Short Communication

DETECTION OF VIRUSES IN APPLES AND PEARS BY REAL TIME RT-PCR USING 5'-HYDROLYSIS PROBES

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SUMMARY

Apple stem pitting virus (ASPV), *Apple stem grooving virus* (ASGV), *Apple chlorotic leaf spot virus* (ACLSV) and *Apple mosaic virus* (ApMV) are common in apples and pears and the main targets of virus elimination from propagation material. The objective of this work was to design primers and probes for a real time RT-PCR protocol for detection of the four above viruses. FAM/TAMRA-labeled probes and primers were designed by searching for highly conserved nucleotide regions in the coat protein gene of the four viruses. Infection levels in analyzed apple samples were 92.6, 96.4, 100 and 88% for ASGV, ASPV, ACLSV and ApMV, respectively. In pears, all pre-existing ASPV infections were detected. Viral infections were confirmed in a selection of commercial cultivars of apples and pear scions, and quince rootstocks, demonstrating the sensitivity and reliability of the designed primers and probes. Real time RT-PCR using 5'-labeled probes is suitable for checking sanitary quality as a routine test in certification programs.

Key words: M. domestica, Pyrus spp., *Cydonia* spp., virus diagnosis.

Virus infections are common in apples and pears grown in Brazil, to levels that may compromise the sanitary status of nursery production. Viruses with economic relevance most frequently found in apples (*Malus domestica*) and pears (*Pyrus* spp.) are *Apple stem grooving virus* (ASGV; *Capillovirus*), *Apple chlorotic leaf spot virus* (ACLSV; *Trichovirus*), and *Apple stem pitting virus* (ASPV; *Foveavirus*) all members of the family *Betaflexiviridae* (Adams *et al*., 2013; Al Rwahnih *et al*., 2004; Jelkmann, 1994; Magome *et al*., 1997; Nickel *et al*., 1999, 2001). These viruses have filamentous, flexuous particles and ssRNA genomes. Isolates of ACLSV type strain, which are widespread in pome and stone fruits, cause phloem necrosis at the graft union and decline of most commercial cultivars grafted on Maruba-kaido (*Malus prunifolia* cv. Ringo) and certain clones of *M. sieboldii*, while ACLSV "Maruba-type" isolates are latent in these species (Yanase *et al*., 1990). Similar symptoms such as phloem necrosis and decline have been observed in cvs Fuji and Gala grafted on Maruba-kaido in Southern Brazil, infected by ASGV (Nickel *et al*., 1999). ASPV is associated with several graft-transmissible disorders of economic relevance such as pear vein yellows, pear stony pit, sooty ringspot and quince fruit deformations (Paunovic and Rankovic, 1998; Paunovic *et al*., 1999) as well as with green crinkle and star crack of apples (Desvignes *et al*., 1999; James *et al*., 2013). A further common virus in apples and other rosaceous species is *Apple mosaic virus* (ApMV), (*Bromoviridae*, genus *Ilarvirus*), a cosmopolitan pathogen with labile quasi-isometric particles, and a tripartite ssRNA genome (Shiel *et al*., 1995).

Tools for the diagnosis of these viruses include biological, serological and molecular assays (Menzel *et al*., 2002). Biological tests are becoming gradually less popular, since they are time-consuming, work-intensive, susceptible to environmental factors, and are often difficult to interpret or inconclusive. Immunoenzymatic assays, display detection efficiency restricted to seasonal windows and low sensitivity due to low virus titer in woody tissues. Moreover, good antisera to certain viruses are often unavailable. Assays such as several variants of RT-PCR, including immunocapture RT-PCR (Candresse *et al*., 1995), the coupling of the reverse transcription to the PCR reaction in a one-step reaction, and non-isotopic riboprobes for specific applications, are currently the most widely used molecular diagnostic instruments (Lopez *et al*., 2003). Development of multiplex RT-PCR protocols has allowed the simultaneous detection of several viruses, reducing the cost and time of testing (Menzel *et al*., 2002; Hassan *et al*., 2006). Use of IC-RT-PCR (Candresse *et al*., 1995) and IC-RT-nested-PCR (Lunello *et al*., 2005) increase the sensitivity of RT-PCR, but the latter requires two sequencial thermocycling runs. The use of crude extracts from *in vitro*-grown plants represents a significant improvement of RT-PCR as a routine test (Kummert *et al*., 2000). Despite of these advancements, conventional RT-PCR main drawbacks remain its low sample processing capability and the laborious post-amplification processing by gel electrophoresis that favors sample contamination by carry over and makes it unpractical for large scale applications.

The search for alternatives has increased the use of virus diagnostic applications based on real time RT-PCR using labeled probes with different fluorescent detection

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Table 1. Sequences, genome positions of primers and probes and size of amplicons.

Primer/probe names*	Primer and probe sequences 5'-3'	Genome position	Amplicon size	GenBank access No.
SGp220	AAGACCCAGTTTCCCGCTGTTGG	220 – 242	69 bp	AF438409
SGs197	TTGCTGTTTTCGGGTCATCT	197 – 216		
SGas266	TAATGAACCGGAGGGGTATCA	246 – 266		
SPp662	AGGTGGGAGTTTATCTGGCTAGGCAT	662 – 687	86 bp	AY572458
SPs635	TGGGAATCCCTGAGCATCAACTCA	635 – 658		
SPas721	ATGTAGACTTGTCTGAGGCGCCAA	698 – 721		
CLp80	ATGGAAGGACAGGGGCAATCCTGG	80 – 103		
CLs51	GTTCCTGGCCGCAGAAGGCAGACCCCT	51 – 77	86 bp	EF138602
CLas137	GCTATGTTCGCGAAGATGGACTCC	114 – 137		
MVp201	CCCGAATGTTGAGCCGAAAATTC	201 – 223		
MVs174	GAGGAGGACAGCTTGGGAGGTTAG	174 – 197	89 bp	GQ131805
MVas263	GCAGTCACTTCTCGACTGCTCAGG	240 – 263		

* SP, ASPV; SG, ASGV; CL, ACLSV; MV, ApMV; s, sense primer; as, antisense primer; p, probe.

chemistries (James *et al*., 2006), which combines the specificity of PCR amplification with DNA hybridization. Real time RT-PCR using fluorogenic 5'hydrolysis probes is performed in one tube, greatly reducing amplicon contamination and avoiding laborious post-amplification handling of samples. Its versatility and suitability for detection of ACLSV, ASPV and ApMV have been reported previously using minor groove binder (MGB) 5'hydrolysis probes (Salmon *et al*., 2002a, 2002b; Marbot *et al*., 2004). To our knowledge combination of primers and non-MGB labeled probes for the diagnosis of the four viruses in question had not been attempted before.

The objective of this study was to design primers and fluorogenic 5' hydrolysis probes and evaluate their suitability for detection of the four viruses in apples, pears and quinces, using real time RT-PCR. Apple, pear and quince samples used in this study included apple cultivars of economic relevance growing in commercial orchards, nurseries and germplasm collections of scientific institutions of southern Brazil, and material from our virus isolate collection. Apple samples had been biologically indexed and/or analysed by conventional RT-PCR, IC-RT-PCR, ELISA and/or Western blots and dot-blots over several years, thus their sanitary status was well known, as previously reported (Nickel *et al*., 2001; Radaelli *et al*., 2006; Silva *et al*., 2008; Fajardo *et al*., 2011). Since methods of analysis, reagents, buffers, plant tissues and time of sampling used in those tests were dissimilar, complete coincidence with results presented here is not expected. The virus status of Asiatic and European pears and pear rootstocks was unknown except for ASPV, which was diagnosed in several field samples, and in a cv. Williams P4 plant exposed to virus removing treatment by meristem culturing (Table 3). Real time RT-PCR requires a meticulous study of published virus sequences, designing of primers and probes based on a large number of cloned sequences to evaluate their variability (Kummert *et al*., 2000), and adequate software support for oligonucleotides suitability analysis. The design of primers and probes was initiated based on the coat protein (CP) gene sequences of regionally occuring isolates of ASGV, ASPV, ACLSV and ApMV (AF438409, AY572458, EF138602 and GQ131805) and sequences from GenBank (www.ncbi.nlm.nih.gov), which were aligned using CLC Sequence Viewer 6 (CLC bio A/S, Denmark) (Table 1). In the most conserved CP sequence regions of the four viruses, primers were selected for best alignment and analysed for suitability by the software Oligo Analyser (Invitrogen, USA). Where no complete sequence homology was found, consensus sequences were used. Oligonucleotides were synthesized commercially (IDT and Sigma-Aldrich, USA). The probes were labeled at the 5' end by the reporter dye FAM (6-carboxy-fluorescein) and carried the quencher TAMRA at the 3' end. Total RNA was extracted from 100 mg of bark scrapings by adsorption of nucleic acids to silicium dioxyde (Boom *et al*., 1990) adapted to woody plants (Rott and Jelkmann, 2001).

Reactions of real time RT-PCR were performed in plates containing 96 wells. StepOnePlus real time PCR System (Applied Biosystems, USA) was used for thermocycling. Data of the reactions were analysed graphically by an amplification plot using StepOne Software v2.2.2, determining the variable threshold cycle (C_q), defined as the cycle at which a significant increase of fluorescence occurs. Working mixtures of oligonucleotides and probe of each virus contained 20 µl of the sense oligonucleotide (100 µmol), 20 µl of the complementary oligonucleotide (100 µmol) and 4 µl of each virus-specific labeled probe (100 µmol), adjusting the mixture to a final volume of 240 µl with RNase-free water, storing it at -20 °C (Osman *et al*., 2007). A typical reaction with TaqMan probes consisted of: (i) 6.1 µl of the master mix (TaqMan Master Mix One-Step RT-PCR kit; Applied Biosystems, USA), optimized for the exonucleatic assay with labeled TaqMan probes, containing AmpliTaq Gold DNA polymerase, dNTPs with dUTP, one passive reference dye (ROX), which is a fluorescent dye used to adjust the basal level of fluorescence, or background, and an optimized buffer; (ii) 0.6 µl of the "working mixture" giving a final concentration

Table 2. Detection of viruses in apples by real time RT-PCR using 5'-hydrolysis probes.

Cultivar	Access Nr.[1]	SP[2]	C_q	SG	C_q	CL	C_q	MV	C_q	Origin of the cv.
Baronesa	M027	+[3]	38	+	30	+	26	+	34	Brazil
Braeburn	M061	+	37	+	31	+	28	+	35	New Zealand
Castel Gala	M193	+	26	+	40*	+	32	+	34	Brazil
Castel Gala	M194	+	34	Nt		Nt		+	26	
Cripps Pink	M080	+	31	–	40	+	29	+	29	Australia
Delicious	M176	+	34	+	32	+	25	+	29	USA
Fred Hough	M190	Nt		+	24	+	32	Nt		Brazil
Fuji	M003	–	40	+	30	+	32	+	33	Japan
Fuji	M057	+	28	+	33	+	24	+	26	
Fuji	M058	+	30	+	31	+	29	+	27	
Fuji	M078	+	32	+	33	+	29	+	27	
Fuji	M200	+	29	+	28	+	32	+	34	
Fuji Kiko	M069	+	32	-	40	+	31	Nt		
Fuji More	M182	+	35	+	32	+	32	+	33	Brazil
Fuji Precoce	M210	+	25	Nt		+	25	+	26	
Fuji Select	M184	+	28	+	31	+	30	Nt		
Fuji Suprema	M095	+	29	+	28	+	32	+	29	
Gala	M175	+	20	-	40	–	40	+	30	New Zealand
Galaxy	M181	+	25	+	34	+	28	+	32	
Granny Smith	M162	+	31	+	28	+	30	Nt		Australia
Imperatriz	M032	+	40*	+	29	+	29	+	37	Brazil
Imperial Gala	M059	+	30	+	30	+	33	+	36	USA
Maxi Gala	M163	+	26	+/–	35	+	30	–	35*	Brazil
Mishima	M185	+	30	+	33	+	28	+	33	Japan
Royal Gala	M012	+	32	+	27	+	25	+	37	New Zealand
Royal Gala	M013	+	39	+	30	+	26	+	37	
Royal Gala	M015	Nt		+	26	+	23	–	37*	
Royal Gala	M018	+	37	+	30	+	29	+	32	
Royal Gala	M053	+	32	+	40*	+	35	–	32*	
Royal Gala	M073	+	28	+	29	+	31	Nt		

[1] Sample access numbers in the plant collection of Embrapa Uva e Vinho; [2] SP, ASPV; SG, ASGV; CL, ACLSV; MV, ApMV; [3] previous sanitary status; * previous record unconfirmed. C_q, quantitation cycle; Nt, not tested; By convention, a C_q 40 means no amplification of the target sequence; C_q values <40 indicate infected samples.

of 415 nM of each oligonucleotide and 85 nM of probe; (iii) 0.3 µl of a mixture containing Murine leukemia virus reverse transcriptase and RNase inhibitor; and (iv) 3 µl (*ca*. 300 ng) of total RNA. The mixture was adjusted to 12 µl with RNase-free water. Cycling conditions were as follows: pre-PCR cDNA synthesis 35 min at 45°C, and 10 min at 95°C followed by 40 PCR-amplification cycles of 15 sec at 95°C and 1 min at 60°C. Values of Cq in tables 2 and 3 are averages of duplicate/triplicate analyses.

To determine the efficiency of the protocol, tenfold serial dilutions of total RNA extracts of the four viruses were submitted to real time RT-PCR. Detection limits were determined by three independent analyses of total RNA of each virus. Evaluation of the intra-assay variability of recorded C_q values was carried out for the four probes and primer sets by standard deviation analysis of three independent real time RT-PCR amplification runs, using RNA extracts from the same sample of each of a group of 12 apple cultivars, i.e. Baronessa, Fuji Suprema, Galaxy, Mishima, Fuji Select, Braeburn, Royal Gala, Fred Hough, Maxi Gala, Fuji Precoce, Castel Gala and Standard Fuji.

The designed primers and probes and the reaction conditions of real time RT-PCR used, allowed reliable virus detection (Tables 2 and 3). ASPV was detected in 27 of 28 (96.4%) and ACLSV in the totality of 29 (100%) analysed samples. Out 22 apple samples recorded as infected with ApMV, all tested positive in real time. Additionally, three trees (M163, M015 and M053) recorded as ApMV-free, proved to be ApMV-infected by real time (Table 2), resulting in 88% (22/25) agreement with previous records. Real time RT-qPCR confirmed the previous virus status of ASGV infection in 26 out 28 (92.8%) samples. Two plants (M193, M053) were negative in real time, one of which (M193), had tested positive by conventional RT-PCR, and the other (M053) was ASGV-positive by biological indexing and RT-PCR. Similar and even lower percentages of ACLSV and ASPV detection efficiency by real time RT-PCR have been reported (Marbot *et al*., 2004). These authors reported confirmation by real time of infections detected by conventional RT-PCR of 57.7% and 71.8%, respectively for *Prune dwarf virus* (PDV) and ApMV, and demonstrated that the failure to detect PDV was caused by a mismatch of one nucleotide that prevented the fluorogenic reaction and induced the false negatives. This failure occurred despite the use of probes that carry additionally an MGB group at the 3' end which increases the melting temperature of the probe, allowing the use of shorter probes (Salmon *et al*., 2002b), thus increasing

Table 3. Detection of viruses in pears and quinces by real time RT-PCR using 5'-hydrolysis probes

Cultivar	Access Nr[1].	SP[2]	C_q	SG	C_q	CL	C_q	MV	C_q	Origin of cv.
Abate Fetel*	P14	–[3]	40	+	35	+	31	Nt		Italy
Abate Fetel*	P15	+	28	+	35	+	27	–	40	
Cascatense	P27	+	37	-	40	+	34	Nt		Brazil
Clapp´s Favourite	P55	+	33	+	31	+	26	+	25	USA
Commice de Doyenne*	P6	–	40	+	35	+	31	–	40	France
Housui*	P23	+	35	–	40	+	34	Nt		Japan
Kousui*	P25	+	28	–	40	+	30	–	40	
Max Red Bartlett	P12	–	40	+	35	+	28	Nt		USA
Max Red Bartlett	P13	+	34	+	31	+	32	Nt		
Nijisseiki	P32	–	40	–	40	+	34	Nt		Japan
Packham's Triumph	P16	+	35	+	35	+	29	Nt		Australia
Packham's Triumph	P17	+	38	+	35	+	30	Nt		
Pyrodwarf*	P1	–	34	+	27	+	29	+	26	Germany
Pyronia veitchii*	P18	–	40	–	40	nt		Nt		
Quince A	P47	–	40	–	40	+	30	+	31	England
Quince A20	P46	–	40	+	34	+	30	+	29	England
Quince Adams	P48	–	40	+	35	+	31	+	31	Belgium
Quince BA-29	P54	–	40	–	40	+	35	–	40	France
Rocha	P26	+	36	–	40	+	34	Nt		Portugal
Red Bartlett*	P9	+	27	+	31	+	21	–	40	USA
Starkrimson*	P56	+	29	+	34	+	31	+	27	USA
Williams*	P7	+	29	+	35	+	29	Nt		England
Williams*	P8	–	40	+	36	+	24	–	40	
Williams*	P4	–	40	–	40	+	32	–	40	
Williams*	P415	–	40	+	33	+	30	Nt		
Yali	P21	+	31	+	34	+	26	+	25	China

[1]Sample access numbers in the plant collection of Embrapa Uva e Vinho; [2]SP, ASPV; SG, ASGV; CL, ACLSV; MV, ApMV; C_q, threshold or quantitation cycle; Nt, not tested; [3]previous sanitary status; *ASPV-status was known and confirmed, except for Pyrodwarf. C_q, quantitation cycle; By convention, C_q 40 means no amplification of the target sequence; C_q <40 indicates infected samples.

Table 4. Standard deviation analysis of data on detection of *Apple stem pitting virus* (ASPV), *Apple stem grooving virus* (ASGV), *Apple chlorotic leaf spot virus* (ACLSV) and *Apple mosaic virus* (ApMV) from apple plants.

Sample	C_q1	C_q2	C_q3	Average	Standard Deviation	Mean Standard Deviation
ASPV						
M027	37	38	39	38	1	± 0.58
M095	26	27	28	27	1	± 0.58
M181	25	26	27	26	1	± 0.58
M185	26	27	28	27	1	± 0.58
M184	27	26	26	26.3	0.58	± 0.33
ASGV						
M061	26	27	29	27.3	1.53	± 0.88
M190	22	23	24	23	1	± 0.58
M012	27	27	28	27.33	0.58	±0.33
M003	28	23	23	24.67	2.88	± 1.67
M181	30	34	34	32.67	2.31	± 1.33
ACLSV						
M027	26	25	25	25.33	0.58	± 0.33
M061	22	22	22	22	0	0
M190	33	32	32	32.33	0.58	± 0.33
M003	27	27	27	27	0	0
M095	25	25	25	25	0	0
ApMV						
M027	34	35	37	35.33	1.53	± 0.88
M061	33	34	35	34	0.53	± 0.58
M193	29	34	35	32.67	3.21	± 1.86
M182	28	28	29	28.33	0.58	± 0.33
M210	31	32	32	31.67	0.58	± 0.33

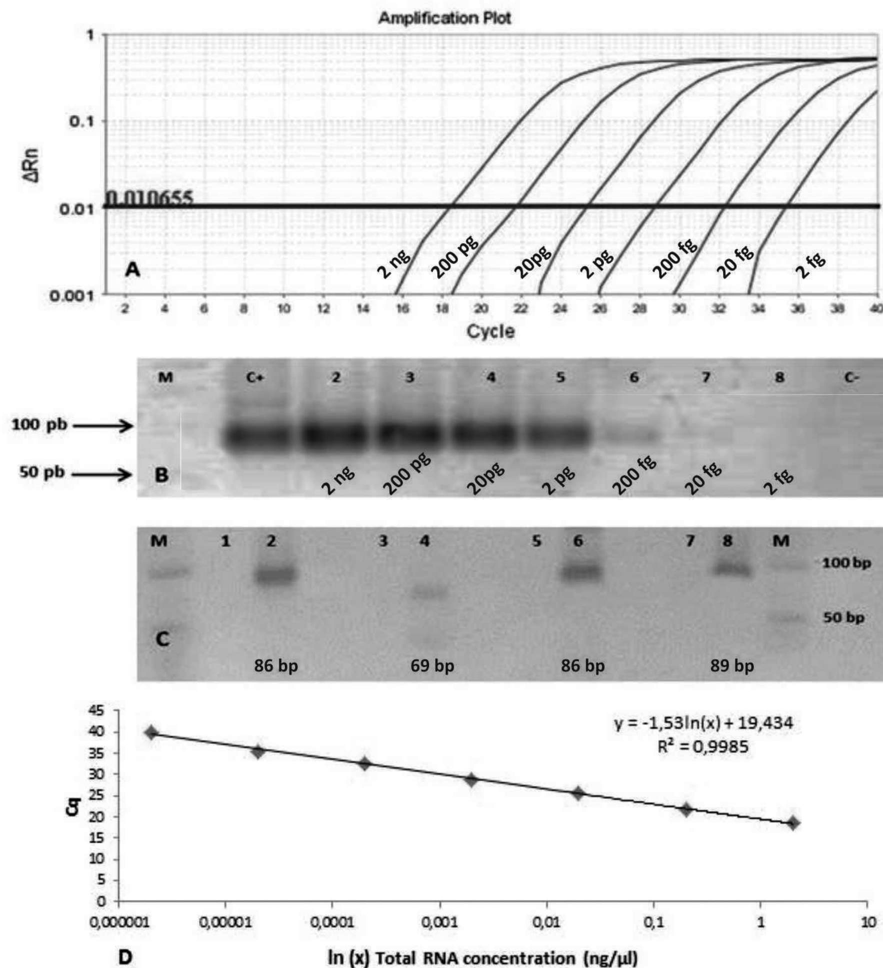

Fig. 1. Efficiency and sensitivity analysis of primers and probes. **A.** Amplification plot showing normalized fluorescence (ΔRn) vs. cycles of RT-qPCR of total RNA dilutions of extracts of ASPV-infected cv. Abatel Fetel P15 ranging from 2 ng to 20 fg/µl. T, threshold. **B.** Analysis of RT-PCR products: M, 50 bp ladder (Ludwig, Brazil); C+, positive control, ASPV-infected cv. Braeburn M061, lanes 2-8, dilutions of total RNA (2 ng, 200 pg, 20 pg, 2 pg, 200 fg, 20 fg, 2 fg/µl) of cv. Yali P21. **C.** Analysis of RT-PCR products: M, 50 bp ladder; Lanes 1, 3, 5 and 7, healthy seedling controls; 2, 4, 6 and 8, respectively, amplification products of ASPV (cv. Red Bartlett P9), ASGV (cv. Fuji Suprema M095), ACLSV and ApMV (cv. Yali P21). **D.** Plot of ln(x) total RNA concentrations of ASPV from cv. Abate Fetel P15 against quantitation cycle (Cq).

the probability of virus detection in situations of high target genome variability. In pears, in which the presence of ASPV was recorded in a few cultivars, recognition of previously detected infections by primers SPs635 and SPas721 and the respective hydrolysis probe SPp662 was complete (Table 3). ASPV had not been detected before in the pear rootstock Pyrodwarf in previous evaluations of this collection of plants.

Viral infections detected in this study occurred in some of the main commercial cultivars of apples, pears and quinces from different geographic origins, demonstrating the specificity and reliability of the primers and probes designed on highly conserved nucleotide sequences of the CP genes of the four viruses. The sensitivity of the protocol measured by the recorded detection limits of ASPV (cvs. Yali P21, Abate Fetel P15 and Red Bartlett P9), ASGV, ACLSV and ApMV (cv. Braeburn M061) fluctuated from 20 to 2 fg/µl total RNA (data not shown), and was comparable to that reported by Salmon *et al.* (2002b).

Real time analyses of serial dilutions of total RNA extracts of ASPV from reference cv. Abate Fetel P15 show quantitatively that primers and probe used are highly sensitive, Cq values correlating accurately with decreasing total RNA amounts from 2 ng/µl to 20 fg/µl (Fig. 1A). Using the same real time primers, it was shown that real time and conventional RT-PCR are equally sensitive based on the limit of detection of ASPV at 20 fg/µl total RNA, in RNA samples (Fig. 1A and 1B). While Salmon *et al.* (2002b) obtained similar results in detecting ASPV, other authors, working with grapevine leafroll viruses, reported a higher sensitivity of real time qRT-PCR as compared to RT-PCR (Osman *et al.*, 2007; Al Rwahnih *et al.*, 2012). Specificity of the primers is supported by the amplified bands of the expected size of, 86, 69, 86 and 89 bp in conventional

RT-PCR runs, respectively, for ASPV, ASGV, ACLSV and ApMV from different cultivars (Fig. 1C). A regression analysis plotting logarithm ln of total RNA concentrations of cv. Abate Fetel against Cq values yielded a $R^2 = 0.9985$ (Fig. 1D). Despite high sequence variation already reported for these viruses, as in ACLSV (Candresse *et al*., 1995; Nemchinov *et al*., 1998; Al Rwahnih *et al*., 2004), ASPV (Radaelli *et al*., 2006; Rodoni and Constable, 2008; Salmon *et al*., 2002b), and ASGV (Magome *et al*., 1997), primers and probes showed a good performance in the detection of isolates of these viruses. Given the high sensitivity of the primers and probes, the detection failures observed in a few samples could be attributed to mismatches of nucleotides between probes and target sequences, as already noted by other authors due to nucleotide sequence variability (Marbot *et al*., 2004; James *et al*., 2006). Analysis of assay variability by mean standard deviation showed an estimation of the low internal variability of the presented data (Table 4). The high percentage of recognition of four main apple viruses from a variety of different clones of several cultivars of apples, pears and quinces by the oligonucleotides and probes used in this study, shows their sensitivity, efficiency, and suitability as diagnostic tools. To our knowledge there has not been a previous report of a collection of 5' hydrolysis probes and primers to support reliable and practical diagnosis of these viruses affecting apples, pears and quinces by real time RT-PCR.

This procedure reduces risk of cross contamination, avoiding labor-intensive gel evaluation steps and UV-exposition, costly and time-consuming protocols that are made unnecessary by the immediate availability of automatic fluorescence readings after thermocycling. The method is highly versatile, allowing large scale sample processing, which is a fundamental requirement for certification programs.

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