Project Title	The improvement of diagnostic capabilities for the soft-fruit industry: the development of rapid and sustainable alternatives for strawberry virus testing
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Project leader:	Dr Rick Mumford
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Key staff:	Dr Wendy Monger
Location of project:	The Food & Environment Research Agency (Fera)*, Sand Hutton, York
Project coordinator:	Dr Paul Walpole
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*Please note: in April 2009, Central Science Laboratory (CSL) merged with several other parts of Defra to become the The Food & Environment Research Agency (Fera).

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The results and conclusions in this report are based on a series of experiments conducted over a two-year period. The conditions under which the experiments were carried out and the results have been reported in detail and with accuracy. However, because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of the results, especially if they are used as the basis for commercial product recommendations.

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AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

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Grower Summary

Headline

A new rapid test for 8 strawberry viruses and 1 phytoplasma has been developed and is available as a commercial service from Fera.

Background and expected deliverables

In the UK, the maintenance of clean nuclear stock (NS) and the provision of disease-free propagation material via the Plant Health Propagation Scheme (PHPS) has resulted in soft fruit crops that are generally free of economically damaging levels of virus. However this status can only be maintained through the continuation of an effective virus-indexing programme.

Recent changes in pesticide and residue regulations mean that the soft fruit industry will need to make changes to its aphid and nematode control programmes, with less reliance on chemical control measures. If control becomes less effective, then viruses spread by aphid and nematode vectors will become more abundant. Climate change could also have an impact on pathogen-vector distribution, increasing disease incidence. These factors make the need for clean planting material even greater.

For testing candidate NS material sourced from within the EU (over 98% of all planting material traded) there are nine viral and virus-like pathogens listed in the PHPS regulations: four aphid-borne viruses namely *Strawberry crinkle virus* (SCV), *Strawberry mottle virus* (SMoV), *Strawberry mild yellow edge virus* (SMYEV) and *Strawberry vein-banding virus* (SVBV); four nematode-borne viruses namely *Strawberry latent ringspot virus* (SLRSV), *Arabis mosaic virus* (ArMV), *Raspberry ringspot virus* (RRSV) and *Tomato black ring virus* (TBRV); and one leafhopper-borne phytoplasma, *Strawberry green petal* (SGP).

Traditionally biological indexing of strawberry for viruses and virus-like pathogens has relied exclusively on slow and laborious leaf grafting techniques, which required skilled staff. However this technology is slow, relatively expensive, tedious to perform and reliant on an ever-decreasing pool of highly skilled staff.

As a result, this project will investigate and develop new diagnostic methods to replace grafting. Of all the diagnostic technologies currently available, real-time polymerase chain reaction (PCR) is the most powerful, offering an exceptional combination of specificity, sensitivity and reliability.

This project has developed a rapid diagnostic package for the reliable detection of nine pathogens (8 viruses & one phytoplasma) known to be a risk from EU propagation material. This will provide an efficient and cost effective means of screening nursery stock to ensure virus-free certification.

Summary of the project and main conclusions

In year one, work focused on designing real-time PCR for all the target pathogens and optimising detection to an equivalent level for all nine viral and virus-like pathogens relevant to material of EU origin.

In the second year, a validation exercise based on samples obtained from world wide collection, was performed to determine the functioning of the molecular approach to detection.

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Methods have been developed and tested that enable the reliable extraction of RNA and DNA from strawberry plants. This work is very important, as it is the first stage of any DNA-based ('molecular') testing and it greatly affects the reliability of the whole test. Strawberry leaves are one of the worst plants for inhibitors and if these are at a particular concentration within the extract they will prevent assays based on PCR from working reliably.

The quality of extracts can be monitored using an internal control assay. Based on real-time PCR, this assay is designed to detect plant RNA/DNA and can be used to show both the quantity of DNA/RNA extracted and its quality (e.g. If inhibitors are present in too large a concentration the assay will fail). Such an assay is being used in the project and will be incorporated into any future diagnostic procedure.

As a result of this work, a rapid strawberry virus/phytoplasma screening service has been made available to growers. This offers reliable detection in days rather than months.

Financial benefits

- This work provides growers with a rapid and reliable screening package. This will ultimately help propagators by improving plant quality and reducing unit costs (e.g. cheaper testing, fewer plants 'lost'). In turn this will benefit those involved in fruit production, who will be supplied with a higher quality product, leading to reduced disease control costs and even less direct losses, through reduced fruit yield and/or quality.

- Given that the new methods will be much faster than existing graft-based testing, it will allow much quicker screening of high-risk material, such as new breeding lines or germplasm material, ensuring that infected material could be isolated more quickly before it could introduce problems into breeding programmes.

- By using an established diagnostic service, this will ensure that any testing methods developed can be delivered to industry, in a way that is sustainable in the long-term.

- As real-time PCR is a generic technology, in the longer term, there is significant potential to integrate these new assays with existing assays for other strawberry fungal and bacterial pathogens, thus developing a highly cost effective, rapid strawberry disease-screening package.

Action points for growers

- A set of real-time PCR assays have been designed to detect the eight most important strawberry viruses, found In Europe. These include viruses transmitted by aphids (*Strawberry crinkle virus, Strawberry yellow edge virus, Strawberry mottle virus* and *Strawberry vein banding virus*) and nematodes (*Arabis mosaic virus, Strawberry latent ringspot virus*, *Tomato black ring virus* and *Raspberry ringspot virus*). These assays have been tested on a large range of isolates from both the UK and abroad.

- A new real-time PCR assay is now available for the detection of phytoplasma diseases. This assay has been designed to detect a broad range of different phytoplasmas from different groups, including strawberry green petal and other phytoplasmas known to infect strawberry crops in other countries.

- Breeders, growers and propagators should now use the rapid strawberry virus/phytoplasma screening service that has been made available to growers, offering reliable detection in days rather than months.

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Science Section

Introduction

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This project has developed a rapid diagnostic package for the reliable detection of nine pathogens (8 viruses & one phytoplasma) known to be a risk from EU propagation material. This will provide an efficient and cost effective means of screening nursery stock to ensure virus-free certification.

1. Virus and phytoplasma pathogens of strawberry

This project has been investigating the detection of nine pathogens:

1.1. The aphid-borne viruses

The four aphid-transmitted virus that infect strawberries all have a limited host range found almost exclusively on *Fragaria* species:

- *Strawberry crinkle virus* (SCV; *Rhabdovirus, Cytorhabdovirus*) a single stranded negative sense RNA virus. This virus is found worldwide and reduces yield and affects fruit quality. It is especially severe when in combination with one or more of the other aphid transmitted strawberry viruses SMoV and SMYEV. Symptoms produced on some strawberry varieties are stunting, vein necrosis, chlorosis and leaf distortion.
- *Strawberry mottle virus* (SMoV; *Picornaviridae, Sadwavirus*) a single stranded positive sense RNA bipartite virus. This is the most widespread virus affecting strawberry plants worldwide. Infected plants are less vigorous and produce fewer strawberries than normal. The virus attacks the foliage of the plants and causes

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scattered, inconspicuous spots on the leaves. Symptoms vary seasonally, even though some strawberry species do not show the typical mottling at all.

- *Strawberry mild yellow edge virus* (SMYEV; *Flexiviridae, Potexvirus*) a single stranded positive sense RNA virus. This is one of the most widespread virus diseases of cultivated strawberry. Most commercial cultivars are tolerant to infection with this virus and they do not show symptoms. However, infections by severe strains and particularly mixed infection with other viruses lead to decline and to the development of symptoms such as dwarfism, stocky plants, mosaic, mottle, and leaf distortion.
- *Strawberry vein-banding virus* (SVBV; *Caulimoviridae, Caulimovirus*) a double-stranded circular DNA virus. This virus is found worldwide with symptoms of discontinuous banding, streaking and spotting of older leaves, twisting of leaflets is also observed.

1.2. The nematode-borne viruses

In contrast to the aphid-transmitted viruses, the four nematode-transmitted viruses that infect strawberries have a very wide host range of plants, including other fruit crops such as raspberry:

- *Strawberry latent ringspot virus* (SLRSV; *Picornaviridae, Sadwavirus*) a single stranded positive sense RNA virus.
- *Arabis mosaic virus* (ArMV; *Comoviridae, Nepovirus*) *a* single stranded positive sense RNA virus.
- *Raspberry ringspot virus* (RRSV; *Comoviridae, Nepovirus*) a single stranded positive sense RNA virus.
- *Tomato black ring virus* (TBRV; *Comoviridae; Nepovirus*) a single stranded positive sense RNA virus.

1.3. The leafhopper-borne phytoplasmas

A number of phytoplasma diseases have been identified in strawberries. The most common and only one identified in the UK to date is Strawberry Green Petal (SGP). However, it is unclear exactly which phytoplasma species/group causes SGP, with reports of different phytoplasmas being associated with these symptoms. This situation is not uncommon with diseases caused by these pathogens, as so little has been known about them until recently. Phytoplasma are presently identified and classified using the ribosomal 16S and 23S genes. Using these genes it is possible to design assays that can detect all phytoplasmas. Given the uncertainty regarding which specific phytoplasmas may be involved with disease in strawberry, assays designed to detect a broad-range of species are ideal for detection purposes.

2. TaqMan® real-time PCR technology

The main nucleic acid-based detection methods are the conventional PCR or RT-PCR and the Real-Time RT-PCR methods. The latter method (specially the TaqMan® chemistry) uses an internal primer located between a specific pair of primers that is labeled at opposites ends with a reporter and quencher dyes. The intact probe is in a non-fluorescence state, as the quencher absorbs fluorescence emitted by the reporter. During amplification, the reporter is separated from the rest of the probe resulting in fluorescence, which can be detected. This fluorescence is proportional to the quantity of amplified product (ΔRn). The cycle threshold (Ct) value is related to the log of the number of target RNA molecules in the reaction. This method has been applied to the detection of various plant pathogen including viruses and has been shown to be the most sensitive detection method presently in use (Mumford et al., 2004; Boonham et al., 2002,2004; Korimbocus et al., 2002).

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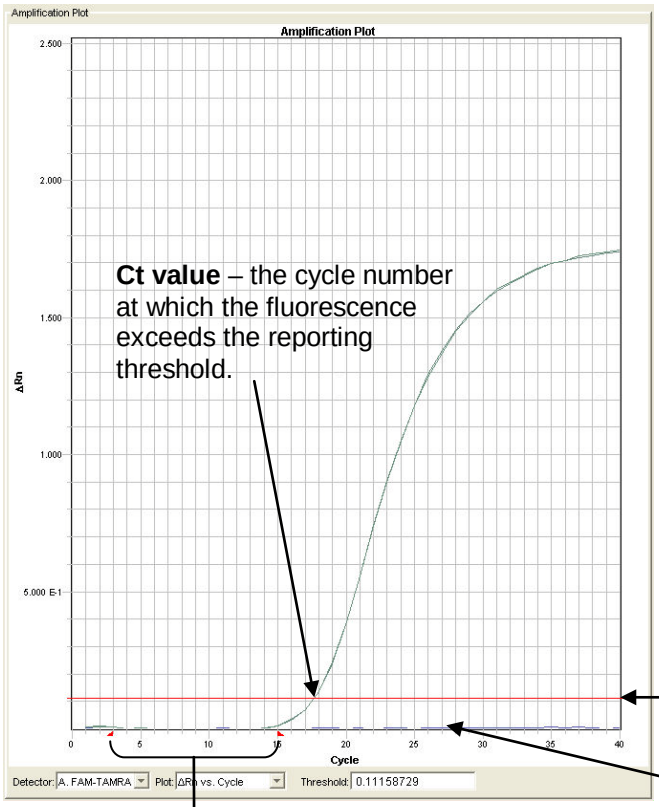
Figure1: TaqMan® amplification plot



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Materials and Methods

1. Nucleic acid extraction

1.1. RNA extraction from strawberry leaves

Three methods were compared:

- RNeasy plant mini kit (Qiagen cat. No. 74904).
- CTAB RNA extraction method (adapted from Lodhi *et al* 1994), with the overnight precipitation step performed with an equal volume of 4M lithium chloride and not isopropanol.
- Fera Kingfisher RNA extraction method (in-house method utilizing magnetic beads)

1.2. DNA extraction from strawberry leaves

Two methods were compared:

- CTAB total nucleic acid (TNA) extraction method (adapted from Lodhi *et al* 1994). This method extracts both RNA and DNA.
- Fera Kingfisher DNA extraction method (in-house method utilizing magnetic beads; different buffer system from RNA method)

2. Real-time PCR assay design

Assays (TaqMan®) were designed using ABI Primer Express software, using sequences obtained from the NCBI database (www.ncbi.nlm.nih.gov). The details of the different assays that were designed are described below, under the Results section.

3. Real-time PCR

Real-time PCR (TaqMan®) was performed using generic conditions, essentially as described before (Mumford *et al*., 2000), using TaqMan® core reagent kits (Applied Biosystems; Cat. No. 430 4441). Primers are used at a working concentration of 7.5pmol/µl and probes at 5pmol/µl, in each 25/µl reaction. Assays were run on Applied Biosystems (ABI) 7900 machines.

Machine program for a RNA template:
30min at 48°C, 10min at 95°C, then 40 cycles of 95°C for 15sec and 60°C for 1min.

Machine program for a DNA template:
2min at 50°C, 10min at 95°C, then 40 cycles of 95°C for 15sec and 60°C for 1min.

4. Other detection methods

In order to confirm the presence of pathogens in samples other detection methods, conventional PCR and ELISA, were also used. Antisera used in ELISA was available commercially and supplied by either Loewe (Germany) or PRI (the Netherlands).

5. Strawberry grafting

Strawberry grafting was performed with the stock plants UC4, UC5, UC6 and UC11 and using standard grafting procedures, as established at Fera. Grafted plants were maintained in a mist cabinet and not in polythene bags, for the first 2 weeks after grafting.

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Results and Discussion

1. Real-time PCR (TaqMan®) assay design and validation

1.1. Virus assay design

For the eight target viruses, a summary of which sequences were used to design assays is presented in Table 1. Between 5 and 20 sequences were aligned and used for the design of each assay. Details of all the assays designed are presented in Table 3.

Regions of the virus genome were selected that had the most sequences available; to ensure that the assays designed were capable of detecting the broadest range of isolates, thus reducing the risk of false negative results. For this reason, in most cases, assays were designed to the viral coat protein gene.

In some cases, in particular for viruses where published sequence data was limited, further conventional PCR primers were designed; laying either side of the TaqMan® assay region. This enabled additional sequence data to be collected for specific virus isolates.

Table 1: Summary of virus assay design data

Virus	Gene sequence used	No. sequences available on Database	Comments
SCV	RNA polymerase gene	9	
SMoV	3' untranslated region (UTR)	13	This region is conserved on both RNA 1 and 2, and amongst all isolates
SMYEV	3' UTR	6	
SVBV	Coat protein	6	
ArMV	Coat protein	9 plus addition 11 sequences obtained from the Netherlands	Highly variable at the nucleotide level, which probably relates to its large host range
RRSV	Coat protein	14	
TBRV	Coat protein	5	
SLRSV	Coat protein	9	Given variability, two primer sets have been designed around a 'universal' probe

1.2. Aphid-transmitted virus assays

The sequence available on the database and sequence obtained during this project showed these viruses to have well conserved genomes and highly suitable for the application of TaqMan® technology.

The assays were tested against the isolates obtained (Table 2), with between 6 and 11 isolates tested for each of these assays. In nearly all cases the isolates were clearly detected. Where an isolate failed to give a positive result or the result was poor, conventional PCR primers were used to firstly confirm if the virus was present and secondly to look at the sequence in the area of the assay. In total only one SVBV and one SMYEV isolate obtained from other sources were not detected by the designed assays. However, in both these cases conventional PCR also failed to detect virus and ultimately the source of these isolates confirmed doubts that the virus was still present due to lack of testing or failure of previous tests. These isolates are not included in Table 2.

1.3. Nematode-transmitted virus assays

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Sequences for these viruses are available on the database and additional sequence was obtained from PCR products produced during this project and colleagues in the Netherlands. These viruses were found to be more variable in sequence and therefore more problematic in the development of TaqMan® assays. They are also comparatively rare in strawberry; hence only one SLRSV isolate tested and one sequence available for ArMV had strawberry listed as their original host plant, all other available sequences and isolates were taken from other hosts.

The assays designed were tested with isolates obtained from colleagues around the world (Table 2). Although only 2 isolates were available to test with the RRSV assay there were 14 sequences available for the initial design. As before, in the case of a negative result, the suspect isolate was re-tested by conventional PCR and/or by ELISA. Initial testing did show that certain isolates were not detected/poorly detected using the real-time PCR assays first designed. As a result, the assays for the viruses ArMV, TBRV and SLRSV were all redesigned to give more efficient detection of isolates. In the case of SLRSV and TBRV, these assays are composed of 2 forward and 2 reverse primers and TBRV also has a minor grove binding probe. This allows for a shorter region of sequence to be used for the probe design. Ultimately all four assays were able to detect all the isolates, confirmed as being virus positive.

Table 2: Isolates of strawberry viruses obtained from collections worldwide

Source of isolates	**No. of isolates obtained for each virus**							
	SCV	SVBV	SMYEV	SMoV	ArMV	RRSV	TBRV	SLRSV
DSMZ (Germany)					1	1		1
Joseph Postman (USA)	1	1	1	2	1			
Claudio Ratti (Italy)	4	6	4	5				
Lisa Ward (NZ)*	1	1	1	3	1	1	5	
Robyn deYoung (Canada)					1		1	
Fera		2		2	8		1	3
Total	6	10	6	12	12	2	7	4

* Some of this material from this source was originally from USA/Canada/Scotland or DSMZ, the isolates listed were confirmed as being different from those already obtained from other sources.

1.4. Phytoplasma assays

For the detection of phytoplasmas, two suitable TaqMan® assay have been designed: an established assay designed to 16S rDNA sequences and a new assay designed to 23S rDNA sequences*. Both are 'universal' assays designed to detect all phytoplasma species. This approach has major advantages over attempting to try and design assays to specific pathogens e.g. SGP. In particular, as little is known about which specific phytoplasmas cause diseases in strawberries, with the possibility that it could actually be a range of different species. Therefore with our current understanding using species-specific assays could lead to false negative results. The established assay has been previously shown to detect all phytoplasmas and would overcome the issue of false negative results. However, 16S rDNA sequences can be too broad-spectrum and as a result, this assay (and others based on this gene) can sometimes detect other bacteria, such as saprophytes that may be contaminating the sample, resulting in potential false positive results. Therefore the second, more specific (based on 23S sequences) was designed and validated. In total 28 23S rDNA sequences, representing all 13 phytoplasma groups were aligned and assays designed from this alignment, in regions that were shared between all species. Alignments of 7 closely-related non-target bacteria, including saprophytes, were also carried out to ensure that the regions selected to detect phytoplasmas were not conserved for these other non-pathogens and hence would not detect them. The universal phytoplasma assay (JH) is given in Table

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3. Details regarding the design and validation of this assay can be found in paper recently accepted for publication (Hodgetts *et al.,* 2009).

As initial testing also confirmed that the new 23S assay was more efficient (and hence more sensitive) than the existing 16S assay (results not shown), the 23S assay was selected for further validation (any further data presented on phytoplasma detection in this report refer to this assay).

In order to validate the new 23S universal phytoplasma assay, it was then tested using 29 representative phytoplasma isolates, covering 12 of the 13 groups currently designated. All the isolates were reliably detected (Hodgetts *et al.,* 2009). In contrast, there was no detection of the closely-related bacterial species *Burkholderia gladioli* using the new assay. This species was chosen as it had the greatest DNA sequence similarity to phytoplasmas and as *Burkholderia* species naturally infect plant hosts. This provides strong evidence that the new assay is indeed specific for the genus *Phytoplasma*.

In addition, to demonstrate the phytoplasma-specific of the new assay, further testing was conducted using 45 general plant clinic samples, representing 10 different plant species. These samples were all either uninfected or infected with a non-phytoplasma pathogen e.g. a viral pathogen. Given the age and condition of these samples, mostly sent through the post and stored for several days/weeks before phytoplasma testing, it was considered likely that these would have been harboring high levels of non-pathogenic saprophytic bacteria and therefore prove a reliable test of assay cross-reaction with non-target bacteria. However, none of these samples tested positive, providing further supporting evidence that the new 23S assay is phytoplasma-specific.

***Note**: much of the design and validation of the new 23S assay was carried out as part of a parallel project on the taxonomy of phytoplasmas funded by Defra Plant Health Division and was carried out by Jennifer Hodgetts, University of Nottingham; co-supervised by Dr Matt Dickinson.

1.5. Internal control assay

An existing plant cytochrome oxidase gene (COX) TaqMan® assay has been used as an extraction control assay. This assay efficiently detects strawberry DNA/RNA.

2. Test development and validation

2.1. Nucleic acid extraction

RNA extraction

The Qiagen RNeasy kit is an expensive method that also co-extracted the greatest amount of inhibitors. Hence this method was discarded early on. Both the CTAB and Kingfisher RNA extraction methods produced RNA which could be amplified by both pathogen and control assays. As the Kingfisher method yields better results (with extracts producing lower Ct values indicating higher RNA yields) and is automated, it has been selected as the method of choice and was adopted for the rest of the project.

DNA extraction

Both the CTAB and Kingfisher DNA extraction methods do yield amplifiable DNA from strawberries, although it was found that the Kingfisher DNA extraction method co-extracted more inhibitors, leading to less efficient detection. As a result, this method often required the resulting DNA to be diluted to remove the effect of the inhibitors, prior to a successful TaqMan® test. The CTAB method has been used effectively for phytoplasma detection from a wide range of samples, including strawberries (work carried out previously; not shown).

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In terms of virus detection, the only DNA virus is SVBV; the virus genome is expressed as a RNA copy and therefore RNA extractions can be used to detect this virus. A comparison experiment was performed whereby the TaqMan® assay for SVBV was performed on both RNA and DNA extractions of infected material. The virus could be detected in both types of extract. The RNA extracts gave better results than the DNA extracts, probably because the DNA extracts were more diluted. Therefore it is unnecessary to use a different extraction method for SVBV and the same RNA extraction method can be used to test for all viruses. This will reduce work and subsequent costs. In total, RNA extractions from 8 infected strawberry plants successfully detected SVBV.

Table 3: Primer sets for TaqMan® assays

Primer & probes	Sequences 5'-3'
SMV-F	GTA GTT TAG TGA CAA TCC AAG CGG A
SMV-R	ACT ACC AAG AGC CGG TGT CCT AC
SMV-P	AGA CGC TGG GCG CCG ACA GTT C
SCV-F	AAC AAT CAT TAA AAC AAG (AG)AG (AG)AC TGT AAT
SCV-R	TCT GGG ATT CAG TGT (AT)GT ATC TTC C
SCV-P	TCA CCA GAG AAG AGA AGA GGA GTC ATC AGG TG
SMYEV F	CAT C(CA)G TGG A(TA)T AGG CTT AAA ATG G
SMYEV R	TCA CTT CAT GGC ACT CAT TGG
SMYEV P	TTT CTT CTC ACT TCT TCT ACC CAG C
SVBV-2538F	GCT AGA AAC AGG TTA GTC AAA TTA CAA ATT
SVBV-2658R	CTT CAA TAT ATT TGG GCC ATT CTT C
SVBV-2593P	TTT TCT GTG ACT ATG AAA CCA ATC TTC
SLRSV-194F	CAT CTC CAA A(AG)T GCT C(AC)T TTC A
SLRSV-192F	ACC TCC TTC AAA GTG TTC CTT TCA
SLRSV-271R	G(CT)C C(AG)C TAG CTT CTG CCT C(AG)C
SLRSV-275R	TGT AGT CCA CTC GAT TCT GTC TCA C
SLRSV-224P	TTG GGT G(CT)C C(AG)T GCA A(AG)C AGC ATA CT
ArMV-TAQ-F2	(CT)AC TTA TGG CAT AGT CCT AAG AGT TGT TAG
ArMV-TAQ-R2	ACA (CT)CC GGG GTA TTT AAA (CT)A(AG) TT
ArMV-TAQ-P2	AAT GGA ACG GGG TCA CTA ACA ACT GGA A
RRSV-1699F	GTT GTG TTG CTT CCC AGG GTA T
RRSV-1780R	(CT)AA AAC CA(AG) (GC)GG TGC ATA TTC TTT
RRSV-1723P	TGC AGA CCT GGG AAA AGG AGG TTA ATC CT
TBRV-UTR-F1	GGC GA(CT) AAT CAA GGT TTG TCC T
TBRV-UTR-F2	GCG ATG CAT AGG GTT TGT CCT
TBRV-UTR-R1	GAA AAC ATT TTA TCA TAT ACA AAA GAA AGA GAA
TBRV-UTR-R2	GAA AAC ATT TTA TCA TTT ACA AAA GAG AAA ATC
TBRV-UTR-P*	CTA TGT TGG ACA CAA AAA
JH-P uni*	AAC TGA AAT ATC TAA GTA AC
JH-F 1	GGT CTC CGA ATG GGA AAA CC
JH-F all	ATT TCC GAA TGG GGC AAC C
JH-R	CTC GTC ACT ACT ACC (GA)GA ATC GTT ATT AC
COX F	CGT CGC ATT CCA GAT TAT CCA
COX R	CAA CTA CGG ATA TAT AAG RRC CRR AAC TG
COXSOL 1511T	AGG GCA TTC CAT CCA GCG TAA GCA

*These probes include a minor grove binding protein.

Extraction optimisation

Over 130 extractions of mostly strawberry material have been performed in the course of this project. On only 14 occasions, based on the COX control assay results, have extractions from strawberry been deemed to have failed and needed to be repeated. In all these cases it was found that efficient detection could be achieved by performing a simple dilution of the extract. As a result of this, the following modifications have been made to the standard Kingfisher RNA extraction method to ensure reliable detection:

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A. One leaflet of a strawberry leaf is taken for extraction; this should not exceed 1.5 X 1.5 cm in area.
B. The leaflet is extracted in 2 ml of Kingfisher working buffer.
C. After extraction, the nucleic acid is eluted into 400µl of water not the standard 200µl.

*2.2. Assay performa*nce

Sensitivity

Using the optimised extraction method, accurate and specific detection was obtained for all 8 viruses across the range of isolates tested. A summary of the range of Ct values obtained is shown in Table 4, with further details are given in Appendix 1. These results show clear and sensitive detection with all isolates tested. Given that an increase in Ct of 3 cycles is equivalent to a 10-fold dilution of the target, the results show that there is sufficient sensitivity to detect the virus present in most of the samples after multiple 10-fold dilutions. By extrapolation detection should be possible in the majority of samples, even if they contain 100-1000 times less virus. Even for the 'weakest' positive (i.e. one ArMV isolate at Ct 35) dilution of between 10-20 times would have been detectable.

To further demonstrate assay sensitivity, a dilution series experiment was run using virus-infected strawberry. Sap from leaf material infected with SMoV was diluted in uninfected strawberry leaf sap, extracts made from each dilution and tested using real-time PCR. The results (shown in Appendix 2) show that virus is readily detected at dilutions down to 1:800, but not at 1:1600 or below. This fits extremely well with sensitivity end-points estimated above and does indeed demonstrate that the new assays have a high degree of sensitivity, when detecting virus from strawberry leaf.

Overall this increased sensitivity is very important, in both reducing the potential for false positives (due to low virus concentrations) and allowing the testing of 'bulked' 'leaf samples (i.e. testing multiple leaves per plant or combining leaves from different plants).

Table 4: The range of Cycle threshold (Ct) values obtained using the 8 virus assays designed

Assay	Typical Ct values for positive samples
SMoV	12-31
SCV	20-26
SVBV	13-31
SMYEV	12-25
SLRSV	15-28
TBRV	12-28
ArMV	16-35
RRSV	14-17

Mixed infections

Given that mixed virus infections are common in strawberry, the ability to reliably detect and distinguish between the individual viruses found in these mixes is important. The results presented in Table 5 show the results obtained from 8 mixed infection plants, where two viruses were present. In all cases both viruses are readily detected.

Table 5: Plants with a mixed virus infection

Plant Source	Ct values for SMYEV	Ct values for SMoV
East Malling collection 1	25/25	22/22
East Malling collection 2	23/23	21/21
East Malling collection 3	24/24	22/22
East Malling collection 4	23/23	21/21

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East Malling collection 5	25/25	24/24
East Malling collection 6	25/25	31/31
East Malling collection 7	21/21	20/21
NSA	24/24	23/23

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2.3. Virus testing

Unfortunately during the duration of the project the levels of virus infection seen in the field declined significantly. For example, no samples were received from PHPS field inspections at all in year one, due to the virtual absence of virus from propagation material in the 2007 season. While this is ultimately good news for the UK strawberry industry, this lack of field samples has meant that validation has focused more on overseas isolates from virology collections, rather than current UK isolates.

However, in year two additional strawberry material was obtained from Sue Baker/Sarah Troop (Nuclear Stock Association) and via EMR (David Yohalem). This material was screened using the virus assays. Typical results obtained can be seen in Figure 1.

In total 103 plants were received via the NSA, much of it mother stock material. Most of this material was clean but 8 plants tested positive for SMYEV. Included in this material were two known graft-positive plants: one infected with SMYEV and one that was exhibiting virus symptoms, but where the pathogen(s) had not been identified. In both these cases virus was successfully detected: the known SMYEV-positive tested positive for SMYEV and the plant showing unknown symptoms was found to be a mixed infection of SMYEV and SMoV.

Additional strawberry material was obtained from EMR (received winter 2008), in the form of their strawberry virus collection, which was passed to Fera following the completion of the Defra SCV project. Unfortunately, given the age of the collection, the origins of much of the material was obscure and could not be effectively used. Despite this, screening of 16 plants took place, of which 14 infected plants were successfully identified: 1 infected with SCV, 2 infected with SMYEV, 4 with SMoV and 7 plants were found to have both SMYEV and SMoV. Some of this material was subsequently used for graft testing.

In addition to the testing carried out at Fera, the SCV assay was also transferred to David Yohalem, EMR, for use in the Defra SCV project (HH3229SSF; Incidence and epidemiology of strawberry crinkle disease). In this project the SCV real-time PCR assay was effectively used by EMR to detect virus in both plants and aphids (David Yohalem, Pers. Comm.)

2.4. Phytoplasma testing

To date several different phytoplasma have been identified infecting strawberry plants, including members of the 16Sr groups 1, 3, 6 and 12. Of the isolates tested, all these four groups were represented and were detected by both assays. This included one isolate listed as strawberry green petal phytoplasma (this is a plant from an existing collection, maintained by grafting in vinca). This isolate tested positive with the new real-time assay and sequencing confirmed this as ca. Phytoplasma asteris (16SrI aster yellows group). Aster yellows are amongst the most ubiquitous of all phytoplasmas, being found in a wide range of different hosts. Given this result and the extensive validation against aster yellows, if SGP in the UK is indeed caused by phytoplasmas of this group, then reliable detection can be achieved.

Unfortunately, despite attempts to source field-infected samples, none were received during the project. This reflects the overall rarity of phytoplasmas in UK strawberry crops. Given the extensive validation work carried out using other hosts, there is no reason to suspect that reliable detection of phytoplasmas in strawberry cannot be achieved. However, further parallel testing must be carried out to confirm this (see below).

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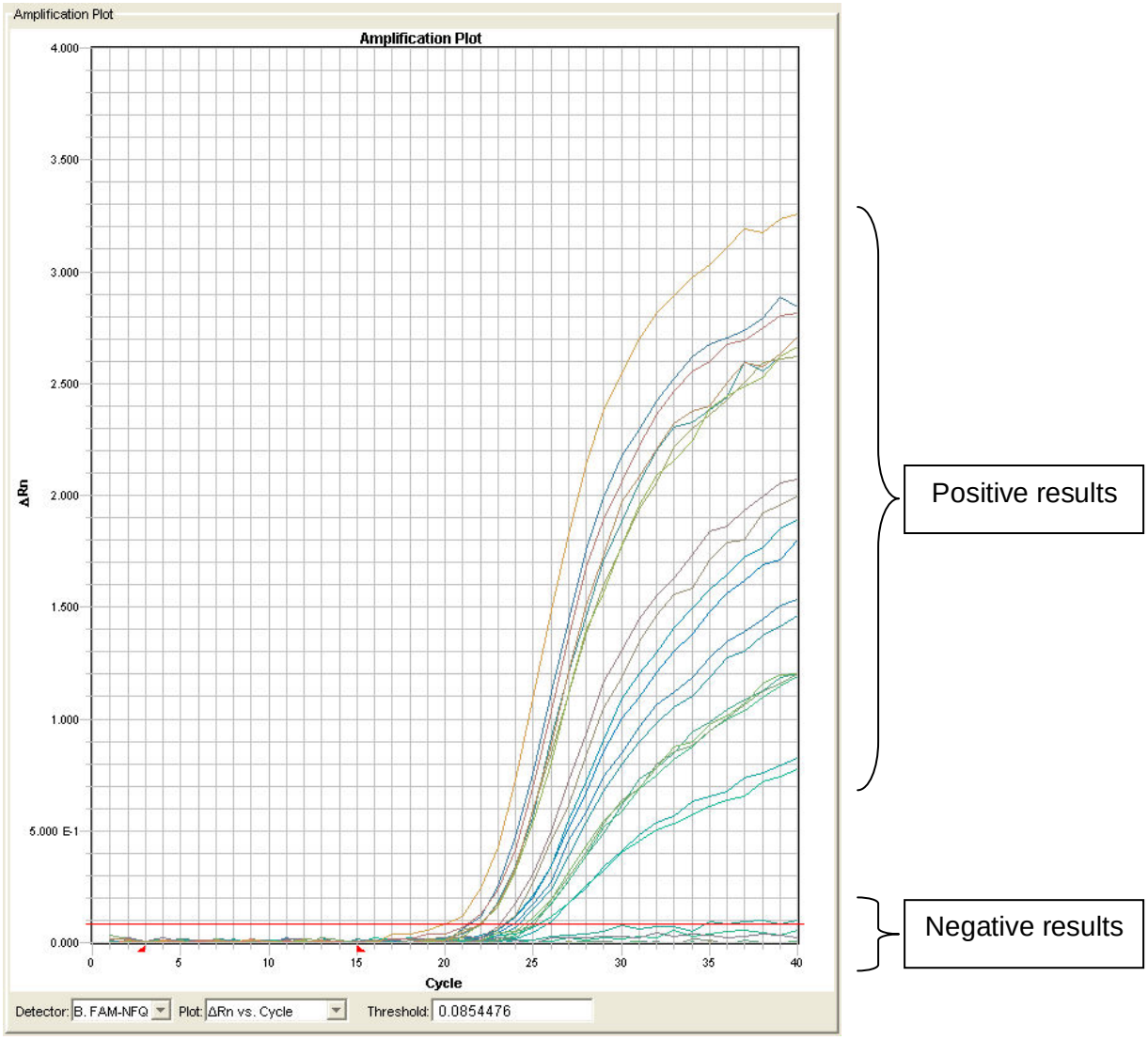
Figure1: Typical results obtained using real-time PCR. The results show the amplification plots obtained using the SMYEV TaqMan® assay, after testing 14 plants received from EMR. Of those 14, 9 were positive for SMYEV and 5 were negative. Tests were carried out in duplicate.



2.5. Comparison with existing methods

Comparison between ELISA and real-time PCR assays for nematode-borne virus detection

ELISA assays are available for all of the four nematode-borne viruses and given the overall simplicity of this method, it could potentially be deployed as a simple initial screen for these viruses. However it is suspected that given the variation in nepoviruses, ELISA testing could fail to detect certain isolates and therefore give false positive results. As a result, this concept was tested using the collection of isolates assembled in this study.

Where sufficient material was available, a limited number of nematode-borne virus isolates, were tested by both ELISA and TaqMan®. From the results obtained (Table 6), it can be seen that overall using real-time PCR is more reliable than ELISA. Of the seven isolates tested, while all were detected using real-time PCR, ELISA detected only 5. For the negative ELISA results, in the case of the undetected ArMV isolate, sensitivity is probably the issue, where even the real-time PCR gave a high Ct value (34), indicating a relatively weak positive (i.e. low concentration of virus). It is well established that real-time PCR is

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considerably more sensitive than ELISA for virus detection, routinely being 3-4 orders of magnitude (e.g. 100-1000 times) more sensitive. This improved sensitivity is evident when looking at the RRSV test results: although ELISA did detect virus in the sample, the results are borderline (only 2 x healthy control sample), compared to the strong real-time PCR positive (Ct 17). In the case of the SLRSV isolate tested here, the strong TaqMan® positive (Ct 16) suggests that the lack of ELISA detection is linked to serological variation in the isolate, not being detected by the antiserum in question.

Table 6: Comparison of ELISA and TaqMan® assays

Isolate	TaqMan®Ct average	ELISA *
ArMV PV0215 (DSMZ)	16	0.853/0.780 (9 x healthy)
ArMV 9002 (USA)	27	0.277/0.253 (3 x healthy)
ArMV (New Zealand)	34	Not detected
TBRV PV0179 (DSMZ)	19	0.469/0.459 (3 x healthy)
TBRV PV554 (DSMZ)	17	1.040/1.051 (7 x healthy)
SLRSV PV0247 (DSMZ)	16	Not detected
RRSV PV0429 (DSMZ)	17	0.230/0.213 (2 x healthy)

*An ELISA result is considered positive when is twice the healthy control reading

Comparison between grafting and real-time PCR assays for virus detection

Strawberry grafting was performed using clean stock plants of the indicator varieties UC4, UC5, UC6 and UC11. Healthy and SMoV, SVBV and SMYEV infected strawberry leaves (from plants that had tested positive and showing symptoms) were grafted onto each of the 4 indicators. At the time of the testing, no SCV-infected plants were available.

In total 22 plants were tested by grafting. After 10 days the plants were examined. Two plants were found to have their grafted leaf turn brown and hence failed. A further 3 plants were found to have browning of the petiole of the grated leaf and were also considered failures. All remaining plants were kept for further testing.

At three weeks post-grafting, all plants were re-examined for visual symptoms and were tested by real-time PCR. For the latter test, a leaflet from a non-grafted indicator leaf was taken, RNA was extracted (modified Kingfisher RNA method) and tested using the appropriate TaqMan® assay. In all cases, no symptoms were recorded and no virus was detected. This testing procedure was repeated at 9 weeks post-grafting. While, as before, no symptoms were observed, one plant did test positive for SMoV by real-time PCR.

While overall these results are somewhat inconclusive, they do clearly demonstrate the key issues related to graft testing, in particular the failure of grafts to 'take' (this can vary; in this experiment the figure was 5 failures out of 22 = 23%) and the length of time taken. In this example, there were still no symptoms after 9 weeks, despite the fact that the grafts were carried out from known infected material and testing showed that at least one plant was infected following grafting. It is possible that symptoms might appear eventually and these plants will be maintained to follow this. However, this work does demonstrate that grafting is not an efficient testing method, especially if large numbers of tests are required and/or rapid results are needed.

3. Technology transfer

All the assays have now been transferred to the virology diagnosis sub-team and they are now trained to use the assays. Indeed the new 23S phytoplasma assay has now been adopted as the primary screen for all suspect phytoplasma samples received by the diagnosis team.

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As a result Fera are able to provide a service to the strawberry industry using the new real-time PCR methods. However, while considerable validation work has been conducted, given the lack of suitable infected material, it has been impossible to conduct sufficient comparative testing between the real-time PCR and grafting. For this reason, any testing service offered by Fera will be done in parallel using both technologies, at no additional cost. It is foreseen that this parallel testing service will be in place for at least the first season or until sufficient samples have been received to create a validation pack that would comply with ISO17025 guidelines. Once this has been achieved the real-time PCR testing method will be integrated into Fera's existing generic ISO17025 accreditation for real-time PCR testing.

Conclusions

Overall this project has delivered the development of a suite of real-time PCR (TaqMan®) assays that permit the accurate detection of nine important strawberry pathogens. In terms of assay design, the work to develop TaqMan® assays for the four nematode-borne viruses proved more problematic than it did for the aphid-transmitted viruses. This was due to the high levels of variability seen amongst these viruses, including the existence of diverse strains. However, by using different approaches to probe and primer design, these issues have been overcome and a full set of assays has been designed and validated against a range of isolates, both *in silico* (i.e. published sequences) and *in vivo* (i.e. using isolates obtained from collections from the UK and around the world). Using this approach all assays have been effectively designed against at least 12 isolates but in certain cases over 30 (i.e. ArMV and the phytoplasma assays). When combined with the further testing conducted using infected strawberry material, this gives us confidence in the ability of the assays to reliably detect virus in strawberry plants.

In addition to the design and validation of assays, methods for allowing the efficient extraction of RNA and DNA from strawberry have also been identified. It has been found that all strawberry viruses, including the DNA virus SVBV, can be efficiently tested for using only an RNA extraction method. The selected RNA extraction method (based on an automated and hence cost-effective system) has been further adapted to give reliable extraction specifically from strawberry plants. The quality of both DNA and RNA from strawberries can be effectively tested using an internal control assay; an important consideration for ensuring reliability in routine use.

The major advantage of real-time PCR testing over grafting is time. While grafting can take up to 12 weeks (before symptoms appear), real-time PCR can be completed within days. This offers significant advantages in terms of decisions making, especially where material is infected and new material needs to be sourced. An additional advantage of TaqMan® over grafting is where mixed infections occur. The symptoms seen during grafting are difficult to decipher, and in reality only detection and not identification is possible using this method. In contrast using TaqMan® both detection and identification can be reliably achieved.

Future developments

<u>Knowledge transfer</u>

Further efforts will be required to publicise the work of this project to the industry. Due to timing and the fact that validation work was still ongoing, it was not possible to offer anything at the main soft fruit industry trade shows in either June (Fruit Focus) or October (National Fruit Show) of last year. However it is intended to rectify this situation and we will be in contact with the HDC to offer our attendance at these or other events (as appropriate). No further costs will be requested.

<u>Development of service with industry</u>

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Now that the core-science has been done, it is critical that this new technology is offered to the industry. Fera is currently developing a plan, which they will be presenting to key industry partners in May 2009. Initially this will involve those involved in nuclear stock, including those involved in breeding (e.g. Meiosis and EMR). Further cascading down to propagators is envisaged. In part this would be achieved by making the new testing service available as part of the PHPS. Further discussions will be held with those in the PHSI to see how this might be achieved, to have maximum positive impact and disease control, while not adding a heavy additional cost burden to the scheme.

As mentioned before, this will initially be offered as a no-extra cost, parallel-testing service with both real-time PCR and grafting. Once sufficient comparative validation data is obtained, then a rapid service based on real-time PCR alone will be maintained.

Integration with other real-time PCR tests

With other real-time PCR tests available for the detection of pathogens such as angular leaf spot and crown rot, this presents the possibility to develop a more integrated pathogen-screening package. As described above, discussions will be held with key industry partners to identify exactly what services they require and how an integrated package might be developed.

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Appendix 1: Results for known positive isolates received from reference collections

Tables contain TaqMan® results expressed as Ct values, each isolate was tested twice and has two Ct values. The lower the Ct number the stronger the positive result. Results of Ct 40 are negative.

A. Aphid-borne viruses

Source of isolate	Isolate	Ct values
	SCV	
Joseph Postman (USA)	9014	20/21
Claudio Ratti (Italy)	374-F	31/31
	36-MA5	26/24
	20	23/24
	31	20/20
Lisa Ward (NZ) orig. USA	1	24/24
	SMYEV	
Joseph Postman (USA)	9085	16/18
Claudio Ratti (Italy)	MadGot1	20/20
	MadGot2	19/19
	8	16/16
	30	13/12
Lisa Ward (NZ) orig. USA	2	19/19
	SMoV	
Joseph Postman (USA)	9015	12/13
	9032	16/16
Claudio Ratti (Italy)	8	17/18
	18	12/14
	30	22/21
	31	13/14
	32	13/13
Lisa Ward (NZ) orig. USA	5	22/21
	6	21/21
	11	17/16
Fera	1	22/21
	6	22/24
	SVBV	
Joseph Postman (USA)	9093	25/25
Claudio Ratti (Italy)	5	27/27
	8	17/18
	27	30/31
	30	15/16
	31	13/13
	32	14/15
Lisa Ward (NZ) orig. USA	3	25/25
Fera	A	23/24
	B	25/25

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B. Nematode-borne viruses

Source of isolate	Isolate	CT values
	RRSV	
DSMZ	PV0429	17/17
Lisa Ward (NZ) orig. Scotland	7	14/14
	ArMV	
DSMZ	PV0215	16/16
Joseph Postman (USA)	9094	26/28
Robyn deYoung (Canada)	CPVC-0017	19/20
Fera	20715151	30/29
	14/07/2005	31/28
	20711736	20/22
	20711980	27/29
	12387	28/33
	12386	30/28
	20706379	30/30
	Quinoa	21/22
Lisa Ward (NZ)	10	33/35
	TBRV	
Lisa Ward (NZ) orig.DSMZ	TBRV (DSMZ PV-0521)	26/24
	TBRV (DSMZ PV-0070)	12/12
	TBRV (DSMZ PV-0179)	19/19
	TBRV (DSMZ PV-0191)	11/11
	TBRV (DSMZ PV-554)	17/17
Robyn deYoung (Canada)	TBRV (CPVC-0083, Lucas)	28/28
Fera	Potato 13/10/00	13/13
	SLRSV	
DSMZ	PV0247	15/16
Fera	20515600	15/15
	20514302	28/28
	20512035	15/14

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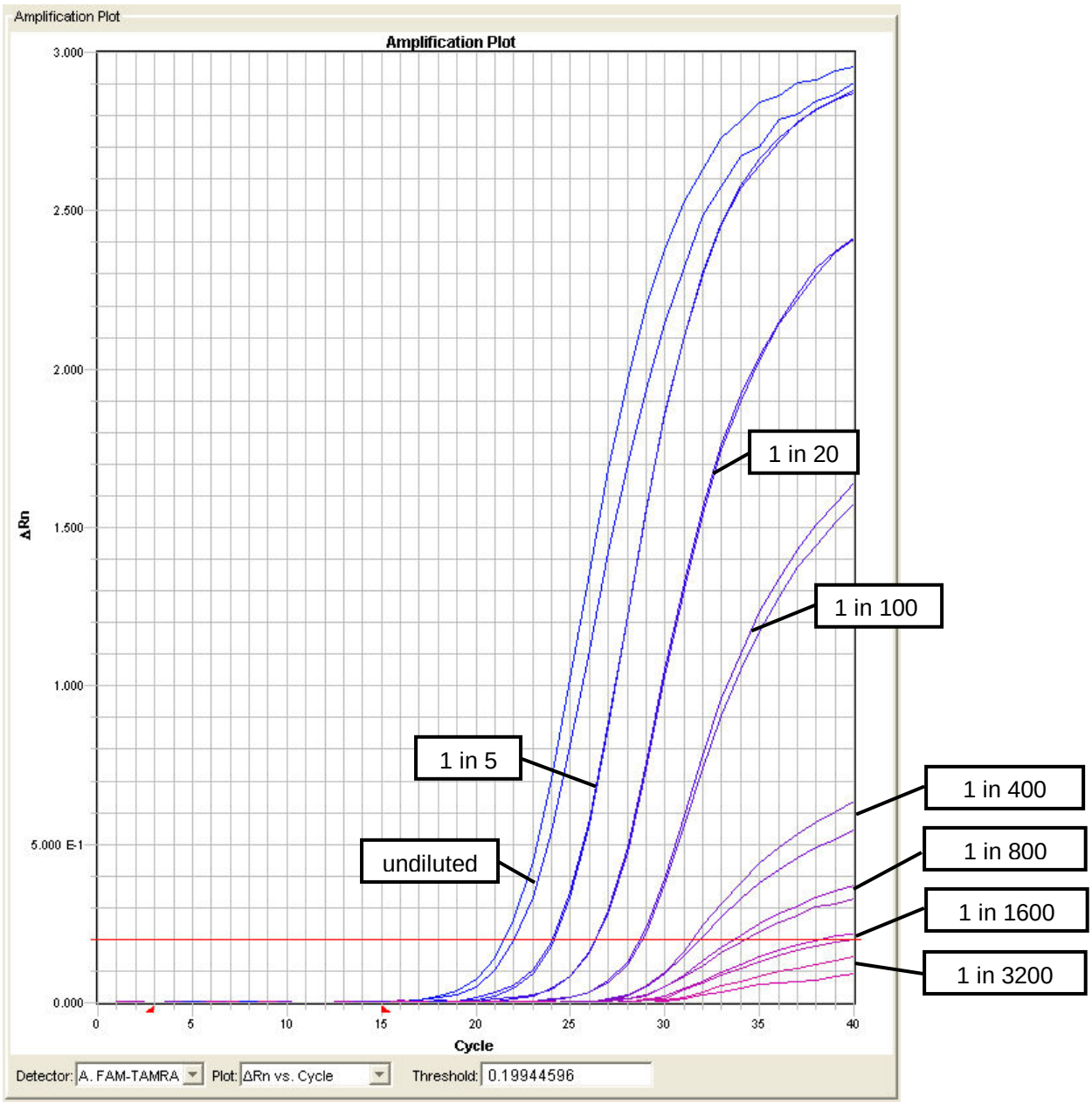
Appendix 2: Results from dilution series using SMoV-infected strawberry leaf material

TaqMan® results using SMoV assay for a 1-5 dilution series produced from infected sap diluted in uninfected sap. All extracts were tested in duplicate. The red line indicates the fluorescence baseline; results below this line after 40 cycles (Ct 40) are negative.



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