaluate the sensitivity of developed TVX real-time RT-PCR.

The sensitivity for the detection of TVX from *Tulip x hybrida* bulbs by ELISA, conventional and real-time RT-PCR was carried out using a 10-fold dilution. Results showed that the four selected samples tested negative by ELISA (Table 4). Two samples gave positive results with different dilution rate (up to 1:1,000) by both conventional and real-time RT-PCR (Table 4.1, Fig. 4.3 and 4.4). A third sample was also detected by real-time PCR only with a dilution rate of up to 1:100 (Table 4.1, Fig. 4.4). Meanwhile conventional RT-PCR showed weak non-specific amplification (Fig. 4.3).

The sensitivity of the detection of TVX real-time PCR using leaves or bulbs as starting material was also assessed and compared with ELISA and conventional RT-PCR. Bulb

samples were not tested by ELISA because the ELISA already shows very low sensitivity using leaves when compared to RT-PCR (Table 4.2). Six and 11 samples from bulbs and leaves respectively tested positive for TVX by real-time PCR while 4 and 10 samples from bulbs and leaves respectively tested positive for TVX by conventional PCR. More samples tested TVX positive when using leaves rather than bulbs for either the conventional or real-time RT-PCR.

Table 4.1. Serial dilution results for the detection of *Tulip virus X* from *Tulip x hybrida* bulbs by ELISA, conventional and real-time RT-PCR.

Sample	No.	Dilution	Results*		
			ELISA	Conventional RT-PCR	Real-time RT-PCR
Tulipa x hybrida 1	1	Original	-	+	+
	2	1:10	-	+	+
	3	1:100	-	+	+
	4	1:1000	-	+	+
	5	1:1,000	-	+	+
	6	1:10,000	-	-	-
Tulipa x hybrida 2	7	Original	-	-	+
	8	1:10	-	-	+
	9	1:100	-	-	+
	10	1:1000	-	-	-
	11	1:1,000	-	-	-
	12	1:10,000	-	-	-
Tulipa x hybrida pink diamond 20	13	Original	-	+	+
	14	1:10	-	+	-
	15	1:100	-	-	-
	16	1:1000	-	-	-
	17	1:1,000	-	-	-
	18	1:10,000	-	-	-
Tulipa x hybrida pink diamond 49	19	Original	-	-	-
	20	1:10	-	-	-
	21	1:100	-	-	-
	22	1:1000	-	-	-
	23	1:1,000	-	-	-
	24	1:10,000	-	-	-

* Results were recorded as detected "+" or not detected "-"

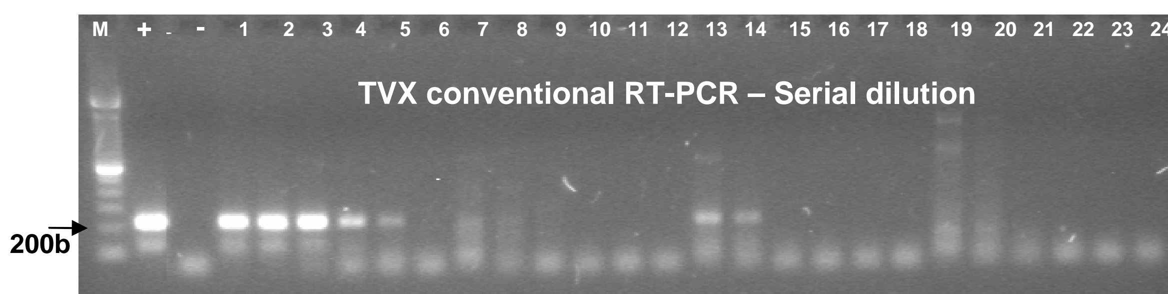


Fig. 4.3. Serial dilution results of the Tulip virus X (TVX) conventional RT-PCR on four Tulipa x hybrida bulb samples. Lanes 1-6, 7-12, 13-18 and 19-24 correspond to samples 1, 3, 15 and 16 respectively listed in Table 5.1 with 10-fold serial dilution.

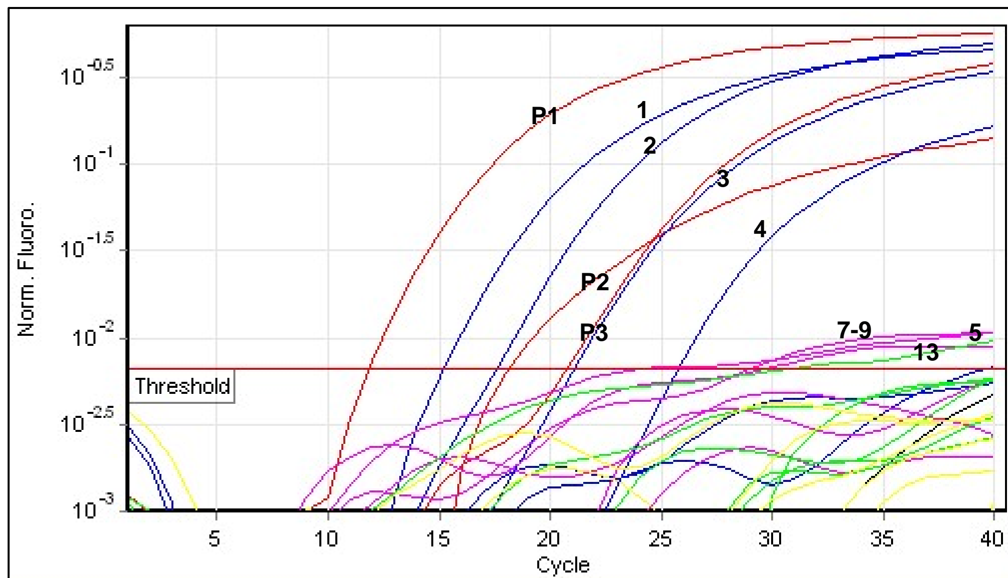


Fig. 4.4. Serial dilution results of the *Tulip virus X* (TVX) real-time RT-PCR on four *Tulipa x hybrida* bulb samples. P1-P3 represents a 10-fold serial dilution of the TVX positive control. Lanes 1-6, 7-12, 13-18 and 19-24 correspond to samples 1, 2, 15 and 16 respectively listed in Table 5.1 with a 10-fold serial dilution.

Table 4.2. Detection of *Tulip virus X* (TVX) by ELISA, conventional and real-time one step RT-PCR using either leaves or bulbs as starting materials of 24 *Tulipa x hybrida* samples.

No	Sample	Bulb			Leave		
		Conventional RT-PCR*	Real-time RT-PCR*	ELISA*	Conventional RT-PCR*	Real-time RT-PCR*	ELISA*
1	*Tulipa x hybrida* 1	+++	++++	NT	-	+	-
2	*Tulipa x hybrida* 2	-	+	NT	-	-	-
3	*Tulipa x hybrida* 3	+++	+++	NT	-	-	-
4	*Tulipa x hybrida* 4	-	-	NT	-	-	-
5	*Tulipa x hybrida* 5	-	-	NT	-	-	-
6	*Tulipa x hybrida* 6	-	-	NT	+	+	-
7	*Tulipa x hybrida* 'Pink diamond' 12	-	-	NT	-	-	-
8	*Tulipa x hybrida* 'Pink diamond' 13	-	-	NT	-	-	-
9	*Tulipa x hybrida* 'Pink diamond' 14	+	++	NT	+	+	+
10	*Tulipa x hybrida* 'Pink diamond' 15	-	-	NT	++++	++++	-
11	*Tulipa x hybrida* 'Pink diamond' 16	-	-	NT	++++	++++	+
12	*Tulipa x hybrida* 'Pink diamond' 17	-	-	NT	++++	++++	-
13	*Tulipa x hybrida* 'Pink diamond' 18	-	-	NT	++++	++++	+++
14	*Tulipa x hybrida* 'Pink diamond' 19	-	-	NT	++++	++++	-
15	*Tulipa x hybrida* 'Pink diamond' 20	+	+	NT	++++	++++	-
16	*Tulipa x hybrida* 'Pink diamond' 21	-	+	NT	++++	++++	++
17	*Tulipa x hybrida* 'Pink diamond' 49	-	-	NT	+	+++	-
18	*Tulipa x hybrida* 'Pink diamond' 50	-	-	NT	-	-	-
19	*Tulipa x hybrida* 7	-	-	NT	-	-	-
20	*Tulipa x hybrida* 'Parade' 1	-	-	NT	-	-	-
21	*Tulipa x hybrida* 'Parade' 2	-	-	NT	-	-	-
22	*Tulipa x hybrida* 'Parade' 3	-	-	NT	-	-	-
23	*Tulipa x hybrida* 'Parade' 4	-	-	NT	-	-	-
24	*Tulipa x hybrida* 'Parade' 5	-	-	NT	-	-	-

*RT-PCR and ELISA results were recorded as very strong "++++", to weak "+" and negative reactions were recorded as "–". NT: Not tested.

4.3. CONCLUSION

The real-time RT-PCR developed for the detection of TVX in this study can detect virus from bulb samples with good specificity and sensitivity. It is anticipated that similar results would be obtained for the other real-time RT-PCRs which have been developed.

When assessing the detection of TVX in leaves, ELISA had the lowest sensitivity compared with both conventional RT-PCR and real-time RT-PCR. The sensitivity of conventional and real-time RT-PCR for TVX were similar but the conventional RT-PCR had less specificity and amplified a number of non-specific products giving rise to false negative results.

Both conventional and real-time RT-PCR were able to detect TVX in dormant bulbs. However, the efficacy of virus detection was less in bulbs than leaves with conventional RT-PCR detecting the virus in 4 and 10 samples respectively, and real-time RT-PCR detecting the virus in 6 and 11 samples respectively. This implies that approximately half the number of infected plants would be detected if they were tested as dormant bulbs rather than as growing plants.

The conclusion that detection of TVX in dormant bulbs whatever the method used is less reliable than detection in growing plants suggests that it may be more appropriate to test growing plants in situations where a high degree of confidence of detection is required. Previous research has drawn similar conclusions. Sharma *et al*. (2005) reported that in lily, five viruses (CMV, LMoV, LSV, SLRSV and TBV) were detected in the inner scales but not the outer scales of the bulbs of 4 out of 16 plants and in 10 plants, viruses were detected in the leaves but not the bulbs. Van der Vlugt *et al*. (1993) reported that when harvested ISMV could only be detected in the basal plate of gladiolus bulbs and not the scales. In dormant tulip bulbs, van der Vlugt *et al.* (1988) reported that there was no evidence of a regular pattern of TRV distribution and that the virus could be reliably detected in the leaves by ELISA but not in the dormant bulb. It is likely that the problems encountered with reliably detecting viruses in dormant bulbs are related to both virus concentration and uneven distribution in the bulb. Further work on the sampling strategy from dormant bulbs for a reliable detection of viruses would be required. One aspect could be to test over a period of time as virus titre may differ during storage. Another aspect could be to try multiple sampling sites on a bulb as often the virus distribution is uneven in dormant bulb.

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6. Appendices

Appendix 1, Primer and probe sequences from previously developed real time RT-PCR protocols.

Target virus	Primer name	Sequence 5' – 3'	Reference
Tobacco rattle virus	TRV-1466F TRV-1553R TRV-1489	ATGCTAACAAATTGCGAAAGC TACAGACAAACCATCCACAATTATTTT ACGTGTGACACCAACCATGTCAGCAACT(probe)	Mumford *et al.*, 2000
Iris yellow spot virus	DQ658242-524F DQ658242-524R DQ658242-552T	TCCAGAATGTGAAAAAAGAAGCACT TGCCATGACTCTTGCAACTTTG TATAAGCAGATTCTCAA CTTA (probe)	Mumford *et al.*, 2008
` *Iris yellow spot virus*	IYSV-465c IYSV-239f	CAGCAAAGTGAGAGGACCACC TGAGCCCCAATCAAGACG	Pappu *et al.*, 2008

Appendix 2. Gibbs & Gibbs extraction protocol

GCP buffer:
200 mM guanidine thiocyanate
1% polyvinylpyrolidone (PVP);
2% (w/v) CTAB (cetyl trimethyl ammonium bromide)
1.4 M NaCl
0.1 M Tris-HCl pH 7.0
Boil, and snap cool, to disperse the PVP
Add β-mercaptoethanol to 1.0% before use.

- Grind 50-100 mg of frozen tissue in 500 µl of GCP buffer.
- Stir vigorously (vortex), centrifuge at maximum speed for 2 mins, immediately collect the fluid phase from between the sedimented and floating solids.
- Add an equal volume of GCP buffer and 100 µl chloroform: isoamyl alcohol (24:1) or 100 µl of phenol: chloroform (50:50).
- Stir (Vortex) to emulsify, centrifuge at maximum speed for 2 mins, and collect the fluid phase.
- Add 1/10th volume of 7.5 M NH_4OAc and 1 volume of isopropanol. Mix well and put in freezer for 5-10 mins to precipitate nucleic acids.
- Centrifuge in a Microfuge at maximum speed for 5-10 mins.
- Pour supernatant from nucleic acid pellet with care – the pellet may detach.
- Put 1 ml of 70% ethanol on the pellet and centrifuge again for 1 min (N.B. nucleic acids can be safely left in 70% EtOH at -20°C. They can also be stored for several years in 95-100% EtOH).
- Pour off the most of the ethanol, centrifuge and carefully remove the remaining ethanol.
- Air-dry the nucleic acid pellet at room temperature and re-suspend in sterile nuclease free water.

Appendix 3. Hot phenol extraction method (Vunsh *et al.*, 1991)

Plant material was quick-frozen in liquid nitrogen and homogenized in a pre-cooled Waring blender. Two (v/w) volumes of an 85°C phenol buffer solution (50% phenol, 50 mM LiCl, 0.5% SDS, 5 mM EDTA, and 50 mM Tris) were added and mixed before addition of a further volume of chloroform. After 30 min shaking at 250 r.p.m. the water phase was again chloroform extracted. The RNA was pelleted in 2 M LiCl and washed twice with 2 M LiCl and 70% alcohol. The RNA was dissolved in a buffer containing 25% formamide, 0.7 M NaCl, 10 mM HEPES, 10 mM EDTA and 0.1% SDS, pH 7.4, heated for 5 minutes at 65°C, and applied three times on a poly(U)-Sepharose column (Pharmacia).The column was extensively washed with the same buffer containing 50% formamide and 0.5 M NaCl, and the poly(A) RNA was eluted in 95 % formamide, 10 mM HEPES, 5 mM EDTA and 0.1% SDS, pH 7.4. The RNA was ethanol precipitated after addition of 0.25 volumes of 10 M NH_4Ac, washed, and dissolved in sterile H_20.

Appendix 4. ssRNA extraction methods (Miglino *et al.*, 2006)

Manual ssRNA extraction (magnetic capture). Plant tissues, 100 mg, were ground in a mortar in the presence of 500 µl of a lysis buffer containing guanidinium isothiocianate as chaotropic salt, and centrifuged at 4000 x g for 30 s afterwards. The supernatant was transferred to a microtiterplate and 10 µl of streptavidin-labelled magnetic beads (Bilatec GmbH, Germany) and 3 µl of 0.1µmol solution of biotin-labelled Potex 4 (Eurogentec) added. Samples were incubated for 10 min at 4ºC, the microtiter plate placed on a magnetic separator, the magnetic beads sedimented and washed several times with a washing buffer according to the manufacturer's protocol. The magnetic bead pellet was suspended in 10µl water and 1µl was used in RT-PCR.

KingFisher Automated ssRNA Extraction (magnetic capture). Plant tissues, 100 mg, were ground in a mortar in the presence of 500 µl of a lysis buffer containing guanidinium isothiocianate as chaotropic salt and centrifuged at 4000 x g for 30 s afterwards. The supernatant was transferred to a KingFisher microplate and 10 µl of streptavidin-labelled magnetic beads (Thermo Lab systems) and 3 µl of 0.1µmol solution of biotin-labelled Potex 4 capturing probe (Eurogentec) added. Samples were placed on a KingFisher extraction processor and the ssRNA purified according to the manufacturer's protocol. The magnetic beads were suspended in 10µl water and 1µl used in an RT-PCR assay.