



Final report

**The validation of virus-specific real-time RT-PCR assays
for commercial use in the potato industry**

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EXECUTIVE SUMMARY

In South Africa, the routine testing of potato tuber samples for virus determination as part of the certification process is currently done by way of the DAS-ELISA method, specifically for *Potato virus Y* species (genus *Potyvirus*, family *Potyviridae*) and *Potato leafroll virus* species (genus *Polerovirus*, family *Luteoviridae*). Although the ELISA method is typically simple and inexpensive, and particularly suited to use in a high-throughput routine testing regime, the reliability of the results is inadequate when testing newly infected, freshly harvested potato tuber material. To substantially improve the level of reliability, tubers are allowed to mature so that dormancy may break and sprouts may develop. Actively dividing material in non-dormant potato tubers supports virus multiplication, which leads to an increase in the virus load, and subsequently a more reliable ELISA detection of the virus. Unfortunately, the dormant period in some cultivars (long-term growers) takes up to two months, and sometimes even longer to pass, which heightens the risk to the grower striving for the production of good-quality planting material.

A more sensitive method would allow for the testing of dormant, fresh tubers, without it being necessary to break dormancy so that virus levels would increase with the growth of sprouts. Such a method is the nucleic acids-based polymerase chain reaction (PCR) method, and variations thereof. A decision was made to design, optimise, validate and implement a hydrolysis probe-based real-time PCR (RT-PCR) method (the so-called Taqman assay) for potential future application in Potato Laboratory Services (PLS), for the testing of freshly harvested tubers in a reliable manner. Based on the validation for commercial use, the application of the test can be extended to leaf material, *in vitro* plants, mini tubers produced in greenhouses, and tubers harvested from the field at different stages of the production process.

This project entailed not only the design, optimisation and implementation of primers and probes suitable for use in a Taqman assay, but also the design, optimisation, implementation and validation of the composition of the reagent mixes, the thermal cycling conditions, the extraction method and the customised process, which resulted in an additional methodology for use in Potato Laboratory Services. Extensive validation was conducted to compare the currently used ELISA regimen with the proposed PCR-based methodology, by testing actual samples of all types using both methods. Briefly, the results are as follows:

- 1 100% correlation between the ELISA and PCR when *in vitro* material is tested for the presence of PVY and PLRV.
- 2 100% correlation between the ELISA and PCR when G0 material harvested from greenhouses is tested for the presence of PVY.
- 3 94.7% correlation between the ELISA and PCR when G0 material harvested from greenhouses is tested for the presence of PLRV. The occurrence of the virus in G0 material is highly unlikely. The testing of a sample that did indeed test positive for the presence of PVY and PLRV aided in the comparative testing of the two methods.
- 4 It was confirmed that in terms of primary (in-season) infection, the PCR is more effective than the ELISA when it comes to detecting virus infection occurring later in the season.
- 5 Where secondary infection (infection that originated from the mother tuber) is evident, the correlation between the PCR and ELISA is dependent on the distribution of the virus in the plant, and the subsequent accurate sampling of infected material. Correlation of 100% is possible and has been indicated.

It can be concluded that the customised PVY- and PLRV-specific PCR-based process, which includes a Taqman assay, is a process with increased sensitivity that is able to facilitate the accurate enumeration of virus-infected tubers in samples. In contrast to the ELISA, dormant tubers can be tested using the PCR. The Taqman assay has the ability to detect late-season infection much more accurately, and in a certification set-up has the advantage of surpassing the ELISA in terms of predicting virus content in seed lots, under production conditions of increased virus and vector pressure.

1. INTRODUCTION

Sustainable potato production in South Africa, as elsewhere in the world, relies on a supply of healthy, good-quality planting material. Parameters that are used to qualify planting material include, but are not limited to, the presence of pathogens. Viral pathogens could disqualify the product's marketability by causing unwanted visible symptoms such as the necrotic lesions of potato necrotic ringspot disease (PNRD), or could disqualify seed tubers due to potential yield losses in the progeny crop. It is therefore important that the virus content of potato seed tubers is predicted as accurately as possible, not only by relying on visual inspection, but also by testing representative samples from tuber seed lots. Visual inspection of the crop in the field is useful for predicting secondary infestation of pathogens that are easily recognisable and identifiable. However, recent cultivars bred for virus resistance and tolerance can lead to a weaker manifestation of typical symptoms, making detection and identification difficult.

The advantageous use of molecular methods in the potato industry, in specific nucleic-based methods such as PCR, applied in the detection of pathogens, is illustrated in the results of several existing research projects funded by Potatoes South Africa (PSA). In addition, the needs and demands of potato growers further accentuate the necessity of having commercially useful and pathogen-specific molecular assays for the accurate and precise detection of potato disease-causing agents (pathogens).

Potatoes are produced in several geographical regions in South Africa, located in areas ranging from a winter rainfall climate to a tropical summer rainfall climate. Different climatic regions make it possible to produce potatoes throughout the year, thus satisfying the market demand for fresh potatoes, and resulting in seed potatoes being required throughout the year. Certification therefore takes place prior to storage, and not after storage as in many other potato-producing regions around the world. Potato seed certification is a process that takes several months to complete, with the duration of this process influencing the availability of planting material over time. In shortening the duration of the certification process by using a test method that can be executed on freshly harvested tubers instead of sprouted tubers (as elaborated on further in this report), the supply of certified planting material can be satisfied. This also leaves the producer with enough time to make management decisions based on market trends.

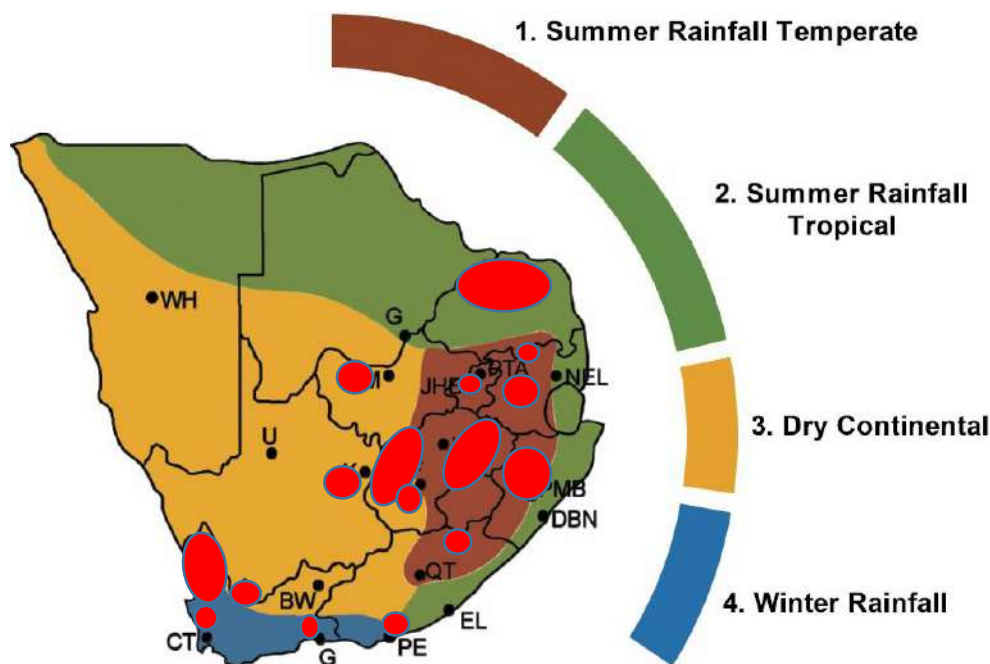


Figure 1. Climatic regions of South Africa, showing the main potato production regions

The invention of the polymerase chain reaction (PCR) method in 1985 by the research group of Kary Mullis (Saiki *et al.*), has revolutionised almost all fields of biological sciences, especially those in molecular biology. In short, PCR can be defined as a series of repeated temperature-dependent reaction steps whereby the DNA or RNA (genetic material of all living things) of an organism (living or dead; animal/human/plant/micro-organism), is multiplied million-fold to detectable levels, enabling the technique to be used to detect small quantities of any such organism. The technique is highly specific and can be used to detect a target DNA copy from a mixture of DNA molecules.

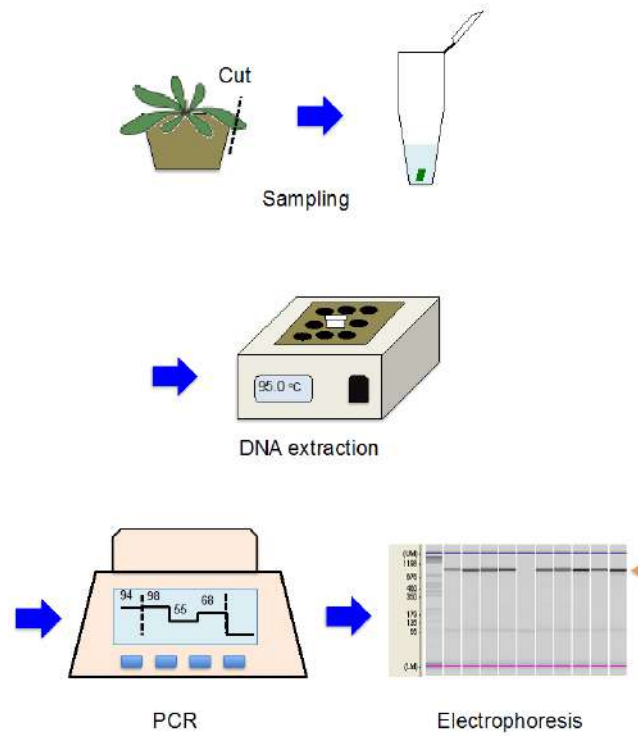


Figure 2. Simplified PCR process

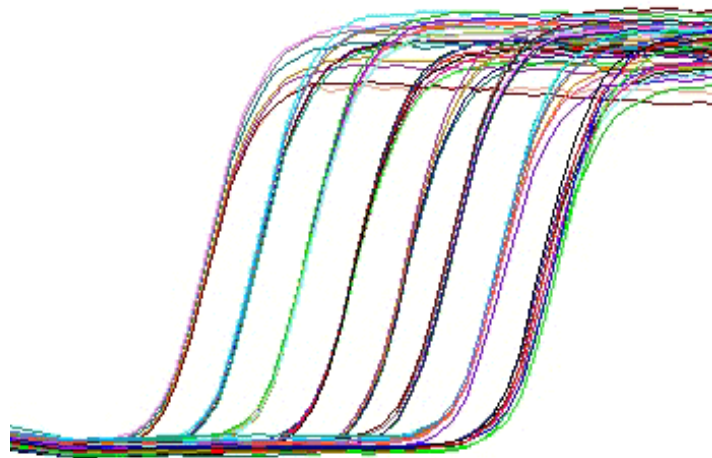


Figure 3. Real-time PCR results depicted as sigmoidal curves, using computer software instead of bands on a gel, as for end-point PCR during electrophoresis

An appropriate extraction method, whereby the DNA/RNA is separated from the other cell components, is employed for the organism in question, as well as the matrix (material in which it occurs, such as leaves/tubers/soil/blood/tissue), from which it is to be extracted. A PCR reaction is performed on the DNA/RNA extracted, which entails the cyclic repetition of temperature-dependent steps in order to multiply the target pathogen's DNA/RNA. The results of the conventional end-point PCR are analysed through electrophoresis (Fig. 2), while the results of the next-generation real-time PCR are monitored in real time, as it happens (Fig. 3). Real-time PCR, also known as quantitative PCR, has been innovatively adapted to various applications, one of which is the Taqman™ PCR, whereby hydrolysis probes are employed to allow fluorescent detection of the target DNA/RNA over time (Fig. 4).

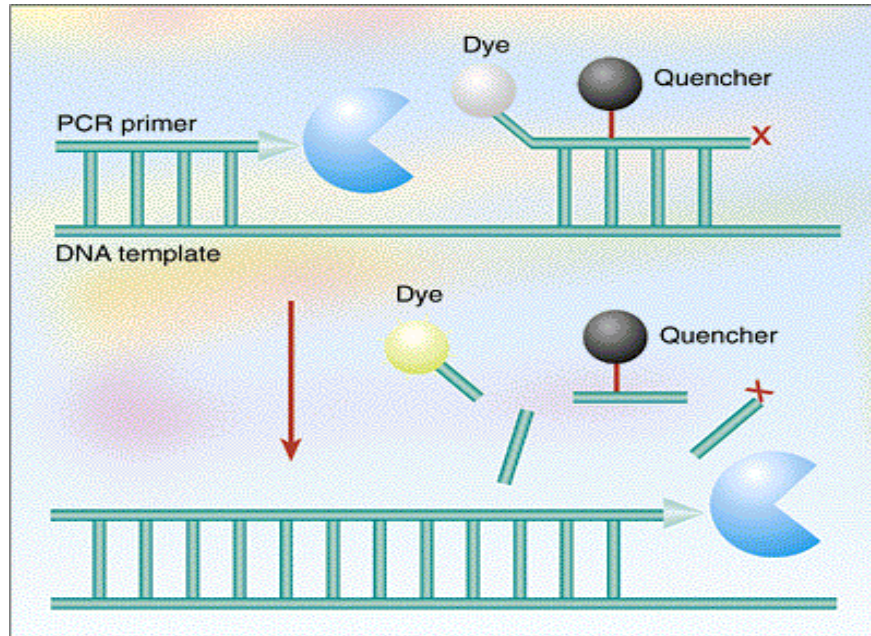


Figure 4. Taqman probes, labelled with a fluorescent dye on one end and a quencher molecule on the opposite end, anneal to the specific template DNA; Upon elongation of the target sequence by the polymerase enzyme (depicted as a blue PACMAN figure), the polymerase causes detachment of the fluorescent dye from the probe, resulting in a fluorescent signal that is detected by the PCR machine and reported during the PCR reaction as a sigmoidal curve (in Fig. 3)

A result can be considered positive when crossing the threshold at the cycle threshold (CT) (Fig. 5). The CT value is a relative indication of the quantity of template in the sample. Real-time PCR allows for a shorter reaction time and more sensitive detection, while the hydrolysis probe used in a Taqman assay improves specificity in addition to the specific primers already used.

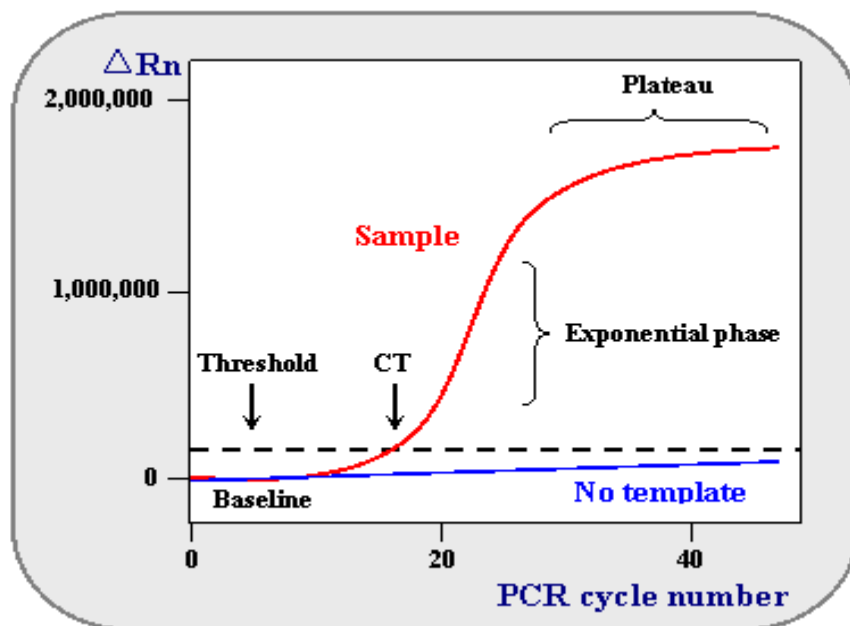


Figure 5. Real-time quantitative plot indicating the development of a fluorescent signal during the course of the reaction; For a negative reaction, no curve would develop; CT value could be used as a relative indication of the concentration of the target DNA/RNA

PCR has become an essential technique in many common research and diagnostic procedures. It has been proven that this technique has a wide variety of applications as a highly sensitive and specific procedure able to detect anything containing nucleic acids (DNA and RNA). For selected applications, due to the specificity, it is necessary to develop and optimise different reaction conditions (i.e. customisation). Customisation demonstrates that with minimal changes to the reaction conditions and procedure, the specificity and sensitivity can be adjusted, making it relatively easy to design a reaction best suited to the specific needs involved.

Currently overshadowing all other needs is the need in the environment of the certification of seed potatoes, to have a fast turn-around of test results relating to the virus status of seed lots. Although the South-African Seed Potato Certification Scheme is not compulsory, ware and seed growers can benefit from a test regimen where results are reported earlier in terms of the supply and demand for good quality planting material in time. For most growers of seed potatoes, the timeframe within which virus tests are currently being conducted is too long. Growers wait longer than 28 days for results to be generated and reported, due to the fact that the dormancy of the tubers must be broken, and sprouts must reach between 3 mm and 5 mm in length, for viruses to reach sufficient intracellular levels to enable the detection and accurate prediction of the virus content of a seed lot. The potato leaf roll virus (PLRV) and potato virus Y (PVY) are two viruses that are tested for on a routine basis. Although producers that market produce as ware potatoes and not seed potatoes are not affected by the turn-around time of tests to the same extent as seed producers, ware producers do submit diagnostic samples to laboratories from time to time. In these cases, tuber samples submitted also need to be sufficiently sprouted before being tested in order for a reliable test result to be reported after testing. Ware producers are also affected by the duration of the test process in that the quality of the ordered seed is on risk during a process that takes too long. In addition to a shorter turn-around of results, the increase in sensitivity of the PCR-based assays, allows a better prediction of virus content in batches of seed potatoes to the benefit of any producer of potatoes.

Since the early 1990s, the PLS group of laboratories has been testing the virus content in representative samples of seed tuber batches, by means of optimised assays developed as part of the group's customised processes. The development and

optimisation of such customised processes was undertaken by role players actively involved in the industry – specifically the certification process and laboratory environment. Certification of seed potatoes can only be completed on receiving the results of the tests executed by the ICCSP approved laboratories (PLS).

When testing tubers by means of the customised ELISA process, the submitted sample is incubated under controlled conditions to allow for the breaking of dormancy, until a minimum of 28 days has passed and the minimum number of tubers required per sample has sprouts that are more than 3 mm long. The rationale behind breaking the dormancy and allowing tubers to sprout is that a cell multiplies viral offspring to levels high enough to facilitate detection of such viruses in actively growing tissue, and by employing a more sensitive method, such as PCR, tubers can be tested fresh (dormancy not yet broken), while still allowing for the virus load to be detected.

During the period of dormancy, tubers are at risk of rot and insect damage, and the grower/seller of the seed tubers is at risk of losing a potential buyer who would rather source the product elsewhere. It is therefore in the best interests of the growers (both seed growers and ware growers) to have virus test results available within a much shorter period of time.

Molecular methods of testing, being in general more sensitive than the ELISA test (able to detect lower levels of the target organism), enable the testing of tubers before dormancy has been broken. This allows growers to trade much earlier, thus reducing the risk of damage, with the added advantage of having a significantly more accurate determination of the virus content. An early decision to market tubers as ware potatoes, rather than as seed, can save the grower a great deal of money.

In order to extend any developed assay from a research environment onto a commercial scale, and to apply it in the potato production industry as a detection method (whether for seed production or ware production), the adequate validation of such assays is essential. Only a properly validated assay can be incorporated in a test process and customised for commercial use, with limited or no risk of falsely interpreting any results or inaccurately predicting the virus content of a seed crop. With regard to RT-PCR, the risks involved in terms of obtaining false results due to the instability of the template, the presence of degrading enzymes and the ease of contamination due to extreme sensitivity, proper validation is critical prior to the use of assays on a commercial scale.

1.1 Motivation

Potato growers need test results at an earlier stage in the potato seed production process. PCR-based methods, which are more sensitive and specific than a range of other tests, can address this need. The aim is not to replace current methods, but to offer an additional method in situations where growers need results as soon as possible after harvest. The overall aim of this project is to provide the potato industry with one or more commercial RT-PCR assays, specific to PVY and/or PLRV, for application in the seed production or ware production of tubers.

The publication of popular and/or technical article(s) is envisaged, to serve as an informative communication tool to advise growers on the use of the different methods. It is important to establish a protocol approved by both the Department of Agriculture and the Independent Council for the Certification of Seed Potatoes (ICCSP), to be used by the members of the PLS group of laboratories under the supervision of Plantovita, the controlling laboratory in the potato industry.

An additional long-term advantage inherent to the use of these assays is the growth in knowledge related to the host, pathogen/s, and disease/s tested for and studied at PLS.

1.2 Objectives

Designing and developing the assay:

- a. Primer designs (if an unpublished assay is the method or route chosen).
- b. Reaction mix set-up.
- c. Designing the time-temperature profile.
- d. Choosing an extraction method based on knowledge of the pathogen and host (matrix of extraction).

Optimisation of the assay:

- a. Fine-tuning the reaction mix constituents.
- b. Optimising the time-temperature profile.
- c. Evaluating, comparing and optimising the extraction method of choice, based on performance.

Validation of the method:

- a. Determining analytical sensitivity.
- b. Determining specificity.
- c. Determining robustness.
- d. Determining repeatability.

Evaluating the performance of the method in a commercial set-up, in comparison to routinely used ELISA assays, in order to accurately interpret results and form opinions relating to pathogen status (method and process validation).

2. MATERIALS AND METHODS

2.1 Taqman primer and probe design

For both PVY and PLRV, genome sequences available from NCBI and GENBANK, including the international variation of currently known strains, were used to design primers and probes. Selected whole genome sequences were aligned using the Basic Local Alignment Tool (BLAST), and conserved areas were selected for primer and probe design. Primer and probe combinations were designed, and proposed individual sequences for the primer and probe sets were sent to TiBMolBiol (Berlin, Germany) for the synthesis of these oligonucleotides. The primer set specific to PVY was designed to anneal in conserved areas in the P3 gene (Figure 6), while conserved areas in the PLRV ORF3 sequence (Figure 7) were selected for the PLRV-specific primers and probes.

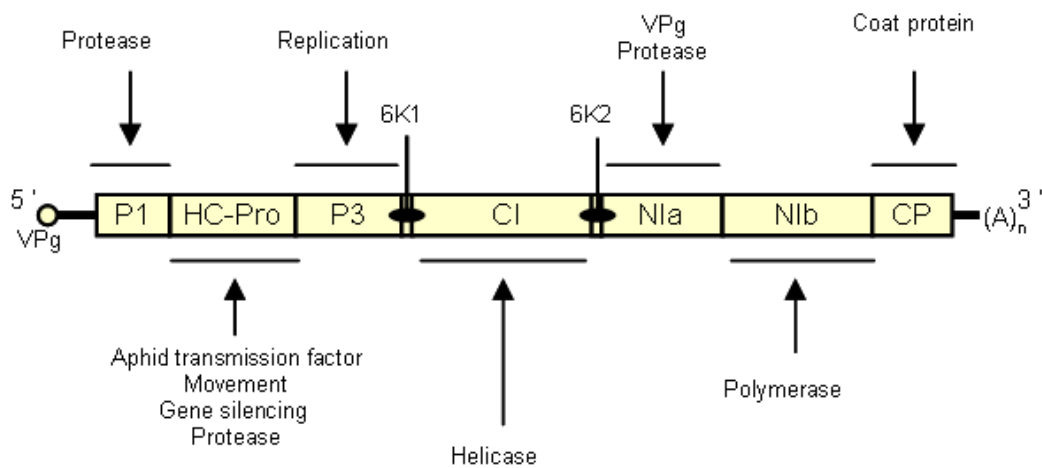


Figure 6. PVY genome organisation

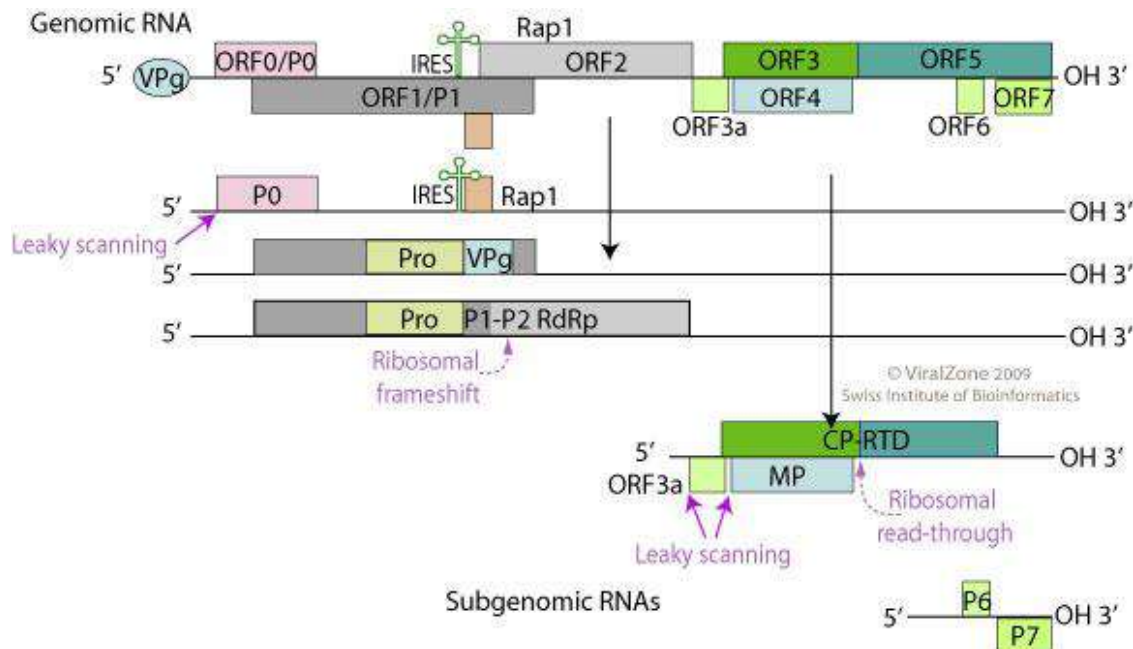


Figure 7. PLRV genome organisation

PVY-specific forward primers, reverse primers and probe are respectively: ALL F, ALL S, ALL A, ALL R and All Tm. PLRV-specific forward primers, reverse primers and probe are respectively: PLRV F, PLRV, S, PLRVA, PLRV R and PLRV Tm. Primer and probe sequences were selected to have the resultant amplicons close to (and less than) 100 bp for optimal amplification and detection. Two sets of primer and probe combinations per virus were evaluated experimentally.

The primers and probe sequences used as internal controls were as designed specifically for the 18S rRNA gene of the *Solanum* spp. by Du *et al.* (2006).

2.2 Reaction mix set-up and optimisation

Initial reaction mix components were as recommended (by the supplier of the LightCycler 480 RNA Master Hydrolysis Probe kits, Roche Diagnostics GmbH, Mannheim, Germany) in the manual accompanying the kit. The kit contains the reagents of choice to optimise and implement the real-time RT-PCR hydrolysis probe-based assay. The kit makes use of hot-start Tth DNA polymerase, while the reaction contains an Enhancer™ reagent that can, if so decided, be included in the Master Mix when annealing and amplification of the template is complicated – for example when a GC-rich template is chosen as the target template. The effect of the Enhancer™ on the outcome of the assays was investigated by including it in the initial setup and subsequently omitting it after completion of the reaction mix set-up.

Although a one-step RT-PCR was envisaged in a 10µl reaction volume, a decision was made to follow the recommendations of the supplier and thus save time on the validation process – hence, a volume of 20µl was used. Subsequent to the optimisation of the reaction mix set up and the achievement of effective amplification, the volume was reduced to 10µl as a means of lowering the cost per routine reaction.

A decision was made to use both nuclease-free water (no template control) and an uninfected material control as the negative controls. Primers specific to the target pathogen, as well as the internal control 18S rRNA primers and probes, were added so as to enable the monitoring of the effectiveness of the extraction procedure on the negative material control. Positive controls were always extracted template RNA instead of cloned sequences, in order to protect the process from the risk of cross-contamination when using plasmid controls.

The assay was set up using a LightCycler 480 (Roche Diagnostics GmbH (Mannheim, Germany), with the intention of executing the project in a 96 well format, allowing for comparison with the ELISA assay currently in use.

2.3 Design and optimisation of the time-temperature profile

Cycling conditions were initially set up as follows: 63°C for 3 minutes to allow for the reverse transcription of the RNA template to DNA, followed by the denaturation of the template at 95°C for 30 seconds, then the actual PCR in 45 cycles (95°C for 15 seconds, 60°C for 30 seconds), and ultimately the final step - cooling the block of the instrument at 40°C for 10 seconds. According to observation, no significant amplification occurred after 35 cycles, hence the decision to reduce the number of cycles to 35 per assay.

Final optimised cycling conditions were set up as follows: 63°C for 3 minutes, then 95°C for 30 seconds, followed by 40 cycles at 95°C for 15 seconds, and finally at 60°C and 72°C for 1 second respectively. On completion of the cycles, a cooling step was included for 10 seconds at 40°C, while annealing temperatures of 56°C, 58°C and 60°C were investigated.

Both the one-step method and the two-step method (where the reverse-transcriptase step was executed separately from the PCR) were considered for routine use. The two-step method included the use of the Roche Diagnostics GmbH (Mannheim, Germany) Transcriptor First Strand cDNA Synthesis Kit to prepare cDNA from the viral RNA, as well as the Lightcycler 480 Probes Master Kit for the amplification of DNA.

In performing the two-step method, the cDNA synthesis step was carried out by firstly preparing the 13 µl primer mix (chosen as the forward primer in each assay) per reaction on ice in volumes adequate for the number of samples. Once the primer had been prepared, the primer mixes were denatured at 95°C for 1 minute. The Master Mix was prepared by adding together the concentrated buffer until achieving a final concentration of 8 mM MgCl₂, RNase inhibitor at 20U final amount per reaction, nucleotide mix at 1 mM final concentration and the reverse transcriptase at 10U final amount per reaction, all in a final reaction volume of 7 µl. The Master Mix was added to the primer mix and samples were subsequently added, after which incubation occurred for 30 minutes at 55°C. The reverse transcriptase reaction was inactivated by means of an incubation step at 85°C for 5 minutes.

Inactivation was halted by placing reaction vessels on ice. The cDNA so obtained was stored at -20°C after use. The PCR step was then performed using the cDNA as the template and the LightCycler 480 Probes Master as the kit. As per the method prescribed for the LightCycler Probes Master Kit, 5 µl of the cDNA was added to 10 µl of the double-concentrated kit reagent buffer and 2 µl of the 10x primer stock solution. To the reaction was added 3 µl nuclease-free water, for a final reaction volume of 20 µl. The PCR step consisted of a denaturation step at 95°C for 5 minutes, followed by 50 cycles at 95°C for 10 seconds, at 60°C for 30 seconds, at 72°C for 0.1 second, and finally a cooling step at 40°C for 5 minutes.

2.4 Development and optimisation of the extraction process

The application of the RT-PCR hydrolysis probe-based assay was intended to enable the large-scale testing of potato tubers for specific pathogens, as well as the *ad hoc* testing of samples containing limited amounts of plant material, such as mini-tuber samples (*ad hoc* amounts), *in vitro* plants and diagnostic samples. The availability in the laboratory of extraction methods that would facilitate both *ad hoc* samples and high-throughput samples was therefore essential. Several potential extraction methods were therefore evaluated for use. The extraction of *ad hoc* samples can be performed manually with little or no mechanical intervention, while the high-throughput samples (so-called field samples) can only be extracted with the intervention of an automated or semi-automated system.

Three methods of extraction were considered, namely a simple manually prepared buffer-based method, a commercial buffer-based method, and a semi-automated method, as listed below:

- 1 GES method (Bellstedt *et. al*, 2010), whereby a glycine-EDTA-sodium chloride buffer is prepared in-house as the basis of lysis and extraction, with an additional grinding buffer.
- 2 Tri-Pure (Roche diagnostics GmbH (Mannheim, Germany), as an isolation reagent method.
- 3 Magnetic bead-based Kingfisher™ system (Thermo Fisher Scientific), using suitable buffers included in the kit. In evaluating the Kingfisher™ system, two different kits were employed, namely the Kingfisher™ Pure RNA Plant Kit, and the Kingfisher™ Total RNA Kit.

In the case of all these methods, nuclease-free water was used as the eluate.

The sample material was all naturally infected (through either the primary infection or secondary infection of potato plants). The tuber material included in the evaluation of extraction methods was fresh or sprouted, while the leaf material used in this particular stage was of a known virus status and previously freeze-dried.

The effectiveness of the extraction methods was evaluated by comparison based on OD (absorbance) values of the extraction products, as well as the efficacy of the amplifications on the extracted RNA as measured by a comparison of found cycle threshold (CT) values. For RT-PCR, a reaction is declared positive when a fluorescent signal accumulates after a number of cycles above a threshold. The threshold is, in simple terms, the background level of fluorescence (intrinsic fluorescence of the probes). The CT (cycle threshold) is defined as the number of cycles required for the fluorescence signal to cross the threshold. CT levels are inversely proportional to the concentration of the target sequence in the sample.

A MaestroNano spectrophotometer (NanoDrop Technologies) was used to measure the absorbance values of total RNA post-extraction at ratios of A_{260}/A_{230} and A_{260}/A_{280} , in order to test for contamination by polyphenols, carbohydrates and proteins in the eluate.

During potato production, and specifically during certification, tubers are harvested from the field and subsequently sampled. In the laboratory, a working sample is taken from the submitted sample by removing specific virus-containing parts. The distribution and titres (amounts) of PVY through the tuber are generally more even than in the case of PLRV, although using the PLRV-specific ELISA, PLRV allows for better detection in dormant tubers (Cox & Jones, 2011; Mortimer-Jones *et al.*, 2009; Rek, 1987). The stolon end of each tuber was removed as part of the working sample since both PVY and PLRV is phloem-bound viruses. A piece was removed from the stolon end of each tuber using an in-house-designed and locally manufactured, spring-loaded coring tool. The weight of the working sample from each tuber was on average 0.1g. Pieces from five tubers were pooled into a sieve-sectioned, labelled Bioreba sampling bag (Bioreba, Basel, Switzerland). All items of equipment used were dipped before and after sampling in 3% NaOCl, then rinsed in distilled water, to prevent cross-contamination between sub-samples.

Submitted leaf material from *ad hoc* samples (diagnostic samples) was cut to obtain a working sample through the removal of symptomatic tissue. For statistical and official samples, the entire submitted composite leaf sampled in the field was homogenised and used as the working sample.

Subsequent to sampling, a homogenisation buffer (developed in-house) was added to all working samples in Bioreba bags, to prevent oxidisation of the sample and to limit degradation of the target nucleic acid. Homogenisation was investigated using 1 ml, 2 ml, 3 ml and 5 ml homogenisation buffers; by omitting the anti-foam emulsion, and by replacing the homogenisation buffer with nuclease-free water. The effect on the material of β -mercaptoethanol (Sigma-Aldrich, South Africa) as an antioxidant, as well as the inclusion of guanidine thiocyanate (Sigma-Aldrich, South Africa) as a chaotropic agent to denature any degrading enzymes, was also monitored.

The homogenisation buffer was composed as follows, with the pH set to pH 8 prior to use:

- 0.25M Sodium chloride (Merck)
- 0.05M Tris-Cl (Merck)
- 0.02M EDTA (Merck, added from a pH 8 stock solution of 1M, or added as dry powder)
- 1% SDS (w/v; Sigma-Aldrich, South Africa)
- 4% PVP (360 000MW, w/v; Sigma-Aldrich, South Africa)
- 2% (w/w) β -mercaptoethanol added immediately before use
- 0.01% (w/w) anti-foam emulsion (Sigma-Aldrich, South Africa) added immediately before use.
- 6M guanidine thiocyanate added immediately before use to a final concentration of 4M

After adding the homogenisation buffer to the working sample, the contents of the Bioreba bags (sub-samples) were macerated using the Homex 6 grinding device, also from Bioreba (Basel, Switzerland).

The homogenate was then used in all subsequent extraction steps, with the exception of the GES method, where the grinding buffer was used as part of the published method. The grinding buffer used in the GES extraction method was composed as follows:

- 15 mM Na₂CO₃
- 35 mM NaHCO₃
- 2% PVP (w/v)
- 0.2% BSA (w/v)
- 0.05% Tween 20 (v/v)
- 1% Na₂S₂O₅.

2.5 Validation of the designed assay

The extraction method of choice, to allow for the high-throughput testing of samples and the comparison of the ELISA vs. the RT-PCR, was the Kingfisher™ Pure RNA Plant Kit (Thermo Fisher Scientific). The high throughput and quality of the product made it the obvious choice.

The extraction of naturally infected material was done using the in-house-prepared homogenisation buffer before performing the magnetic bead-based method using the Pure RNA Plant Kit. To validate the designed assay, the specificity, sensitivity and repeatability were determined using this method, with the optimised reaction composition and the optimised thermal conditions. Repeatability was investigated by extracting three repeats of a naturally-infected leaf sample and including seven repetitions of each of the extracted repeats in the RT-PCR.

Dilution series were prepared using total RNA extracted from naturally infected leaf material until an effective dilution of 10⁻⁸ was achieved. Five repetitions were performed to determine the average CT value for each dilution.

To investigate the specificity of the assay, infected and non-infected leaf and tuber material was tested using both the ELISA and the newly designed RT-PCR. Furthermore, tubers that were naturally infected with strains of PVY (PVY^{NTN}, PVY^{WILGA}, PVY^O, PVY^N), and/or freeze-dried leaf material containing mixed infections of PVY^O, PVY^N, were subjected to extraction and RT-PCR analysis.

2.6 Validation in terms of the commercial use of the process

To validate the commercial application of the RT-PCR hydrolysis probe-based assay, 154 individual tubers were tested with both the ELISA and the RT-PCR, planted individually in pots in the greenhouse at Plantovita, after which leaves were sampled and tested for both PVY and PLRV using the PCR. This was done with material that was produced under high virus pressure, thus the assumption could be made that the majority of the positive results were the result of primary infection (in-season infection caused by the vectors), while secondary infection (from the mother tuber) in a smaller number of tubers could be expected. Tubers were sampled from plants with or without visual leaf symptoms of PLRV and/or PVY. Furthermore, 320 tubers that had already been tested by ELISA in another regional laboratory were planted in the greenhouse, after which leaves were sampled and subjected to the RT-PCR. In total, 474 tubers were subjected to the RT-PCR, either by testing of the tubers or testing the leaf material from tubers that had already been tested using the ELISA currently used in PLS.

In addition to the tubers individually tested, samples of tuber material that were already certified as early-generation material were obtained from growers. This material was used to optimise the handling and processing of tubers and to compare the results from testing the fresh tubers with the results of tests when the leaves of these tubers were sampled and tested after planting and harvesting in the greenhouse.

In addition to testing tubers individually, the ELISA results and the PCR results had to be compared where tuber pieces were pooled. Working samples were taken from sprouted tubers, and both an ELISA and a PCR were performed for these samples. In this way, the exact same tubers could be used for both assays. Seven samples of sizes ranging from 20 to 400 were tested in this way. This means that the tubers were sprouted before testing could commence, otherwise the ELISA would not have complied with the customised process and the comparisons would not have been realistic.

In comparing the ELISA and PCR results when fresh tubers were tested (direct tuber testing), parallel testing was performed using the ELISA and PCR on different submitted samples from the same seed lot. Quantities of tubers in these 15 samples ranged from 200 to 400 tubers.

In total, of the 32 samples tested, 17 parallel tests were performed on sprouted tubers, 15 were performed on fresh tubers, and for 13 samples the exact same tubers were used for both tests. Of the 32 samples, 18 were planted in the greenhouse after the initial RT-PCR, so as to subsequently sample leaves for the repetition of the RT-PCR. The purpose of planting previously tested tubers in the greenhouse was to investigate whether the RT-PCR on the tubers would falsely predict the percentage of tubers giving rise to infected plants.

3. RESULTS

3.1 Taqman primer and probe design

Two sets of primers and a probe for both PVY and PLRV were synthesised by TibMolBiol using suggested accessions in GENBANK and proposed regions of annealing. Based on the primer set and the location of these on the genomes of the target viruses, corresponding hydrolysis probes were also synthesised. As a first measure of specificity, a BLAST analysis of the primer and probe sequences, against internationally available sequences of known strains, indicated only complementarity to target sequences on the genomes of PVY and PLRV respectively.

It was found that both sets of primers with the sequence specific probe could lead to effective amplification and detection of the target sequences, with no significant difference between the efficacy per reaction in the different sets. As singleplex assays, the pathogen-specific probes were labelled in both instances with 6-FAM fluorescent dye at the 5' end of the oligonucleotide, while for PLRV the probe was labelled on the 5' end with a Black Hole Quencher (BHQ™). In addition, the PVY-specific probe was double-quenched with IOWA BLACK quencher (IABkFQ)™ at the 3' end, as well as a ZEN™ quencher in the centre of the probe. The result was better signal development and even less noise bleeding through for the PVY assays. The resultant signal-to-noise ratio was much lower. The combinations of choice are indicated in Table 3.1.

An internal control was chosen to be the 18S rRNA gene of *S. tuberosum*. The primer and probes designed specifically for the 18S rRNA gene of *S. tuberosum* resulted in efficient amplification as a singleplex assay. By including the internal control in an assay, the efficacy of the extraction could be monitored. True negative results can therefore be distinguished from false negative results, which could be the result of unsuccessful or ineffective extraction of RNA or ineffective synthesis of cDNA. The 18S rRNA-specific probe was labelled by the Cy5™ at the 5' end of the oligonucleotide with a Black Hole Quencher (BHQ™) on the 3' end.

Although all reactions are currently performed in singleplex, the labelling of probes with different fluorophores allows for multiplex assays to be set up without much difficulty. Using the fluorophores as indicated, signal detection in the wavelength range of 520 nm and 668 nm respectively for pathogen-specific probes and the internal control probe is possible.

These primer and probe combinations (Table 3.1) would allow for the specific detection of strains of PVY and PLRV currently known, while including adequate controls to monitor the efficacy of the extraction procedure performed on the sample.

Table 3.1. Oligonucleotide details for use in hydrolysis probe-based PVY- and PLRV-specific real-time RT-PCR assays

OLIGO	Length (bp)	Tm (°C)	Annealing (°C)	Amplicon length	GC content
All F	25	59.8 (58.5)	60	159 bp	46%
All R	26	56.6 (56)	60		36.5%
All Tm (double q)	30	64.6 (62.4)	60		45%
PLRV F	24	59.4 (56.7)	60	90bp	41.7%
PLRV R	25	59.4 (55.9)	60		44%
PLRV Tm	22	66.7 (61.8)	60		59.1%
18S rRNA F	19	57.5	60		57.9%
18S rRNA R	25	55.6	60		40%
18S rRNA Tm	29	62.8	60		48.3%

3.2 Reaction mix set-up and optimisation

For both the PVY- and PLRV-specific reactions, initially generic reaction mix set-ups were used to execute the real-time RT-PCR reactions in a one-step format. This generic set-up was proposed in the manual of the LightCycler 480 RNA Master Hydrolysis Probes Kit. A proposed two-step generic format was also investigated (first reverse transcription, after which the PCR was performed in a second tube/well). Both gave comparable results, with inconsistent and insignificant advantages of the two-step above the one-step method. Since the assays will be conducted in a commercial setup, the accompanying risks of contamination when a two-step assay is performed must be considered. For application in the routine diagnostic set-up, the **one-step format** is the format of choice, since handling steps in the execution of the procedures increases the risk of cross-contamination. Optimisation of the one-step format is therefore preferred above the implementation of a two-step method.

The final reaction mix setup was optimised to consist of Mn(OAc)_2 at 3.25 mM, primer concentration of 0.5 μM each and probe concentration of 0.25 μM , using the recommended amount of polymerase and 40-60 ng total RNA per reaction of 10 μl . The Master Mix is divided into the wells of a white 96 well LightCycler 480 Multiwell plate after preparation thereof.

Primer and probe concentrations can have an influence on the efficacy of the reaction, therefore variations in the concentrations of primers (both forward and reverse) and probes were investigated. Primer and probe concentrations, both as low as 0.16 μM for PLRV, did lead to amplification of the target. However, the signal-to-noise ratio was lower than for the final concentrations of primer and probe, of 0.5 μM and 0.25 μM respectively. Primer and probe concentrations of 0.25 μM and 0.12 μM respectively for PVY led to amplification and signal development, but as in the case of PLRV, the noise was such that the efficacy of the reaction was significantly diminished. It was decided that initial concentrations of 0.5 μM of both primers and 0.25 μM of the probes for PLRV and PVY would be used.

Different quantities of components, and reaction volumes of 10 μl and 20 μl , were also evaluated. Performance of the reaction in a 10 μl volume was just as effective as the performance in a 20 μl volume. The concentration of cations in the polymerase reaction buffer and Activator™ proved to be sufficient for effective amplification. Specific and sensitive amplification illustrated that for these particular assays, omission of the Enhancer™ (containing additional components that would facilitate the amplification of GC-rich templates or templates rich in secondary structures) did not adversely affect the results, and that these particular reactions can be performed in the absence of Enhancer™.

The hot-start polymerase facilitates the sensitive detection of the target RNA sequences by overcoming secondary structures in the template and by minimising mis-priming during the annealing step. The use of the hot-start polymerase (as a component in the LightCycler 480 RNA Master Hydrolysis Probes Kit) enabled the very specific amplification of the target genes of both viruses, as indicated by the binding of each sequence-specific probe and the single amplification product on an agarose gel of the expected size (results not indicated).

Due to the cost of implementing a robotic volume-handling system to pipette extremely small volumes, the 96 well system was chosen over the 384 well system. Reaction volumes and the concentration of components were adjusted to suit the 96 multiwell plate format.

3.3 Design and optimisation of the time-temperature profile

Generic thermal cycling conditions were used initially. To standardise the performance of assays, minor changes were made to the time-temperature profile. Noise, particularly in the PVY-specific assay, was resolved by including a signal detection step of 72°C for one second during the PCR, as well as double-quenched probes (Integrated DNA Technologies (IDT), www.IDTDNA.com). This additional step did not adversely affect the PLRV assay. Although 56°C and 58°C proved to also be annealing temperatures that could lead to specific amplification of the target, 60°C was chosen. This decision was based on the future use of these conditions for other pathogens, when running assays in parallel with the PVY and PLRV assays in this probe-based format, in an effort to keep the cost per assay as low as possible.

Even though the initial conditions included 45 cycles, no amplification after 35 cycles was detected. The amplification of very low concentrations of the target served to indicate later on in the validation process that cycles should rather be optimised to 40 to allow clear development of signal and clear appearance of product, while limiting noise – especially for the PVY specific assay. The 72°C for 1 s step was included to allow better capturing of the fluorescence signal.

These thermal cycling conditions will enable the user to conduct PVY- and PLRV-specific and sensitive RT-PCR assays simultaneously in singleplex (separate wells/tubes), with the ultimate aim being to establish an efficient multiplex assay in routine use, which would significantly reduce the costs involved, as well as the amount of time taken to run assays.

Table 3.2. Thermal cycling conditions for PVY- and PLRV-specific real-time RT-PCR assays

THERMOCYCLING CONDITIONS			
63°C; 3min			RT step
95°C; 30sec			Denaturation of cDNA
95°C; 10sec	35 x	Denaturation	Amplification
60°C; 30sec		Annealing/Elongation	
72°C; 1sec		Signal capturing	
40°C; 30 sec			Cooling block

3.4 Development and optimisation of the extraction process

An extraction method is an integral part of any PCR assay, therefore the choice and optimisation of such a method is crucial in the development of an assay. Since a magnetic beads-based assay results in the best quality DNA and RNA extracted, and allows for a higher throughput of samples, this was the method of choice. Evaluation of the manual methods (GES procedure, as well as the TriPure method) showed the potential of these methods for use on an *ad hoc* basis for small-scale samples occasionally received.

The **Kingfisher™ system** was chosen for routine high-throughput extractions of tuber and leaf material due to the low cost per individual well, the throughput capacity and the quality of the resultant product. Two individual kits were evaluated, as being compatible in this system (resultant yields of more than 1 ug and with A260/A280 ratios indicating the absence of or limited amounts of proteins and DNA). The TriPure method, based on the use of phenol and chaotropic salts, was compared with the magnetic beads to allow for a method to be on hand should individual samples be processed. Also, the TriPure

extraction method performed well, yielding good-quality RNA of 1.1 ug total yield and A 260/A 280 ratios above 1.8. The quality of the RNA aids in the effectiveness of the RT-PCR reaction, as well as the stability of the RNA during storage. The more stable the RNA in the extraction product, the longer it keeps during storage, allowing assays to be performed on the material in the future, such as in the resolution of disputes.

The spectrophotometric data indicates that contamination of the nucleic acids after extraction using the GES method is high. This can potentially lead to a decrease in the stability of the RNA during storage, and this product could degrade much sooner than the products of other extraction methods evaluated.

Table 3.3. Comparison of RNA extraction methods for use in the commercial application of real-time RT-PCR hydrolysis probe-based assay specific to PVY and PLRV respectively; Yield indicated as µg/ml

EXTRACTION METHOD	EXTRACTION METHODS											
	PVY control						PLRV control					
	AU 260/280	YIELD	RANGE		Average CT	Average CT	AU 260/280	YIELD	RANGE		Average CT	Average CT
			MIN	MAX					MIN	MAX		
TriPure	1.898	75.87	1.771	2.137	24.11		1.871	116.81	1.548	2.014	27.65	
GES	0.64	249.87	0.609	0.788	27.29		0.659	230.08	0.643	0.692	30	
KF Total RNA	1.429	107.41	1.018	1.866	-	-	1.429	107.41	1.018	1.866	-	-
KF Pure RNA Plant	1.35	82.94	1.006	1.527	29.32	29.04	1.616	24.49	1.071	1.72	26.41	26.3

After evaluation of the Kingfisher™ kits, the intra-assay repeatability of the Pure RNA Plant Kit was determined (Table 3.4) for both virus-specific assays. With the extracted material positive for PVY, the repeatability was better than for the PLRV-infected material, with the lowest variance at 0.588 and the highest at 4.27. In the assumption that every 3.3 CT value represents 1 log difference (10x difference) in the amount of target sequence, the data in Table 3.3.4 indicates that the amount of PLRV target in the material on which the intra-assay repeatability was performed, was higher than for PVY. Also, the variation in CT values was not even 10x in difference, indicating reliable repeatability.

Table 3.4. Intra-assay repeatability of real-time RT-PCR hydrolysis probe-based assay specific to PVY and PLRV, using the template obtained from the Kingfisher™ Pure RNA Plant Kit Method

KF PURE RNA PLANT INTRA-ASSAY REPEATABILITY								
PVY	CT		CT		PLRV	CT		CT
KF 1.1	30	KF 2.1	30		KF 1.1	25.68	KF 2.1	25.99
KF 1.2	30	KF 2.2	28.98		KF 1.2	28.87	KF 2.2	29.27
KF 1.3	29.77	KF 2.3	28.86		KF 1.3	24.32	KF 2.3	24.48
KF 1.4	29.43	KF 2.4	29.67		KF 1.4	25.5	KF 2.4	24.45
KF 1.5	29.09	KF 2.5	29.03		KF 1.5	30	KF 2.5	30
KF 1.6	27.61	KF 2.6	26.76		KF 1.6	25.33	KF 2.6	24.66
KF 1.7	29.33	KF 2.7	30		KF 1.7	25.21	KF 2.7	25.27
Average CT	29.32		29.04			26.42		26.30
Std. Dev.	0.83		1.12			2.13		2.35
Var.	0.588		1.068			3.896		4.727

To facilitate efficient extraction of total RNA, *the in-house extraction/homogenisation buffer was adapted* to aid the homogenisation of leaf and tuber material before proceeding with the extraction process. The homogenisation buffer did improve the ease with which the samples were homogenised, and aided in the efficacy of the total extraction process. A decrease in the oxidation of the input material (tubers and leaves) was visually observed. By including 3 ml of the homogenisation buffer, enough material could be recovered from the 0.5 g tissue excised from the tuber to add to the kit's lysis buffer, enabling standardisation. The addition of the anti-foam emulsion significantly prevented the formation of foam during homogenisation, thereby facilitating the pipetting of adequate amounts of homogenised material into the lysis buffer of the Kingfisher™ Pure RNA Plant Kit. The addition of guanidine thiocyanate immediately before use was discontinued, since the lysis buffer already contained sufficient amounts to inactivate the degrading enzymes in the sample. However, the 2% (w/w) β-mercaptoethanol was routinely added to prevent oxidation of the material and also to act as a reducing agent to aid in the inactivation of existing degrading enzymes such as RNases.

3.5 Validation of the designed assay

Dilution series were performed to investigate the sensitivity of the assays (diagnostic sensitivity describes the number of false negatives generated). Theoretically, a PCR assay is 1000x (3 log units) more sensitive than the ELISA. The sensitivity of any assay, however, is influenced by the type of matrix from which it is extracted, and the type of target sequence involved. The sensitivity determination of both methods, using identical input material, indicated that the sensitivity of the PVY- and PLRV-specific ELISA assays ranged from the 2×10^{-3} dilution, while the PCR enables detection lower than the 10^{-5} dilution. This difference of 1000x was expected, and better, since the PCR is theoretically 1000x more sensitive in general. Obtaining this level of sensitivity in the matrices used within the customised process enables the detection, in most instances, of virus present in fresh tuber material.

The specificity describes the amount of false positives generated. To investigate this, material infected with different races of PVY viruses and PLRV-infected material originating from different regions in RSA, representing all local variations, were tested. The assay is intended to detect only the target. In the assays performed, only the target sequences for both viruses were detected. Sequence data of strains of PLRV and PVY currently known to infect potato (PVY^{NTN}, PVY^N, PVY^O, PVY^{Wlga} and recombinations; PLRV strains) were used to design the primers and probes. The results indicated that the assays employing the designed primers and probes are specific solely to the PVY strains and PLRV strains respectively. All four primer sets proposed to, and designed and synthesised by, the service provider, proved to be specific to the target sequences and all annealed at 60°C, as indicated by amplification of the target and insignificant noise-to-target ratio for the virus sequences. Since the reactions are done in singleplex at this stage, cross-talk between fluorescence channels is not being considered.

Inter-assay reproducibility (monitored by repeatedly using the extracted material between assays on different occasions) and intra-assay repeatability (monitored by repeatedly using the extracted material in repeats on the same occasion) illustrates the repeatability, reproducibility and robustness of the assay. Both assays are repeatable and robust. Repeated freeze-thaw cycles of less than 15 x is recommended for especially the PVY RNA to prevent ultimate degradation of the template.

Negative controls containing RNA from healthy material did not give CT values below 40 cycles.

The use of plasmid material containing cloned target gene sequences is not advised in a routine laboratory setup, since the use of plasmid material as positive controls can dramatically raise the risk of cross-contamination during the process.

3.6 Validation in terms of commercial use of the process

To compare the ELISA and hydrolysis probes-based RT-PCR methods, and to validate the assays for potential use in the potato industry on a commercial scale, it was necessary to go much further than the conventional validation of an assay (such as a simple diagnostic assay validation). The actual sample comparison using both ELISA and PCR methods proved critical in the interpretation of the results for use in the potato production environment. Since the process of sampling tubers to arrive at a working sample from the submitted sample differs for each assay, the comparison of the assays ultimately had to be done using both types of assays and sample preparation methods.

The planting of tubers in a greenhouse, and the retesting of the leaves, gives an indication of false negatives generated during an initial test (for reasons such as sensitivity, specificity, virus distribution, condition of the matrix, etc.).

The following steps were included in the commercial validation process:

- 1 Testing samples on a tuber-for-tuber basis when sprouted.
- 2 Testing samples (fresh and sprouted), planted in a greenhouse, and testing the leaves to optimise the process in terms of sampling from a tuber, composition of the homogenisation buffer, pooling of tuber bits, and volumes of input material.
- 3 Testing samples using exactly the same tubers for both the ELISA and PCR, with sprouted material used for purposes of executing the ELISA.
- 4 Using both methods to test samples from the same seed lot, with the PCR performed on fresh tubers, and the ELISA on sprouted tubers.

- 5 Testing *in vitro* plants, G0, field generations (field samples and post-control samples) to include all kinds of samples and national variations that may arise during potato production, whether seed potato production or commercial production, including variation in terms of sample types in the potato production process and the certification of seed tubers.

The testing of samples on a tuber-for-tuber basis (using the same material in the case of both both the ELISA and PCR), per potato tuber, served to confirm that the PCR's sensitivity could detect in-season infection more easily, since the selected tubers originated from both symptomatic plants and asymptomatic plants from the same field. Both the ELISA and PCR successfully detected secondary infection with ease, but the primary infection (in-season infection caused by the transmission of virus by the vectors) was generally only detected using the PCR. The results of the PCR on these were confirmed by planting the tubers in the greenhouse and retesting the leaves after emergence. The role of the distribution of viruses through the plant became evident during the comparison of PCR results, in that when two tubers were tested per plant, only an average 45% for PLRV and 26% for PVY tested positive both times. In addition, the role of virus distribution through the tubers became clear, since different haulms from a mother tuber displayed differences in virus status. Infected and uninfected haulms emerged from one tuber. It was also evident that buds/sprouts removed to obtain working samples in the first test resulted in a negative outcome when leaves from the same tuber were sampled and tested.

It was possible to confirm the results of the PCR performed on the sprouted tuber. Moreover, in the case of very recent tuber infections, the tuber test can still give rise to negative results, while the emerged leaf material can give rise to positive results. The PCR result in the tuber test is, however, significantly closer to the number of positive readings obtained from the emerged leaf material.

The effects of pooling (adding pieces of a few working samples together in a well to decrease the size of the actual analytical sample, allowing for more economic testing of samples containing a high number of tubers), using pieces from five tubers, were seen when the *ad hoc* samples were tested. The tubers were subsequently planted, and the pooled leaves tested again. The condition of the tubers caused some to not emerge, thereby affecting the total number of tubers represented and tested and in the end also the calculated percentage of tubers predicted to be infected. This illustrated that when pooling small samples, the percentage predicted infection is skewed. Pooling of larger samples was not affected as much when the percentage of infection was predicted by the number of positive readings.

In testing actual sample types using both the ELISA and PCR, 100% correlation was obtained when *in vitro* material was tested, with 61/61 samples testing negative for both assays (Table 3.5). G0 tubers (mini tubers) were tested using both methods (sprouted). A total of 209 pooled subsamples were analysed, representing 1045 tubers. For PVY, 100% correlation was obtained. All tubers and subsamples tested negative for the presence of PVY. When the same tubers were tested for the presence of PLRV, two of the samples tested positive using both methods. The number of subsamples that tested positive with the ELISA was one for each sample, while the number of positive readings for the PCR was 9 and 4 respectively. This equates to a 94.7% correlation when the number of subsamples is considered. It should be noted that all subsamples that tested positive in the PCR could be linked to ELISA readings either above the cut-off value or slightly below the cut-off value. This served to prove the sensitivity of the PCR in terms of detecting low levels of virus.

Table 3.5. Comparison of PVY- and PLRV-specific ELISA assays with PVY- and PLRV-specific real-time RT-PCR assays using *in vitro* plants

In vitro Samples	Individual Plants	Samples	ELISA	PCR
1	160	4	0	0
2	120	3	0	0
3	18	9	0	0
4	220	22	0	0
5	140	23	0	0
		61		

Table 3.6. Comparison of PVY- and PLRV-specific ELISA assays with PVY- and PLRV-specific real-time RT-PCR assays when testing G0 material (mini tubers)

PVY READINGS

G0 Samples	Individual Tubers	Subsamples	ELISA	PCR
1	145	29	0	0
2	50	10	0	0
3	70	14	2	2
4	130	26	0	0
5	325	65	0	0
6	325	65	0	0
		209		

PLRV READINGS

G0 Samples	Individual Tubers	Subsamples	ELISA	PCR
1	145	29	0	0
2	50	10	0	0
3	70	14	1	9
4	130	26	1	4
5	325	65	0	0
6	325	65	0	0
		209		

In comparing the ELISA and PCR, the inclusion of actual samples taken from the field was crucial. Both sprouted and fresh material was tested. When testing exactly the same samples using both methods, sprouted tubers were selected for use as the official means of processing for the approved ELISA method. The results of this comparison are given in Table 3.7.

Samples were taken from growers located in regions with increased virus and vector pressure, so virus content was therefore expected for most of the samples. The results confirmed the ELISA detection of virus, and it was only in isolated instances (presumably in cases of very recent infection) that the ELISA and PCR results differed significantly. Keep in mind that the non-emergence of rotten tubers, the removal of infected haulms during the ELISA process, and the pooling of tuber pieces can all have an effect on the prediction of the percentage of infected tubers.

Table 3.7. Comparison between PVY- and PLRV-specific ELISA assays with PVY- and PLRV-specific real-time RT-PCR assays on samples taken from the field and processed after sprouting

SAMPLES	PVY ELISA %	PVY PCR %	PVY PCR GREENHOUSE %	PLRV ELISA %	PLRV PCR %	PLRV PCR GREENHOUSE %
1	0.00	3.25	4.46	0.00	0.00	0.00
2	0.00	0.00	0.00	0.00	0.00	0.00
3	0.00	0.00	0.00	0.00	0.00	0.00
4	0.00	1.02	0.00	0.00	0.00	0.00
5	0.00	0.50	0.50	0.00	0.00	0.00
6	1.00	0.50	0.50	0.00	0.00	0.00
7	-	0.00	0.00	-	0.00	0.00
8	1.00	1.50	0.00	2.20	2.70	4.50
9	0.25	0.75	1.25	0.25	7.86	2.00
10	0.50	0.00	0.00	2.50	6.43	5.10
11	0.00	0.00	0.00	0.25	1.80	0.50
12	0.00	0.50	-	0.00	3.30	0.55
13	0.00	0.00		0.25	1.25	

More samples from the field, which were not planted in the greenhouse, were processed, tested and then compared using only the ELISA and PCR on tubers (fresh and sprouted). A single sample was tested, making use of the same tubers, with the results reflected in Table 3.8.

As previously mentioned, samples were taken from growers located in regions with increased virus and vector pressure. Virus content was therefore expected in most samples. Table 3.8 depicts the two samples from a seed lot tested in parallel, showing the virus status prediction in each case, applying both methods to samples from a field, with the customised processes being followed. Table 3.8, for example, shows sample 3 having a major difference in the predicted amount of infected tubers of 1% using the ELISA, and 20,59% using the PCR, which can be explained by the small sample used and by the pooling of five tubers, which skewed the statistics. For sample 18, marked differences are seen due to the fact that when using the same tubers, but not testing in parallel (the PCR and a second ELISA was executed much later than the initial ELISA),

virus-containing tuber tissue was removed in the initial ELISA. Virus content in samples with significant differences in the percentage of tubers predicted to have infection was expected, since these samples originated from regions and locations under very high virus and vector pressure and with evidence of in-season infection already seen in the field.

Table 3.8. Comparison between PVY- and PLRV-specific ELISA assays, with PVY- and PLRV-specific real-time RT-PCR assays on samples taken from the field and processed either fresh or sprouted; Samples not planted in the greenhouse

SAMPLES	PVY ELISA %	PVY PCR %	PLRV ELISA %	PLRV PCR %
1	0.00	0.00	0.00	0.00
2	0.00	0.00	0.00	0.00
3	3.08	3.08	1.00	20.59
4	0.00	0.00	1.00	3.30
5	0.00	0.00	0.00	0.00
6	0.00	0.00	0.00	0.00
7	0.00	3.25	0.00	0.00
8	0.00	0.00	0.00	0.00
9	0.00	0.00	0.00	0.00
10	0.00	1.02	0.00	0.00
11	0.00	0.50	0.00	0.00
12	1.00	0.50	0.00	0.00
13	-	0.00	-	0.00
14	1.00	1.50	2.20	2.70
15	0.25	0.75	0.25	7.86
16	1.50	0.50	1.50	9.50
17	0.00	0.00	0.41	3.25
18	8.50	6.43	2.25	3.50
19	0.50	0.00	2.50	6.43
20	0.00	0.00	0.25	1.80
21	0.00	0.50	0.00	3.30
22	0.00	0.00	0.25	0.00
23	0.00	0.00	0.25	1.25
24	0.50	0.00	0.00	0.00
25	0.00	0.00	0.00	4.75
26	0.00	0.00	0.00	0.00

4. DISCUSSION AND CONCLUSION

The advantage of hydrolysis probe-based assays lies in the fact that the generic time-temperature profile (actual PCR reaction) allows for the general execution of reactions that are specific to different target sequences on the same PCR plates at the same time. The same reaction conditions can therefore be used for a number of different target sequences. At PLS, this would allow for the simultaneous amplification and detection of PVY and PLRV in the same assay, even when run in singleplex. Above and beyond these pathogens, other target pathogens can also be included in the design of assays, which can be executed based on exactly the same conditions. This would have tremendous practical and economic implications in the long run. For pathogens with the same kind of genome (either RNA or DNA), multiplexing becomes easier, facilitating even more significant advantages in terms of practical performance and cost per run. It is based on these considerations that the so-called Taqman assay was identified as the method of choice. Technically speaking, another advantage lies in the target-specific probe, which significantly increases the specificity and sensitivity of the assay.

When seeking to test fresh tuber material in particular, without losing the ability to detect low quantities of virus that would have been able to multiply naturally in the host during the sprouting process, the use of a highly sensitive method, such as the PCR-based method, is essential. Such a method allows one to significantly reduce the waiting period involved in obtaining virus results from tuber tests, thus benefiting growers that are in need of early results. Since the PCR-based methodology is significantly more expensive than the ELISA method currently in use, it would not serve as a replacement method at present, but PCR would be a viable addition to the list of methods available to the Potato Laboratory Service (PLS) in its efforts to provide a service to potato growers in an increasingly challenging environment.

In terms of having an assay approved by Industry and the Department of Agriculture, Forestry and Fisheries (DAFF), particular attention must be given to the proper validation thereof. In this project, the validation process ultimately provides the industry with a method and process customised for use in this unique set-up.

In pursuit of this validation project, as per the specified objectives, PLS has been provided with an assay that will enable, most importantly, the detection of PVY and PLRV in fresh tubers using a testing regime very similar to the one used in the current ELISA process, making comparison possible and the interpretation of results easier. The added advantage of an increase in sensitivity, in this case at least, is 100-fold, enabling a laboratory to not only test fresh tubers, but also detect in-season infection at a later stage in the growth season (very recent infection at the time of the test), compared to what was previously possible. When material is produced in systems with little or no virus or vector pressure (in vitro labs, greenhouses, isolated virus-free and vector-free regions), 100 % correlation was expected and was found between the ELISA and PCR. Also, when only secondary infection was expected in a planting, correlation of ELISA and PCR close to 100 % was expected, which was also found. By testing individual tubers from samples, it was noted that the correlation of close to 100 % for secondary infection could be confirmed. When primary infection (in-season infection) was evident, the difference in results between the ELISA and PCR increased. Therefore, the virus and vector pressure in a region/location is closely reflected in the difference between the ELISA and PCR results for both PVY and PLRV.

The sample sizes used in the certification environment (depending on the generation of seed tubers planted – either 400 or 200 tubers) prevent the samples from being tested by means of the processing of individual tubers. A testing regime in which tubers are pooled in five-tuber subsamples was employed, and was also adopted in the PCR testing regime. The interpretation of the results (predicted number of tubers infected) is reflected in statistical tables. The pooling can skew the results, however, making any differences in the infection rate appear to be more significant. Minor differences between the ELISA and PCR can also be explained as an effect of the pooling of five tubers per subsample.

The establishment of molecular technology for the detection of PVY and PLRV in PLS, specifically at Plantovita, paves the way for the potato industry to make major advances in the detection methodology specific to potato pathogens. This validation project has proven the hydrolysis probe-based RT-PCR method to be a method capable of facilitating the testing of fresh tuber samples, taken from the field and from greenhouses at any stage in the production process, as well as leaf samples taken from the field, for use as a management tool and for diagnostic samples, as well as the testing of *in vitro* material for the presence of PVY and PLRV.

5. REFERENCES

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6. OUTPUTS

1. Protocols approved by industry and the Department of Agriculture, Forestry and Fisheries for routine use by Potato Laboratory Services in the certification of seed potatoes.
2. Report-back at meetings of the regional seed committees, the Potato Seed Growers' Forum and the National Seed Committee, as well as the Independent Council for the Certification of Seed Potatoes, regarding the progress of the Validation Project.

Anticipated

1. Informative/popular articles/presentations for publication in industry-specific press, reporting on the outcome and possibilities of the use of these validated methods.
2. Proposed participation in the EAPR Virology Section meeting in 2016, with results being presented either as a poster or orally.