Final Project Report

Project Title: Development and validation of reliable, effective and affordable diagnostic technologies to enhance productivity and international competitiveness of Ontario grape and wine industries

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EXECUTIVE SUMMARY

First off, we would like to thank OGWRI, members of its technical committee, and members of the Board of Directors for entrusting us and for the support you have provided on this project. We feel that we have achieved great success with the project and accomplished each and every objectives set out in the proposal. We hope the technologies we have developed will be adopted by OGWRI and the grape and wine industry for the benefit of the many growers and wineries that have to ensure losses inflicted by viruses and the devastating diseases they cause.

In Ontario, grapes rank as the second largest fruit crop, with nearly 500 grape growers, annual production of 80,400 tonnes, and a farm gate value of about $100 million annually. Ontario wines have garnered prestigious recognition in the international market, and produce an economic impact estimated at $3.3 billion annually. Viruses are highly detrimental pathogens, and are responsible for great economic losses to grape and wine production. Since 2013, the economic impact of viruses and viral diseases in relation to the health, productivity and sustainability of vineyards and quality of fruits and wine products in Ontario has been clearly recognized by grape growers, wineries, government agencies and the research community. There is no cure for viral diseases once plants become infected. While the use of insecticides provides certain levels of control for the spread of viruses that are transmitted by insect vectors, use of virus-free propagating materials remains the only ultimate solution available. Clean stocks are obtained through selection and virus elimination, both of which require the use of highly sensitive and reliable methodologies for virus testing. There is an urgent need in Ontario and other provinces where grapevine is grown for the development and implementation of highly effective and affordable technologies for use in virus diagnosis and the elimination of all major viruses from infected grapevines.

Throughout this project, we have developed both nuclei acid- and serology-based methodologies, produced highly specific antibodies for the detection of important grapevine viruses. We believe that the technologies we have developed have served and will continue to serve as an essential tool for the successful implementation of a clean stock program in Ontario and several other provinces and will prove to be pivotal to the grape and wine industries in the province. Below is a synopsis of what we have achieved in this project.

SYNOPSIS OF MAJOR ACHIEVEMENTS

1) We have successfully established the nucleic acid-based test systems including single PCR (and RT-PCR), multiplex RT-PCR and real-time PCR (and RT-PCR) and demonstrated that they are highly effective, reliable and suitable for the detection of all viruses that were targeted in this project and several other viruses.
2) We successfully expressed and purified recombinant capsid protein of GLRaV-1, GLRaV-2 and GLRaV-3 using insect baculovirus-based expression system. We

successfully expressed and purified recombinant capsid protein of GRBaV using pMAL™ Protein Fusion & Purification System using bacterial cells.

3) We successfully produced polyclonal antibodies against recombinant capsid protein of GLRaV-1, GLRaV-2, GLRaV-3 and GRBaV. We have demonstrated that the GLRaV-1, GLRaV-2 and GLRaV-3 polyclonal antibodies have a very high titer and are effective in detecting the corresponding virus in infected grapevines in Western blotting.

4) We successfully developed and optimized an antibody-based method, DAS-ELISA, for detecting GLRaV-1, -2 and -3 from grapevine leaves. We succeeded in the purification of the immunoglobulin (IgG) from the in-house antisera of GLRaV-1, GLRaV-2 and GLRaV-3 by using anion chromatography and confirmed that the in-house GLRaV-1 and GLRaV-3 antibodies we produced in this project are better than the commercial antibodies from BioReba, the gold standard for serological reagents for the diagnosis of grapevine viruses. We succeeded in the production of in-house GLRaV-3 conjugate and confirmed that the GLRaV-3 conjugate made in this project is comparable to the commercial conjugate from BioReba.

5) We detected by multiplex RT-PCR the six major viruses in 136 grape samples and revealed that the most prevalent viruses in Ontario vineyards are *Grapevine leafroll-associated virus* 3 and *Grapevine red blotch-associated virus*.

6) Recommendations have been made on how to use the nucleic acid-based and serological methodologies we developed in this project for commercial scale testing. Technology transfer and publication of the findings have been initiated.

DETAILED DESCRIPTION OF THE PROJECT

a) Objectives and Project Input

Project Objectives:

(1) To establish and validate nucleic acid-based methodologies [reverse transcription (RT)-PCR and quantitative RT-PCR (RT-qPCR)] for the detection of important grapevine viruses individually and in a multiplex format (Milestones 1, 2 & 3)
(2) Production of highly specific antibodies for use in serological detection of four major grape viruses (Milestones 4, 5 & 6)
(3) To develop and validate serology-based methodologies (dot-ELISA) for the detection of these viruses individually and in a multiplex format (Milestones 7 & 8)
(4) To compare and evaluate the efficacy and cost-effectiveness of both of these methods for the detection of the target viruses and to transfer the technologies for adoption for large-scale applications (Milestones 9)

Project Inputs (i.e. funding level, staff resources, cash and in-kind and other resources utilized in the project to date)

Matching funds:	OGWRI: $89,542 (plus indirect cost at $35,817) OMAFRA-UoG Partnerships Program: $78,223
Staff resources:	Dr. Huogen Xiao, Postdoctoral Research Fellow. Dr. Baozhong Meng, Professor, Principal Investigator. Dr. McFadden-Smith, collaborator, Specialist of OMAFRA. Personnel at the Campus Animal Facility, University of Guelph. Several undergraduate and graduate students.

b) Project Activities and outputs

Identify key activities undertaken to achieve the project objectives (link these activities to the outlined milestone schedule)

I. Development and optimization of nucleic acid-based methods for the detection of grapevine viruses (Milestones 1, 2 and 3)

I-1. Identification of samples infected with various viruses to be used in this project (Milestone 1)

To identify the samples infected with various viruses to be used in this project, we collected grape samples with disease symptoms from Niagara peninsula, the

major grape production region in Ontario. We isolated total RNA using our established RNA isolation method and detected virus infection using PCR and RT-PCR.

I-2. Optimization of conditions for single RT-PCR for individual viruses (Milestones 1 and 2)

We set up and fine-tuned experimental parameters for reverse transcription (RT) and polymerase chain reaction (PCR) for detecting individually GRBaV, GLRaV-1, GLRaV-2 and GLRaV-3 in Group A and GRSPaV, GVA and GVB in Group B. These included primer selection and testing, amount of template RNAs to be used in reverse transcription, amount of the resulting reverse transcription products (cDNAs) to be used in PCR, annealing temperature, concentrations of primers, dNTPs, and the enzyme Taq DNA polymerase.

I-3. Optimization of conditions for multiplex RT-PCR for simultaneous detection of multiple viruses (Milestone 2)

Multiplex RT-PCR was conducted to test the possibility of simultaneous detection of multiple viruses in a single assay. We tested multiplex RT-PCR for two groups of viruses. Group A included GRBaV, GLRaV-1, GLRaV-2 and GLRaV-3 and Group B had GRSPaV, GVA and GVB. Because different amplicons will have different efficiency in PCR amplification, we tested various primer combinations, annealing temperature, concentrations of essential components of the PCR, including concentrations of primers, dNTPs, Mg^{++}, and the amount of Taq polymerase.

I-4. Optimization of conditions for quantitative RT-PCR (RT-qPCR) for individual viruses (Milestone 3)

We set up the protocol and optimized conditions required for the quantitative RT-PCR for each of the seven viruses in Group A and B. We used SYBR Green as the dye and the qPCR machine that is available in the Advanced Analysis Centre of the University of Guelph. We tested various primer combinations, annealing temperature, concentrations of essential components of the RT-qPCR as described above.

I-5. Optimization of conditions for RT-qPCR for multiplex detection of viruses (Milestones 3)

We tried to set up a protocol for the simultaneous detection of four viruses from Group A and three viruses from Group B. Similar to multiplex RT-PCR described above, we tested various primer combinations, annealing temperature, concentrations of essential components of the RT-qPCR, especially the concentration of primers.

II. Expression of recombinant coat proteins of the four most important viruses and generation of 'in-house' polyclonal antibodies (Milestones 4, 5 and 6)

II-1. Cloning and sequencing of the capsid protein genes of four target viruses (Milestones 4 and 5)

We designed and custom-synthesized the oligo nucleotide primers for the amplification of the capsid protein genes of GLRaV-1, GLRaV-2, GLRaV-3 and GRBaV. The capsid protein gene of each virus was amplified using PCR (for GRBaV) or RT-PCR (for the other three viruses) from total nucleic acid preparations extracted from grape samples infected with each of these viruses based on the data obtained from the first year of this project. The amplification products were cloned into the intermediate vector pGEM-T Easy vector. Sequence of the capsid protein for each virus was confirmed through DNA sequencing at the Genomics Facility at the University of Guelph (see Appendix 1).

II-2. Generation of recombinant baculoviruses expressing the polyhistidine-tagged capsid proteins of four viruses (Milestones 4 and 5)

We used the recombinant baculovirus-based expression system to express the capsid proteins of GLRaV-1, GLRaV-2, GLRaV-3 and GRBaV. The positive recombinant clones in pGEM-T Easy corresponding to the capsid protein gene of each virus were subcloned into the expression shuttle vector pFastBac™ HTA (see Appendix 2) via restriction digestion followed by ligation. The vector pFastBac™ HTA contains sequence encoding an N-terminal polyhistidine tag which will be fused to the vial capsid protein upon expression. The recombinant pFastBac constructs containing capsid protein gene for each virus were then transformed individually into *E. coli* strain DH10Bac for recombination into the bacmid plasmid. The resulting Bacmid DNA containing capsid protein gene of each virus was then isolated from *E. coli* cultures. Highly pure Bacmid plasmid DNAs were transfected into insect cells of SF-21 cell line, to produce recombinant baculoviruses. The expression of recombinant capsid protein for each of the four viruses was confirmed by SDS-PAGE and Western blot with antibody His-H8 (Pierce) specific for the polyhistidine tag. These resulting Baculoviruses were harvested from the transfected SF21 cell cultures and stored at -80^{o}C for further uses. The conditions for the expression of the recombinant capsid proteins of four viruses were optimized through generating a high-titer P2 virus stock with a titer range between 2 to 5 x 10^8 pfu/ml (plaque forming units per millimeter of culture) and testing different time points at which to harvest infected cells and to obtain the recombinant capsid proteins.

II-3. Purification of recombinant capsid proteins for these viruses (Milestones 4 and 5)

In order to obtain quality antibody, we first needed to obtain pure preparation of each of the recombinant capsid proteins for these four viruses. Two lysis

methods and several buffers were tested to lyse the insect cells so that the recombinant proteins would be released for subsequent purification. These lysis methods we have tested were the freeze and thaw method, and the French Press method. The three lysis buffers tested were BugBuster (Novagen), CytoBuster (Novagen) and an in-house lysis buffer TKMN (50 mM Tris-HCl, pH8.5, 100 mM KCl, 5 mM 2-mercaptoethanol, 1% Nonidet P-40). Ni-NTA affinity chromatography system was used to purify the recombinant capsid proteins for these four viruses expressed in insect cells (see Appendix 3).

II-4. Technical issues encountered in purification of recombinant capsid protein for GRBaV and attempts to resolve these issues (Milestones 4 and 5)

Although we were successful in expressing the recombinant capsid protein of GRBaV at good levels in the insect cells, we have encountered tremendous technical difficulties at the step of protein purification. This was likely due to the fact that GRBaV capsid protein carries a high positive charge (pI at 11), which interfered with purification using the polyhistidine tag purification system. To circumvent this unexpected obstacle, we then employed a different strategy to express and purify that protein. The strategy we have employed was to express the protein using an entirely different expression vector, pMAL-c2 (see Appendix 4), which contains a large tag – the maltose binding protein MBP (43 kDa). The GRBaV capsid protein gene from the positive recombinant clone in pGEM-T Easy was subcloned into the expression vector pMAL-c2, generating the expression construct pMAL-c2-GRBaV-CP. The resulting construct was then transformed into protein expression strain *E. coli* Rosetta-gami B (DE3)pLysS. Transformants were cultured in 5 ml of LB medium containing the required antibiotics until the OD_{600} reached 0.5, then induced with isopropyl-β-D-thiogalactopyranoside (IPTG) as suggested in the pMAL™ Protein Fusion & Purification System (New England BioLabs). Concentrations of IPTG and expression temperatures were tested for maximal solubility of fusion proteins. After optimizing conditions for the expression of the recombinant fusion protein, two liters of bacterial cell cultures were prepared and used to purify the fusion protein. The protein purification was done through affinity chromatography using amylose column as suggested by the vendor (New England BioLabs) (see Appendix 5 for the protocol of purification).

II-5. Immunization of animals (rats) with purified capsid proteins for generation of polyclonal antibodies (Milestone 6)

A number of animal species are commonly used for the generation of polyclonal antibodies, which include rabbits, rats and chicken eggs. Previous research in our lab and many other labs has shown that antibodies produced from rabbits give high background signal resulting from nonspecific reactivity with host proteins. To prevent this potential issue, we have chosen rats for this purpose, as rats have been increasingly used for producing antibodies for use in the detection of various pathogens. The purified recombinant capsid proteins of GLRaV-1,

GLRaV-2, GLRaV-3 and GRBaV were used as antigens to immunize rats, two per antigen. These rats were mature female of the Sprague Dawley strain, approximately 8 weeks old. To ensure sufficient titer of antibodies, we followed the standard protocol from the Campus Animal Facility for the immunization scheme. Briefly, one primary injection plus 2-3 booster injections were administered for each rat. Test bleed was collected at 3-5 days after each booster injection to monitor antibody response and terminal blood was collected at 5 days after the last booster injection (see Appendix 6 for rat antiserum production schedule). Antisera were harvested by centrifuging the terminal bleed at 10,000 rpm for 10 min, aliquotted and stored in a -20^{o}C freezer.

II-6. Evaluation and validation of the polyclonal antibodies in detection of corresponding viruses (Milestone 6)

Indirect ELISA (see Appendix 7 for the protocol) was used to evaluate the titer of antisera for the first and second test bleed, and the terminal bleed. The antigens used were the recombinant capsid proteins and the second antibody conjugate was HRP-conjugated rabbit anti-rat IgG.

Western blotting (see Appendix 8 for the protocol) was used to validate the polyclonal antibodies for their effectiveness to detect the corresponding recombinant capsid proteins and the viruses in infected grape samples.

III. Development of serology-based methods (Milestones 7 and 8)

III-1. Sample collection, inventory of samples and sample processing (Milestone 7)

In order to evaluate the efficacy of serological and nucleic acid-based diagnostic technologies we developed in the project, hundreds of grape samples were collected for the province-wide survey for all major viruses from two primary appellations: Niagara peninsula and Lake Erie North Shore in August and September 2016. We processed each sample by grinding then into fine powder under liquid nitrogen and put the ground materials into a 15 ml falcon tube and stored all the processed samples at -20^{0}C for later use in virus detection with serological and nucleic acid-based diagnostic technologies.

III-2. Virus detection with dot-ELISA (Milestone 7)

We initially tried to develop Dot-ELISA (see Appendix 9 for the protocol) to detect GLRaV-1, GLRaV-2, GLRaV-3 (using antibodies produced in this project), GVA and GVB (using commercial antibodies purchased from Agritest, Italy) from grape leaves. In order to develop this method we have tested various dot-ELISA protocols, protein extraction buffers (buffers for ELISA and Western blot) and additives (PVP, PVPP, urea, sodium diethyldithiocarbamate, etc), membranes (nitrocellulose, nylon), antibodies (GLRaV-1 and GLRaV-3, GVA and GVB from

different sources; antibodies for GRSPaV were used as positive control), conjugates, substrate and infected samples from different sources.

III-3. Virus detection with DAS-ELISA (Milestone 8)

DAS-ELISA (see Appendix 10 for the protocol) was developed to detect GLRaV-1, GLRaV-2 and GLRaV-3 from grape leaves. We first tested the effectiveness of the commercial GLRaV-1, GLRaV-2 and GLRaV-3 antibodies and conjugates from BioReba in DAS-ELISA. We also examined extraction buffers to improve the DAS-ELISA in grape virus testing. We have also tried to develop DAS-ELISA to detect GVA from grape leaves.

III-4. Isolation and purification of immunoglobulin (IgG) from the in-house antisera of GLRaV-1, GLRaV-2 and GLRaV-3 (Milestone 8)

To isolate antibodies- immunoglobulin (IgG) from the antisera of GLRaV-1, GLRaV-2 and GLRaV-3, 1.5 ml of each antiserum was treated with saturated ammonium sulfate to precipitate immunoglobulin. The precipitated antibody proteins were recovered via centrifugation, followed by further purification through anion exchange chromatography with Macro-Prep High Q cartridge (BioRad) in AKTA FPLC system (GE Healthcare Life Sciences). The wash buffer was 20 mM Trs-HCl, pH 8.5 and the elution buffer was 20 mM Tris-HCl, 1 M NaCl, pH 8.5. The elution fractions were collected and checked with a NanoDrop spectrophotometer for concentration, and with SDS-PAGE for protein purity.

III-5. Preparation of in-house GLRaV-3 conjugate (Milestone 8)

In order to see whether the GLRaV-3 polyclonal antibodies made in this project is effective in DAS-ELISA for detecting GLRaV-3 in grapevines we need to produce enzyme conjugate specific for GLRaV-3 from the purified antibody. The conjugate was made through the coupling the purified GLRaV-3 IgG with alkaline phosphatase by using the glutaraldehyde method. About 2 mg of IgG were used to be coupled with 1.3 mg of alkaline phosphatase (Sigma) for 5 hours at room temperature; the conjugate solution was dialyzed overnight at 4 ^{0}C against 3 changes of 1x PBS. The conjugate solution with 0.02% sodium azide (as preservative) was stored at 4^{0}C for later use.

IV.Evaluation, analyses and technology transfer (Milestone 9)

IV-1. Evaluation and comparison of serological and nucleic acid-based diagnostic technologies (Milestone 9)

A comprehensive evaluation of serological and nucleic acid-based diagnostic technologies in detecting grape viruses was done for reliability, cost and suitability for high through-put assays.

IV-2. Technology transfer (Milestone 9)
Optimized protocols we have established throughout this project are included as appendices of this report. We will be happy to provide further help and consultation to OGWRI or its designate to facilitate the technology transfer process. We look forward to the opportunity for further collaboration with OGWRI and GGO. Furthermore, we have also talked with various parties about the transfer of technologies we developed in this project.

C) Reach and communications

Identify primary target of this project (i.e. agricultural producers, processors, rural Ontarians, consumers, youth, farm families)

The primary targets of this project are all stakeholders of the grape and wine industry in Ontario. These include the many grape growers, wineries, nurseries, government agencies related to agriculture and the fruit industry, and diagnostic facilities for plant pathogens such as the Pest Diagnostic Lab of the University of Guelph.

I have presented and discussed what we had done so far with this project to the Board of Directors and the members of the Technical Committee of OGWRI on May 9, 2016. I have also communicated what we have accomplished so far with GGO and some of the grape growers during the Grape Tailgate Tour in August at the invitation of Dr. Wendy McFadden-Smith (poster appended). We have reached out to a number of grape growers and wineries. Through the communication and reach out, we have established several collaborations with wineries, which have resulted in successful application of several Engage projects from NSERC. We plan to communicate with a large audience on basic knowledge on grape viruses and viral diseases through the publication of a series of newsletter articles on the symptomatology, transmission, detection and management strategies of major viruses.

Manuscripts are being written to disseminate the findings from this project and will be submitted to journals for publication upon approval by OGWRI.

Indicate the total number of people reached by this project to date:

We have so far reached 17 wineries and growers. These include people involved in the production, sales and management of several wineries and a number of growers from

which we collected samples. These wineries and growers include Pellers, Cave Spring Cellars, Rancourt Winery, Di Profio Vineyard, Woer Vineyard, Wiens Family Vineyard, Hipple Vineyard, Henry of Pelhem Vineyard, Baranick Vineyard, Funk Vineyard, Lambert Vineyard, Eastman Vineyard, Creekside Estate Winery, Thirty Bench Winery, Vieni Winery, Delaine Vineyard, and Burning Kiln Winery.

We also provided scientific information and technical support to the following agencies on the development of standard protocols and diagnostic kit:

(i) Pest Diagnostic Clinic at the University of Guelph: protocol development for GRBaV detection using PCR and qPCR.

(ii) Norgen BioTek: consultation on project funded by OGWRI on GRBaV qPCR detection using TaqMan technology.

(iii) CFIA: provided infected grapevine samples and technical information for protocol development for the detection of GPGV.

(iv) Agdia Diagnostic company (based in USA): provided infected samples for the development of commercial kits for detection of GPGV.

Indicate how the targeted audience/beneficiaries were reached i.e. research publications, seminars, press releases, and promotional material. Please include samples of any communications material developed for the project and indicate the number printed/distributed.

I have presented and discussed what we had done so far with this project to the Board of Directors and the members of the Technical Committee of OGWRI on May 9, 2016. I have communicated what we have accomplished so far with GGO and some of the grape growers during the Grape Tailgate Tour in August 2016. We have also sent out invitation letters to a large number of grape growers and wine makers to introduce our research on grape viruses and invite them to take part in provincial grape virus survey project (funded by OMAFRA-UoG Partnerships program). Furthermore we plan to communicate basic knowledge on grape viruses, viral diseases as well as their identification and management strategies through the publication of newsletters. We plan to write up some of the key findings generated in this project for publication in Canadian Journal of Plant Pathology.

Indicate when OGWRI was identified as a supporter throughout the period of the project

We have identified OGWRI as a supporter of this project in various meetings with OMAFRA-UoG Partnerships program, collaborating wineries, as well as on the brochure disseminated at the Grape Tailgate Tour in August 2016.

PROJECT OUTCOMES (ACTUAL VS. EXPECTED) AT SHORT AND LONG-TERM

a) SHORT-TERM OUTCOMES

Outline the actual short-term outcome compared to the expected

1. Seven important viruses have been detected in Ontario vineyards (Milestone 1)

One hundred and eleven infected samples of grapevine were collected from nine varieties of *Vitis vinifera* in Niagara region. These samples were tested with PCR or RT-PCR for 11 viruses infecting grapevines including eight viruses targeted in this project. Of the 11 viruses tested, seven viruses (GRSPaV, GRBaV, GLRaV-1, GLRaV-2, GLRaV-3, GVA and GVB) were detected. GRSPaV, GLRaV-3, GRBaV, GLRaV-2, GVA, GVB and GLRaV-1 were detected in 105, 66, 40, 18, 17, 5 and 2 samples, respectively. This small-scale screening not only demonstrated the magnitude of virus infections in grapevines in Niagara Peninsula, but also provided essential materials infected by these viruses required for this project.

2. Highly sensitive and effective nucleic acid-based methodologies [reverse transcription (RT)-PCR and quantitative RT-PCR (RT-qPCR)] for the detection of important grapevine viruses individually and in a multiplex format have been developed and validated (Milestones 2 & 3)

2.1. A highly sensitive single RT-PCR has been established (Milestone 2)

We have developed a very sensitive single RT-PCR for the individual detection of all target viruses in both group A and B through optimizing RT-PCR conditions which included amount of cDNA to be used in PCR, annealing temperature, concentrations of primers and DNA polymerase (see Appendix 11 for the established method and Table 1 for the primers). The detection limits of single RT-PCR ranged from 10^{-4} (GRBaV, GLRaV-1) to 10^{-5} (GLRaV-2, 3, GVA and GVB) (see Figure 1).

2.2. A very effective multiplex RT-PCR has been established (Milestone 2)

We have developed a very sensitive multiplex RT-PCR for the simultaneous detection of multiple viruses in both group A and B (see Appendix 12 for the established method and Table 1 for the primers). Amplification of an internal control-ubiquitin 60S-ribosome gene was included in both groups to show the effectiveness of RT-PCR. Optimization of RT-PCR conditions was done for concentrations of reaction buffer, annealing temperature, concentrations of primers and DNA polymerase. The detection limits of multiplex RT-PCR ranged

from 10^{-2} (GRBaV, GLRaV-1, GLRaV-2 and GVB) to 10^{-3} (GLRaV-3 and GVB) (Figure 2).

2.3. A very sensitive RT-qPCR using SYBR Green has been established for the detection of individual viruses (Milestone 3)

We have developed single RT-qPCR using SYBR Green to successful quantitatively detect GRSPaV, GRBaV, GLRaV-1, GLRaV-2, GLRaV-3, GVA and GVB in grapevines (see Appendix 13 for the established method and Table 1 for the primers). The detection limits of single RT-qPCR ranged from 10^{-4} (GLRaV-1 and GLRaV-2) to 10^{-5} (GRBaV and GVB) to 10^{-6} (GLRaV-3 and GVA) (Figures 3 and 4), which is more sensitive than that of single RT-PCR developed in Milestone TWO (Table 2).

3. Highly specific quality antibodies for use in serological detection of GLRaV-1, GLRaV-2 and GLRaV-3 have been successfully produced (Milestones 4, 5 and 6)

3.1. The capsid protein genes of GLRaV-1, GLRaV-2, GLRaV-3 and GRBaV have been successfully amplified, cloned and sequence confirmed (Milestones 4 and 5)

The capsid protein genes of all four viruses (GLRaV-1, GLRaV-2, GLRaV-3 and GRBaV) have been successfully amplified using PCR (for GRBaV) or RT-PCR (for the other three viruses) and cloned into the intermediate vector pGEM-T Easy vector. The sequences of the capsid protein genes of all four viruses were confirmed by DNA sequencing (see Appendix 1 for the obtained gene sequences).

3.2. The recombinant baculoviruses containing capsid proteins of GLRaV-1, GLRaV-2, GLRaV-3 and GRBaV have been successfully produced (Milestones 4 and 5)

The recombinant baculovirus-based expression system was used to express the capsid proteins of GLRaV-1, GLRaV-2, GLRaV-3 and GRBaV. The cloned capsid protein genes of all four viruses available in the pGEM-T Easy vector were successfully subcloned into the shuttle expression vector pFastBac™ HTA. After transformation of shuttle vectors into bacterial strain DH10Bac, each of the expression constructs contained in pFastBac HT-A were successfully mobilized into the bacmid plasmid through recombination. The recombinant Bacmid DNA with capsid protein genes of GLRaV-1, GLRaV-2, GLRaV-3 and GRBaV were successfully purified and transfected into SF-21 cells. After optimization, SF-21 cell lines with high virus titer of the recombinant baculoviruses containing capsid proteins of GLRaV-1, GLRaV-2, GLRaV-3 and GRBaV have been successfully obtained.

3.3. The recombinant capsid proteins of three viruses (GLRaV-1, GLRaV-2 and GLRaV-3) have been successfully purified (Milestone 4 and 5)

With the use of Ni-NTA affinity chromatography, the recombinant capsid proteins of GLRaV-1, GLRaV-2 and GLRaV-3 have been successfully purified (Figure 5). The amount of the recombinant capsid proteins for the three viruses varies considerably, with GLRaV-3 being the most abundant and GLRaV-1 being the least abundant. Specifically, 12.5, 37.5, and 90 μg of purified recombinant proteins were obtained for GLRaV-1, GLRaV-2 and GLRaV-3, respectively from the insect culture of one large flask (75 cm^2). To purify enough recombinant capsid proteins for antibody production, at least 30 large flasks of insect cells infected with each of the recombinant baculoviruses were required for each protein.

3.4. The recombinant capsid protein of GRBaV has been expressed and purified successfully in bacteria protein expression system (Milestones 4 and 5)

We were able to express the recombinant capsid protein of GRBaV at good levels in the infected insect cells with Bac-to-Bac Baculovirus Expression System. Unfortunately, we encountered tremendous technical difficulties at the step of protein purification. We sought an alternative approach and have successfully expressed the protein in *E. coli* using an entirely different expression vector, pMAL-c2 (see Appendix 4), which contains a large tag – the maltose binding protein MBP (43 kDa). The expressed recombinant fusion MBP-GRBaV-CP protein were successfully purified through affinity chromatography using amylose resin column as suggested in the pMAL™ Protein Fusion & Purification System (New England BioLabs) (see Figure 6).

3.5. Polyclonal antibodies against the recombinant capsid protein of all four viruses have been successfully produced and GLRaV-1, GLRaV-2 and GLRaV-3 polyclonal antibodies are effective and specific in detecting the intended viruses in grape samples in Western blotting and ELISA (Milestone 6)

We have chosen rats for the purpose of antibody production in this project. Each recombinant protein purified was used to immunize two rats and about 4 ml of antiserum have been harvested from each rat. The titers of the antisera from the terminal bleed are over 8000, 32000, 32000 and 16000 for GLRaV-1, GLRaV-2, GLRaV-3 and GRBaV, respectively.

Western blotting have confirmed that antibodies against GLRaV-1, GLRaV-2 and GLRaV-3 effectively detected corresponding viruses in infected grape leaves with high specificity and the optimal dilution of the antibodies was 1:2000, 1:2000 and 1:6000 for GLRaV-1, GLRaV-2 and GLRaV-3, respectively.

4. Effective serology-based methodologies (DAS-ELISA) for the detection of GLRaV-1, GLRaV-2 and GLRaV-3 in grape leaves have been established (Milestones 7 and 8)

4.1. Enormous efforts have been invested to develop a variant of ELISA, dot-ELISA, for detection of viruses in grape leaves (Milestones 7 and 8)

Dot-ELISA, a variant of ELISA, has been used in detecting viruses in plants, especially herbaceous plants which generally have high virus titer and low amount of inhibitors in leaf extracts. We would like to develop this method for the detection of the major viruses in grape leaves. We have tried different dot-ELISA protocols, protein extraction buffers (buffers for ELISA and Western blot) and additives (soluble and insoluble polyvinylpyrrolidone, urea, sodium diethyl dithiocarbamate, etc.), membranes (nitrocellulose, nylon), antibodies (GRSPaV, GLRaV-1 and -3, GVA and GVB from different sources). However, we failed to use this method for the detection of viruses from grapevine leaves.

4.2. Protocols for DAS-ELISA for detecting GLRaV-1, -2 and -3 have been successfully developed and further improved (Milestones 7 and 8)

As we found that dot-ELISA is not suitable for the detection of grape viruses, we then have tried to establish the commonly used method, double antibody sandwich (DAS)-ELISA, which is the most sensitive and reliable method among all the ELISA variants. We have first tried the commercial GLRaV-1, GLRaV-2 and GLRaV-3 antibodies and conjugates from BioReba. The results show that the DAS-ELISA with antibodies and conjugates from BioReba is effective in detecting GLRaV-1 and -3 from grape leaves, but is less effective for GLRaV-2 (see Table 3). As grape leaves contain high levels of secondary metabolites which would impact ELISA assays, we have tested the effects of adding 1% Polyvinylpolypyrrolidone (PVPP) in the BioReba extraction buffer on the effectiveness of DAS-ELISA. We found that it increased the ratio of ELISA readings between positive samples and heathy control by 50%, which translate to an increase in the sensitivity of DAS-ELISA. We have compared the effectiveness of DAS-ELISA with the new extraction buffer in detecting the viruses in infected grape samples and found that the detection limit reached to 1:400, 1:80 and 1:1600 dilutions of the infected leaf extracts for GLRaV-1, -2 and -3 respectively (Table 4).

4.3. In-house GLRaV-1 and -3 polyclonal antibodies made in this project are better than those from BioReba as the first antibody in DAS-ELISA for detecting GLRaV-1 and -3 in grapevines (Milestones 7 and 8)

As stated above, the polyclonal antisera against GLRaV-1, GLRaV-2 and GLRaV-3, which were made from this project, are effective and specific in detecting the intended viruses in grape samples in Western blotting. We then wanted to know whether the three antibodies could be used effectively in DAS-ELISA in detecting the intended viruses in grape samples. To do this, we first isolated antibodies-immunoglobulin (IgG) from the antisera of GLRaV-1, GLRaV-2 and GLRaV-3 by precipitation with ammonium sulfate and then further purified them by using anion chromatography with Macro-Prep High Q cartridge (BioRad). 8.1, 7.7 and 13.1 mg of IgG were obtained from 1.5 ml of antisera of GLRaV-1, GLRaV-2 and GLRaV-3 respectively.

In order to know whether the three antibodies could be used effectively in DAS-ELISA in detecting the intended viruses in grape samples, we have compared these in-house antibodies (antibodies made in this project) as the first antibody, and the conjugates from BioReba, with both antibodies and conjugates from BioReba. The results show that the ratio of ELISA readings between positive samples and heathy control in DAS-ELISA with in-house GLRaV-1 and -3 antibodies as the first antibody increased by about 30% as compared with the BioReba antibodies. This suggests that GLRaV-1 and GLRaV-3 polyclonal antibodies made in this project are better than those from BioReba A as the first antibody in DAS-ELISA for detecting GLRaV-1 and -3 in grapevines. The in-house GLRaV-2 antibody, however, is less effective than that of BioReba.

4.4. In-house GLRaV-3 conjugate is comparable with that of BioReba as the antibody conjugate in DAS-ELISA for detecting GLRaV-3 in grapevines.

As showed above that the GLRaV-1 and -3 polyclonal antibodies made in this project are better than those from BioReba in DAS-ELISA for detecting GLRaV-1 and -3 in grapevines we then would like to know whether the conjugate made from these antibodies are comparable or even better than that of BioReba. We have tested this by making our own GLRaV-3 conjugate through the labeling of the purified GLRaV-3 IgG with alkaline phosphatase. Using glutaraldehyde method about 2 mg of IgG was used to be coupled with 1.3 mg of alkaline phosphatase. The GLRaV-3 conjugate made was assayed with DAS-ELISA. The conjugate works very well and the best working concentration could be between 2000-4000 dilutions. We have compared the effectiveness of in-house GLRaV-3 antibody and conjugate with those of BioReba in detecting the virus in infected grape samples in DAS-ELISA and found that the detection limit can reach to 1:1600 dilutions of the infected leaf extracts for both systems (Table 4). This indicates that in-house GLRaV-3 conjugate is comparable with that of BioReba as the antibody conjugate in DAS-ELISA for detecting GLRaV-3 in grapevines.

5. The developed nucleic acid-based methodologies and serology-based methodologies have been evaluated (Milestone 9)

5.1. DAS-ELISA is less sensitive than RT-PCR in the detection of GLRaV-1 and GLRaV-2, while it is as sensitive as RT-PCR in the detection of GLRaV-3

To validate DAS-ELISA in detecting the intended viruses in infected grape samples, we have tested more samples infected with GLRaV-1, GLRaV-2 and/or GLRaV-3, which were confirmed previously by RT-PCR (Table 5). DAS-ELISA with BioReba GLRaV-3 antibody and conjugate could effectively and specifically detect the virus in all of the GLRaV-3- infected grape samples. DAS-ELISA with BioReba GLRaV-1 and GLRaV-2 antibodies and conjugates could detect the viruses in most of the GLRaV-1 or GLRaV-2- infected grape samples and the

samples that were not tested positive by DAS-ELISA may have had very low virus titer that was under the detection limit by DAS-ELISA.

5.2. One hundred and thirty six composite grape leaf samples have been tested by multiplex RT-PCR

Multiplex RT-PCR has been developed for the simultaneous detection of multiple viruses in a single assay (Milestone 2), which results in time and cost-savings. We have further validated this system in detecting six major viruses (GLRaV-1, GLRaV-2, GLRaV-3, GRBaV, GVA and GVB) from 136 composite grape leaf samples we collected from two primary appellations: Niagara peninsula and Lake Erie North Shore in August and September 2016 (Milestone 7). Among these six viruses, GLRaV-3 is the most prevalent virus with an infection rate of 54% and GRBaV is the second with an infection rate of 18%, followed by GVB, GVA, GLRaV-2 and GLRaV-1 with an infection rate of 10, 10, 5 and 4%, respectively (Table 6). This testing not only provided proof for the effectiveness of multiplex RT-PCR in grape virus survey; it also produce very useful information for grape and wine industry in developing strategies for managing the grapevine viral diseases in Ontario vineyards.

5.3. Our recommendations on the application and implementation of the technologies developed in this project in the management of grapevine viral diseases

Based on the sensitivity, reliability, cost and suitability of each technology and the targets for high throughput assays, we make the following recommendations on the application and implementation of the technologies developed in this project in the management of grapevine viral diseases.

(i). For testing the propagation materials in the nurseries and for the establishment of a clean stock program, qPCR (or RT-qPCR) should be used as the method has the highest sensitivity (able to detect the intended viruses even at very low levels) and the materials are of high values and critical for the management of viral diseases.

(ii). For routine testing of a small number of samples or testing of a single virus, PCR or RT-PCR should be should be used as the method has a high sensitivity and are available in common diagnostic facilities.

(iii). For large-scale virus survey and testing of multiple viruses, multiplex RT-PCR should be used as the method has a high sensitivity to detect multiple viruses in a single assay, which would save time and costs.

(iv). The serology-based methods, such as DAS-ELISA, could be used to test symptomatic samples that are negative by the nucleic-based methods as the antibodies used in DAS-ELISA are of generally broader spectrum than primers

used in PCR or RT-PCR so as that DAS-ELISA could usually detect the strains that would evade detection by PCR-based methods.

Identify the public good/benefit of the project

Viruses and the disease they cause have emerged as a major impediment to the sustained production of quality grapes and wines in Ontario and the competitiveness of Ontario grape/wine industry on the world stage. Viral infections and diseases are responsible for significant economic losses due to poor performance and reduced productivity of vineyards, deteriorating quality of grapes and wine products, as well as shortened production span of vineyards. Survey for the type of viruses and the magnitude of viral infections across all major grape production regions in Ontario and the generation and implementation of clean stocks for province-wide use is the key to the sustainability, profitability and international competitiveness of Ontario grape/wine industry.

In the first two reporting periods, we have developed highly effective nucleic acid-based diagnostic technologies for the detection of all major viruses that are present in Ontario vineyards. These nucleic acid-based technologies offer the possibility of reliable, highly sensitive, and affordable detection of all major viruses and viral strains when combined with the use of broad-spectrum primers for each of these viruses. Through the use of multiplex assays, several viruses can be detected simultaneously, resulting in significant savings in costs and processing time. The extremely high sensitivity of these tests makes it possible to run composite samples, which will translate into high-throughput and reduction in costs. We have demonstrated that two test systems, conventional PCR and RT-PCR, and the more advanced quantitative PCR and RT-qPCR, are both effective for virus detection. The former system is performed on a conventional thermocycler and assessed through electrophoresis on agarose gels. The latter system is more sensitive and has the advantage of being able to quantify the titer of viruses in the infected vines. However, it requires the more expensive real time PCR machines and high resolution melt curve analysis software to ensure the distinction of different amplification products representing different viruses included in the tests.

In the third and fourth reporting period, we successfully cloned and expressed recombinant capsid protein for each of the four target viruses using the Bac-to-Bac baculovirus expression system in insect cell cultures. We optimized highly conducive conditions for the expression of each recombinant capsid proteins and obtained quality recombinant capsid proteins for three of the four viruses. Prevented by technical difficulties of purifying recombinant GRBaV capsid proteins from insect cell expression system, we sought the alternative of producing GRBaV capsid protein as a fusion to maltose binding protein in bacterial cells. Prevented by technical difficulties during purification of recombinant GRBaV capsid protein from insect cell culture system, we sought the alternative of expressing this protein as a fusion to the maltose binding protein tag in bacterial cells. We have completed on the immunization procedure in animals for the production of polyclonal antibodies against all four viruses. We have confirmed that the polyclonal antibodies against GLRaV-1, GLRaV-2 and GLRaV-3 are

effective in detecting the corresponding recombinant capsid proteins expressed in insect cells and the intended viruses in grape samples in Western blotting. We have successfully developed a protocol for Indirect ELISA for detecting GLRaV-3. We have collected, databased and processed 136 composite grape leaf samples that will be used in the next phase of the project-validation of antibodies and provincial grape virus survey. We have purified GRBaV protein that will be used as antigen to immunize experimental animals for the generation of polyclonal antibodies.

In the fifth reporting period, we have surveyed the 136 grape samples for the six major viruses and found that the most prevalent viruses in Ontario vineyards are GLRaV-3 and GRBaV, which are useful information for grape and wine industries to develop strategies in viral disease management. We have established a sensitive and reliable serological method, DAS-ELISA, for detecting GLRaV-1, GLRaV-2 and GLRaV-3 from grapevines. We have also confirmed that the in-house polyclonal antibodies against GLRaV-1, GLRaV-2, and GLRaV-3 as well as the GLRaV-3 conjugate made in this project are effective in detecting the intended viruses in grape samples in DAS-ELISA, which could reduce the cost for virus testing through replacing the commercial antibodies with these in-house antibodies.

The benefit of the project has been reflected in the success of the province-wide grape virus survey (University of Guelph and OMAFRA partnership project), in which we have surveyed more than 3500 vines for a total of 19 viruses. Importantly, the power of the technologies we have developed has been well recognized by others in the grape/wine research community. For example, at the request of Dr. Lorne Stobbs at Vineland, we helped in the verification of GRBaV in the experimental materials to be used in their project on vector transmission of Grapevine red blotch associated virus in Niagara region. More recently, we have helped Dr. Wendy McFadden-Smith for her project for the survey of GRBaV supported by OGWRI and Dr. Andrew Reynolds from Brock University for his project for the testing of several viruses associated with grapevine leafroll supported by OMAFRA. I have also received numeral inquiries for helping in virus testing. We also provided scientific information derived from this project and technical support to several agencies (The Pest Diagnostic Clinic at the University of Guelph, CFIA, Norgen and Agdia) during the development of standard protocol s or diagnostic kit). I clearly see the prospect that our test systems will receive even broader utilizations by other researchers and diagnosticians in the years to come, as the industry ventures into the next era of viticulture in line with the world.

Speaking of the potential commercial values, I clearly see that both systems could be adopted by the industry through the establishment of a diagnostic facility. This facility will play an essential role for the virus surveys, diagnostics, as well as for the maintenance of virus-free planting materials in the years to come. We will be happy to help out with this endeavor should OGWRI opt to pursue this line of commercialization work.

b) LONG-TERM OUTCOMES

Indicate the key indicators you will be using to measure the project success in the long-term.

The establishment of quick, highly sensitive and affordable diagnostic technologies using conventional and quantitative PCR and RT-PCR and DAS-ELISA will serve as an excellent foundation not only for the province-wide survey on the distribution of all major viruses in Ontario vineyards (OMAFRA-UoG Partnership projects) but also for the generation and implementation of clean grapevine stocks for the grape and wine industry moving forward. We foresee a significant return from this project as the technologies will greatly enhance productivity, quality, sustainability, and international competitiveness of Ontario grape and wine industries through the implementation of clean stock programs.

Some of the key indicators will include:

1) The adoption of our diagnostic technologies by OGWRI or its designate.
2) The adoption of our diagnostic technologies and the knowledge we have generated by diagnostic facilities such as the Pest Diagnostic Clinic at the University of Guelph.
3) Provincial and national surveys for all major viruses in Canada.
4) The development, implementation and continued monitoring system of grapevine clean stock program.
5) Enhanced production of grape and wine production in Ontario and other provinces.

FINAL COMMENTS AND CONCLUSIONS

We feel that great success has been achieved in this project. We have accomplished each and every objective as proposed in this research project. Thank you for entrusting us with this project and the wonderful opportunity you provided to us.

Since the commencement of this project three years ago, we have diligently performed the activities as proposed in the project. As a result, we have done exceedingly well with the objectives proposed for this project. We have established highly effective nucleic acid-based detection systems for all major viruses present in Ontario vineyards. These systems are highly sensitive, reliable, cost-effective and suitable for the purpose of large-scale tests. These systems can be used for the detection of individual viruses through traditional PCR and RT-PCR or quantitative PCR and RT-qPCR. These systems can also be used for the detection of multiple viruses simultaneously through multiplex format. The high sensitivity and the capacity of multiple tests significantly speed up the testing process as well as driving down the costs related to virus diagnosis due to the possibility of testing composite samples and multiplex testing. We have conducted a preliminary survey involving a number of grape growers and wineries and demonstrated the effectiveness of our nucleic acid based detection technologies. Furthermore, we have successfully cloned the capsid protein genes into a Bacmid vector for all four target viruses and expressed recombinant capsid proteins for each virus using the baculovirus/insect cell culture or bacterial system. We have also successfully purified quality recombinant capsid proteins for four viruses using affinity chromatography. We have produced polyclonal antibodies against GLRaV-1, GLRaV-2, GLRaV-3 and GRBaV. These polyclonal antibodies are effective in detecting the corresponding recombinant capsid proteins expressed in insect cells and the intended viruses in grape samples in Western blotting and ELISA. We have surveyed 136 grape samples for the six major viruses and found that the most prevalent viruses in Ontario vineyards are GLRaV-3 and GRBaV, which are useful information for grape and wine industries to develop strategies in viral disease management. We have established a sensitive and reliable serological method, DAS-ELISA, for detecting GLRaV-1, GLRaV-2 and GLRaV-3 from grapevines. We have also confirmed that the in-house polyclonal antibodies against GLRaV-1, GLRaV-2, and GLRaV-3, as well as the GLRaV-3 conjugate made in this project are effective in detecting the intended viruses in grape samples in DAS-ELISA. We have evaluated both the nucleic acid based and antibody-based test systems in terms of reliability, cost and suitability for high through-put assays and made several recommendations on how to use these technologies in the large-scale virus testing.

We will continue our efforts to transfer and promote the technologies we developed to the grape/wine research community. At the same time we will also continue to develop more advanced technologies, such as next generation sequencing, for the diagnostic of grapevine viral diseases. The use of these technologies together will guarantee a successful generation and implementation of clean grapevine stocks for the Ontario grape and wine industry, which will safeguard the productivity, quality, sustainability, and international competitiveness of Ontario grape and wine industries.

Deviations: Unexpected technical difficulties were encountered with the purification of recombinant capsid protein of GRBaV using the baculovirus/insect cell system and with Dot-ELISA. However, we have circumvented both technical issues through the use of alternative approach. For example, we overcame the difficulties pertaining to the purification of recombinant capsid proteins of GRBaV from insect cell cultures through the use of fusion to a different tag, maltose binding protein and expression in bacterial cells. Similarly, the issues with Dot-ELISA were addressed by the development and refinement of DAS-ELISA for the detection of the three viruses associated with leafroll disease.

Table 1 Primers used for RT-PCR, multiplex RT-PCR and RT-qPCR

Target	Primer	Primer sequences 5'-3'	Amplicon size (bp)	Gene
GLRaV1	LR1-502F	5'-TTGAGRGCTCTBATAAAYGAAC-3'	378	Heat shock Protein 70
	LR1-880R	5'-CGTTMARTTCGYCKACSGACA -3'		
GLRaV2	LR2-14568F	5'-RCDATGGAGYTRATGTCYGA-3'	525	Coat protein
	LR2-15092R	5'-AGCGTACATRCTYGCRAACA-3'		
GLRaV3	LR3CP107F	5'-TCTTAAARTAYGTTAAGGACGG-3'	301	Coat protein
	LR3-CP407R	5'-GGCTCGTTAATAACTTTCGG-3'		
GRBaV	GRBaV685F	5'-GAGGGTTGTTTGAAGATAAAG-3'	719	Coat protein
	GRBaV1403R	5'-CCATAATAAACAGCGTGGTC-3'		
GRSPaV	RSP35	5'-AGRYTTAGRGTRGCTAARGC-3'	477	RNA-dependent RNA polymerase
	RSP36	5'-CACATRTCATCVCCYGCAAA-3'		
GVA	GVA6538F	5'-TCTTCGGGTACATCGCCTTG-3'	324	Coat protein
	GVA6862R	5'-TCRAACATAACCTGTGGYTC-3'		
GVB	GVB6448F	5'-ATGGAAAATATATCCCKGATGG-3'	603	Coat protein
	GVB7050R	5'-GTTAACCACCTATATYTCRACAG-3'		
Ubiquitin	UBI-F	5'-CCGCACTCTTGCTGATTACA-3'	144	For Internal control-60S ribosome protein
	UBI-R	5'-GTGCATAACATTTGCGGCAG-3'		

Grapevine rupestris stem pitting-associated virus (GRSPaV), Grapevine red blotch-associated Virus (GRBaV), Grapevine leafroll-associated virus 1 (GLRaV1), Grapevine leafroll-associated virus 2 (GLRaV2), Grapevine leafroll-associated virus 3 (GLRaV3), Grapevine virus A (GVA), and Grapevine virus B (GVB)

Table 2 Comparison of single RT-PCR and RT-qPCR

Sample name	GRSPaV		GLRaV3		GRBaV		Ubiquitin
	RT-PCR	RT-qPCR	RT-PCR	RT-qPCR	PCR	qPCR	RT-qPCR
V1	+	+ / 28.0[a]	+	+ / 26.9	-	+ / 36.7	20.7
V2	+	+ / 28.6	+	+ / 28.4	+	+ / 33.9	21
V3	+	+ / 27.4	+	+ / 28.5	-	+ / 35.8	21
V4	+	+ / 26.3	+	+ / 28.5	+	+ / 29.3	21
V5	+	+ / 25.0	+	+ / 27.6	+	+ / 20.8	20.5
V6	+	+ / 28.3	+	+ / 29.2	-	+ / 33.1	20.9
V7	+	+ / 28.2	+	+ / 27.7	+	+ / 14.0	21
V8	+	+ / 25.4	+	+ / 29.1	+	+ / 15.0	24.7
V9	+	+ / 25.2	+	+ / 32.0	+	+ / 13.5	25
V10	+	+ / 27.6	+	+ / 31.7	+	+ / 14.1	25
V11	+	+ / 26.3	+	+ / 31.2	+	+ / 14.2	25.1
V12	+	+ / 27.9	+	+ / 27.4	+	+ / 13.5	25
V13	+	+ / 29.1	+	+ / 30.0	+	+ / 12.6	25.4
V14	+	+ / 27.7	+	+ / 31.2	+	+ / 13.1	25.2
V15	+	+ / 28.5	+	+ / 32.0	+	+ / 13.9	26.1
V16	+	+ / 26.5	+	+ / 31.2	+	+ / 14.9	25

+ and – : positive and negative respectively in RT-PCR; a: the quantification cycle (C_q) values is showed from RT-qPCR.

Table 3 Detection of grape viruses with DAS-ELISA

Samples	GLRaV-1		GLRaV-2		GLRaV-3	
	RT-PCR	DAS-ELISA	RT-PCR	DAS-ELISA	RT-PCR	DAS-ELISA
49-7	+	**3.4*** +	-	1.8 -	+	**16.7** +
49-10	-	**1.0** -	+	2.0 +	+	**5.6** +
49-13	-	**1.1** -	+	1.3 -	+	**4.1** +
49-15	-	**1.0** -	+	1.1 -	+	**9.5** +
84-15	+	**2.1** +	-	1.3 -	+	**22.9** +

+: positive in RT-PCR or ELISA testing; -: negative in RT-PCR or ELISA testing; *: Ratio of ELISA readings between samples and heathy control; it is a positive "+" when the ratio >2.0. The concentration of GLRaV-3 antibody for coating was 1/1000 dilution. The working concentration of anti- GLRaV-3 IgG alkaline phosphatase conjugate was 1/1000 dilution. 100 mg of grape leaves were extracted with 2 ml of extraction buffer (BioReba).

Table 4 The sensitivity of DAS-ELISA in the detection of GLRaV-1, GLRaV-2 and GLRaV-3 in grapevine leaves

Dilutions [tissue (g) / extraction buffer (mL)]	GLRaV-1[a]	GLRaV-3[b] (BioReba)	GLRaV-3[c] (in-house)		Dilutions [(tissue (g) / extraction buffer (mL)]	GLRaV-2[d]
1 / 40	6.3* +	14.3 +	5.3 +		1 / 10	11.5 +
1 /400	2.0 +	7.4 +	4.56 +		1 / 20	8.3 +
1 / 800	1.3	3.7 +	2.6 +		1 / 40	4.2 +
1 / 1600	1.1	2.5 +	2.0 +		1 / 80	2.5 +
1 / 3200	1.0	1.6	1.5		1 / 160	1.9
1 / 6400	1.0	1.4	1.2		1 / 320	1.5
1 / 12800	0.9	1.7	1.2		1 / 640	1.2

*: Ratio of ELISA readings between samples and heathy control; it is a positive "+" when the ratio >2.0. 200 mg of grape leaves were extracted with 2 ml of extraction buffer (BioReba buffer plus 1% PVPP)-this is a 1/10 dilution, which is used to make other dilutions with BioReba buffer.

a. First antibody is in-house GLRaV-1 antibody and the conjugate is BioReba GLRaV-1 conjugate.
b. Both first antibody and conjugate for GLRaV-3 are from BioReba.
c. Both first antibody and conjugate for GLRaV-3 are produced in this project.
d. Both first antibody and conjugate for GLRaV-2 are from BioReba.

Table 5 The detection of GLRaV-1, -2 and -3 in grape leaves by DAS-ELISA

Samples	GLRaV-1		GLRaV-2		GLRaV-3	
	RT-PCR	DAS-ELISA	RT-PCR	DAS-ELISA	RT-PCR	DAS-ELISA
49-10	**-**	**0.8* -**	+	2.6 +	+	**2.0 +**
49-11	**-**	**0.8 -**	+	3.1 +	+	**>14.4 +**
49-15	**-**	**0.8 -**	+	0.7 -	+	**3.1 +**
ON96	**-**	**0.8 -**	+	1.1 -	-	**0.7 -**
ON101	**-**	**0.8 -**	-	NT	+	**2.0 +**
ON104	+	**2.5 +**	-	NT	-	**0.6 -**
ON108	**-**	**0.8 -**	+	1.1 -	-	**0.7 -**
ON136	+	**4.0 +**	-	NT	+	**>14.4 +**
ON140	+	**2.9 +**	-	NT	+	**>14.4 +**
ON143	+	**2.8 +**	+	12.7 +	+	**8.2 +**
ON144	+	**2.9 +**	-	NT	+	**9.8 +**
ON150	**-**	**1.2 -**	+	2.7 +	+	**>14.4 +**
ON152	+	**0.8 -**	-	NT	-	**0.8 -**
ON230	**-**	**0.8 -**	-	NT	+	**7.1 +**
ON231	+	**1.0 -**	-	NT	-	**1.4 -**
ON273	**-**	**1.1 -**	-	NT	-	**1.0 -**
ON376	-	NT	-	0.9 -	-	**1.0 -**

+: positive in RT-PCR or ELISA testing; -: negative in RT-PCR or ELISA testing; NT: not tested. *: Ratio of ELISA readings between samples and heathy control; it is a positive "+" when the ratio >2.0.

100% correlation was obtained between test results for GLRaV-3 by DAS-ELISA and RT-PCR. For GLRaV-1, 14 of the 16 samples had consistent results between DAS-ELISA and RT-PCR. For GLRaV-2, only 5 of the 8 samples had consistent results between the two test methods.

Table 6 Virus survey with multiplex-PCR from five Ontario vineyards

Vineyards	Samples tested	GLRaV-1	GLRaV-2	GLRaV-3	GRBaV	GVA	GVB
A	19	1	3	3	15	0	0
B	43	6	3	33	1	13	14
C	35	0	0	21	1	0	0
D	30	0	0	12	1	0	0
E	9	0	0	4	7	0	0
Total	136	7	6	73	25	13	14
Percentage of infections		5	4	54	18	10	10

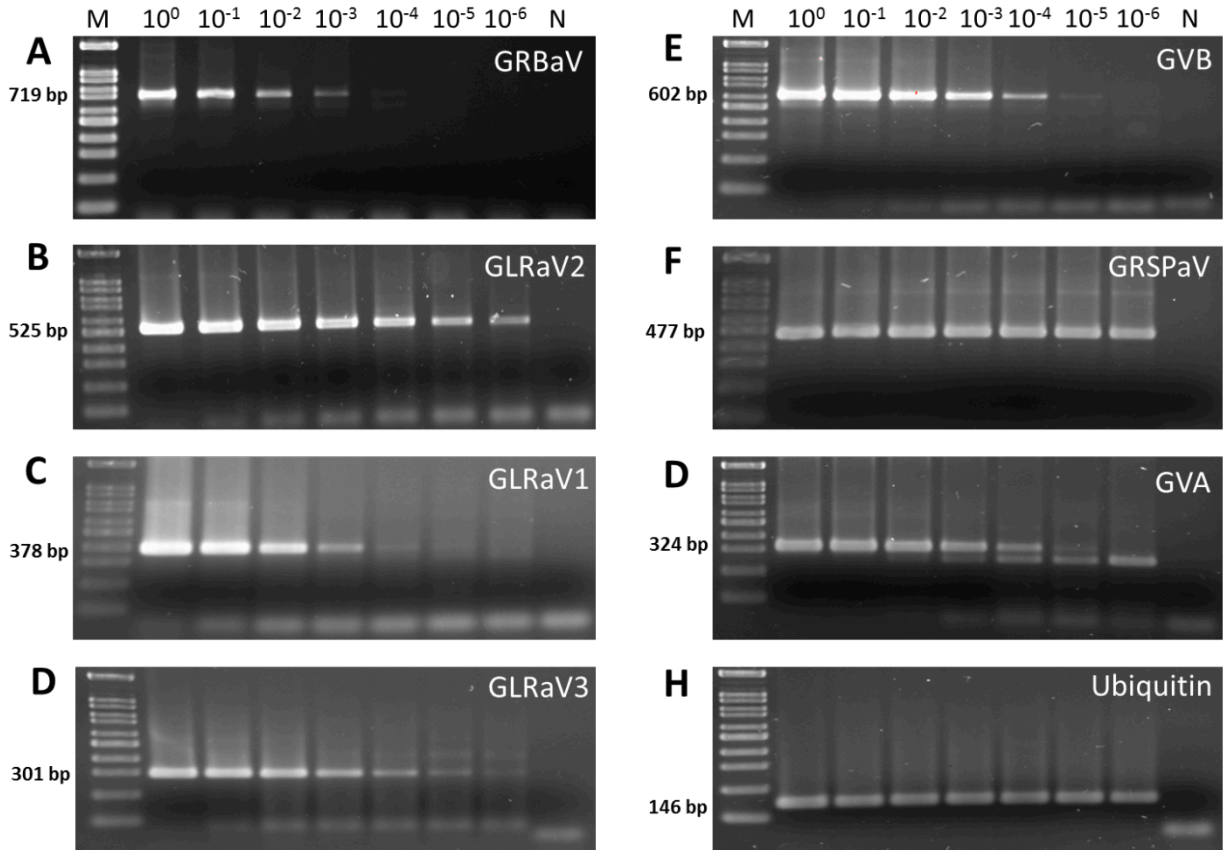

Figure 1 Agarose gel electrophoretic analysis of DNA products amplified by single reverse transcription-polymerase chain reaction (RT-PCR). The RNA was extracted from the positive controls (containing group A viruses-GRBaV, GLRaV-1, GLRaV-2, GLRaV-3, or group B viruses-GVB, GRSPaV and GVA) and serially diluted in RNA extracted from only GRSPaV-infected plants (no virus-free plants were found). Two-steps single RT-PCR for GRBaV (A), GLRaV-2 (B), GLRaV-1 (C), GLRaV-3 (D), GVB (E), GRSPaV (F), GVA (D) and internal control ubiquitin (H). Lane M, 100-bp DNA ladder, lanes from 10^0 to 10^{-6} are 10-fold serial dilutions of the positive control, and lane N, water.

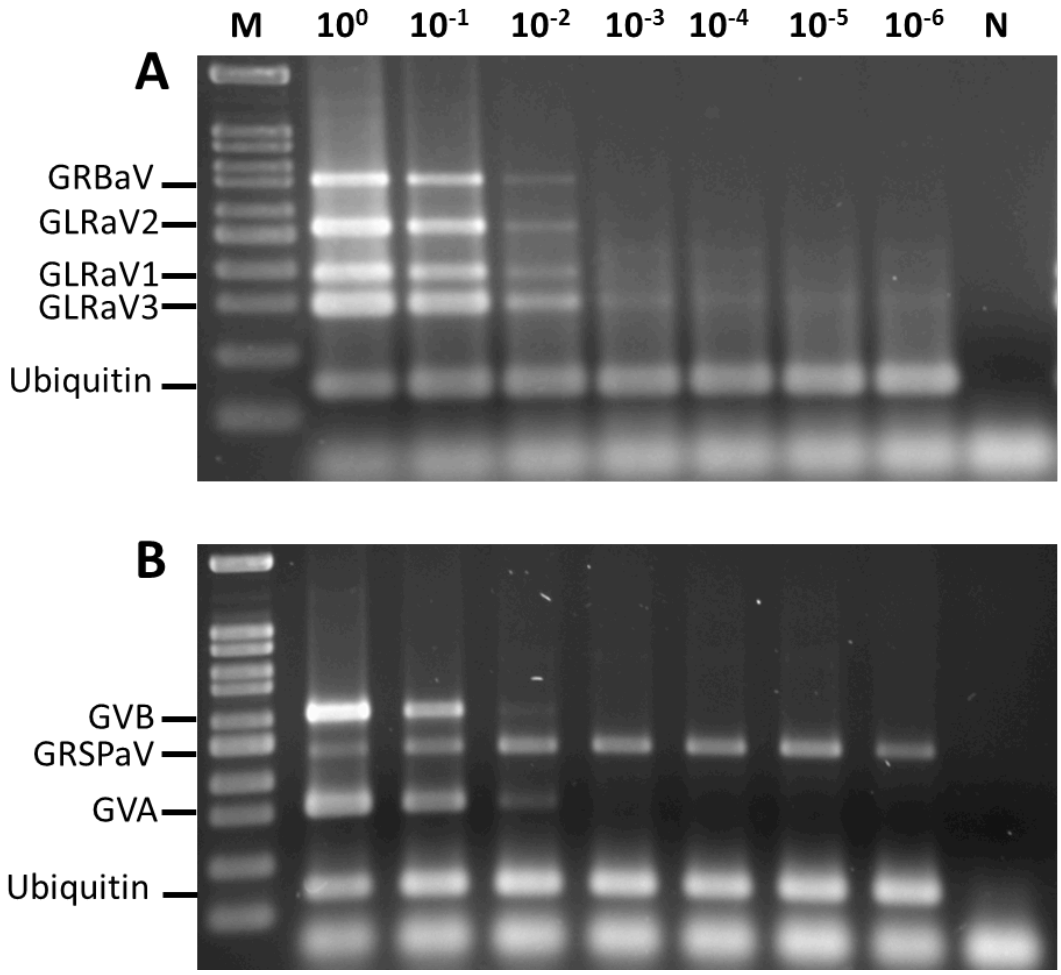

Figure 2 Agarose gel electrophoretic analysis of DNA products amplified by multiplex reverse transcription-polymerase chain reaction (RT-PCR) of group A (A) and B (B) viruses. The RNA was extracted from the positive controls (containing group A viruses-GRBaV, GLRaV-1, GLRaV-2, GLRaV-3, or group B viruses-GVB, GRSPaV and GVA) and serially diluted in RNA extracted from only GRSPaV-infected plants (no virus-free plants were found). Two-steps multiplex RT-PCR were done for Group A viruses (GRBaV, GLRaV-2, GLRaV-1, GLRaV-3 and internal control ubiquitin) and for group B viruses (GVB, GRSPaV, GVA and internal control ubiquitin). Lane M, 100-bp DNA ladder, lanes from 10^0 to 10^{-6} are 10-fold serial dilutions of the positive control, and lane N, water.

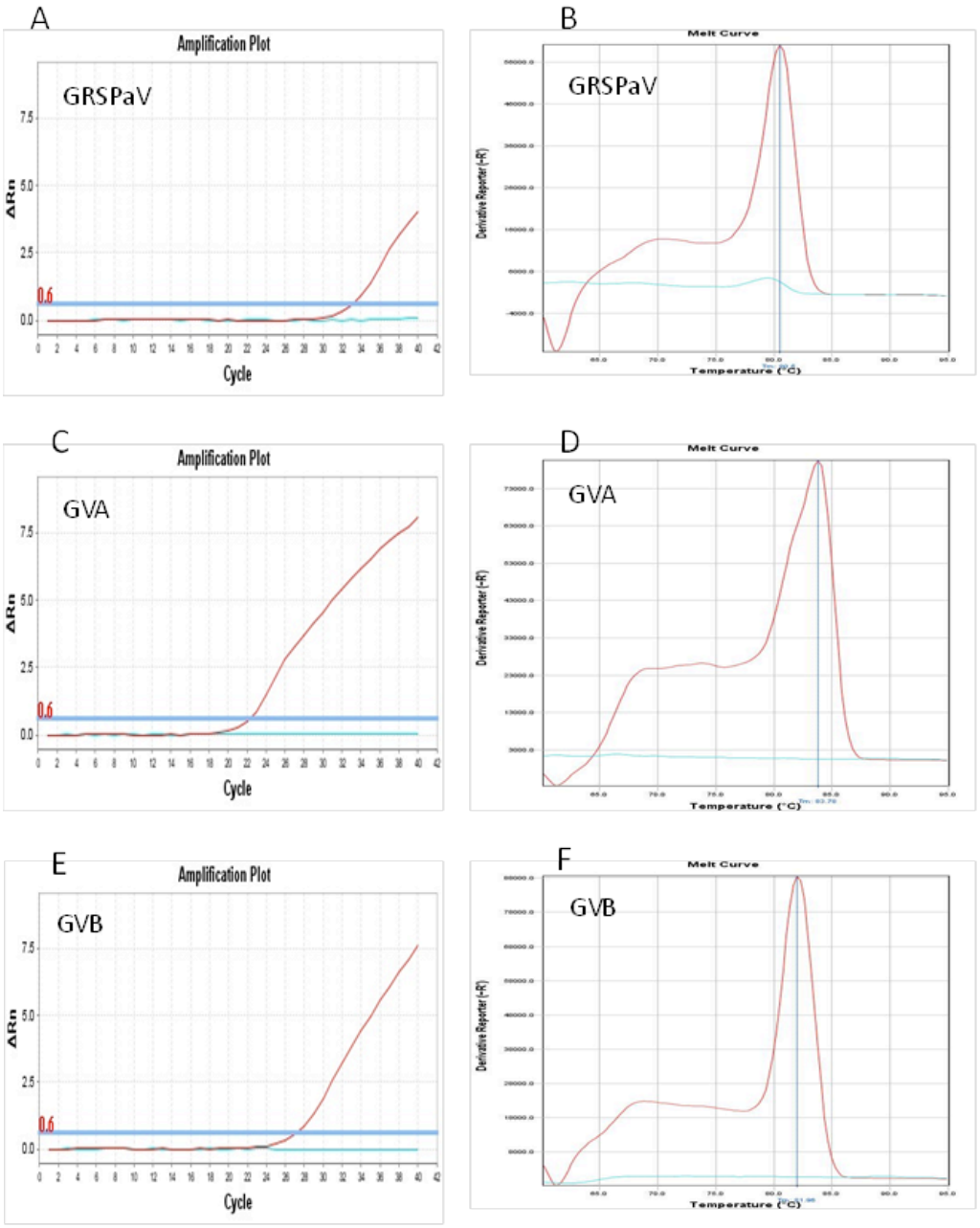

Figure 3 Single RT-qPCR detection of GRSPaV, GVA and GVB
A, C and E on left are Amplification Plot; B, D and F on right are Melt Curve. Positive samples are in red and negative controls are in green. The threshold line at 0.6 is showed in blue in Amplification Plot, and the midpoint of melt phase (T_m) is showed in blue in Melt Curve.

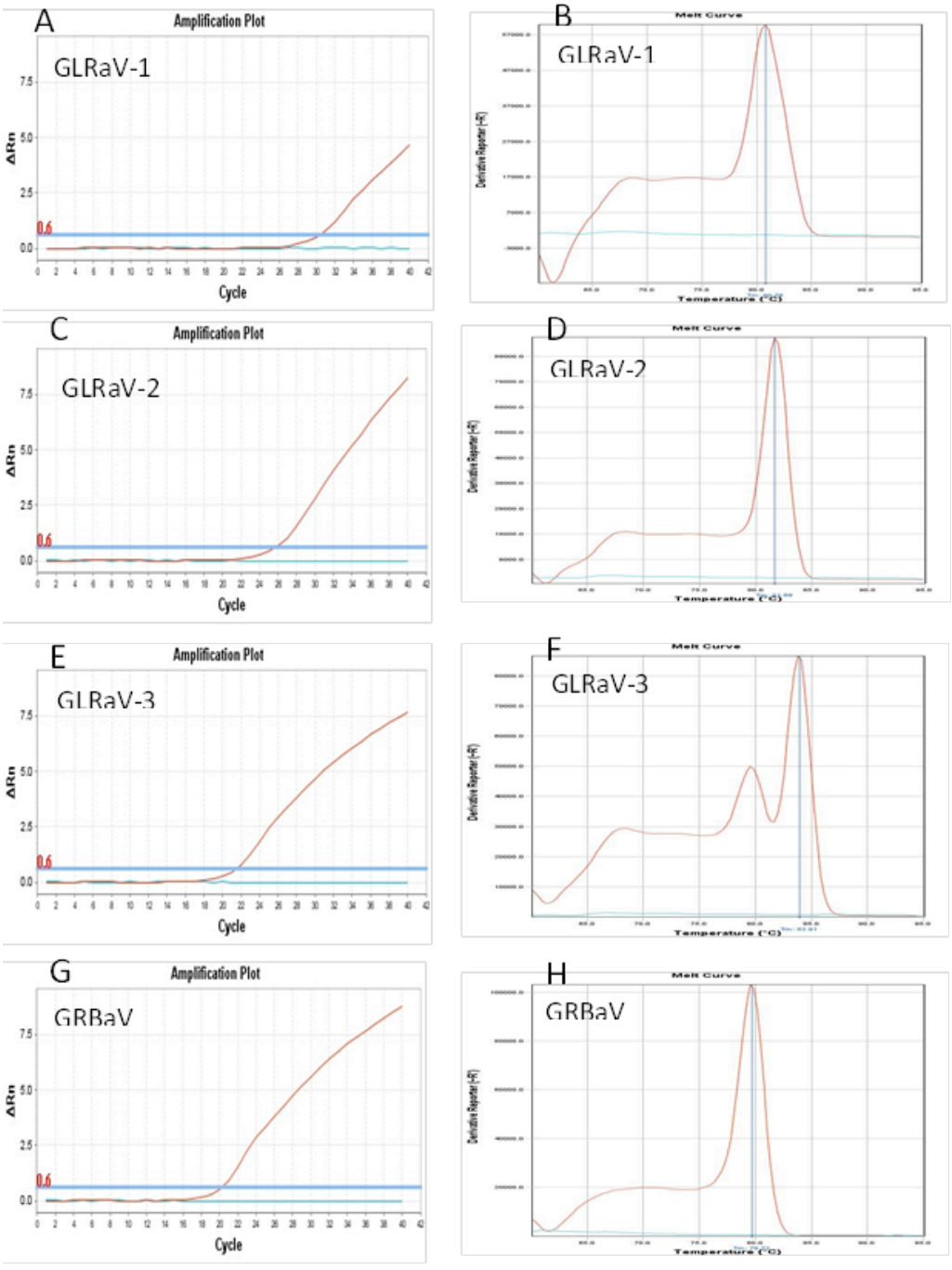

Figure 4 Single RT-qPCR detection of GLRaV-1, -2, -3 and GRBaV
A, C, E and G on left are Amplification Plot; B, D, F and H on right are Melt Curve. Positive samples are in red and negative controls are in green. The threshold line at 0.6 is showed in blue in Amplification Plot, and the midpoint of melt phase (T_m) is showed in blue in Melt Curve.

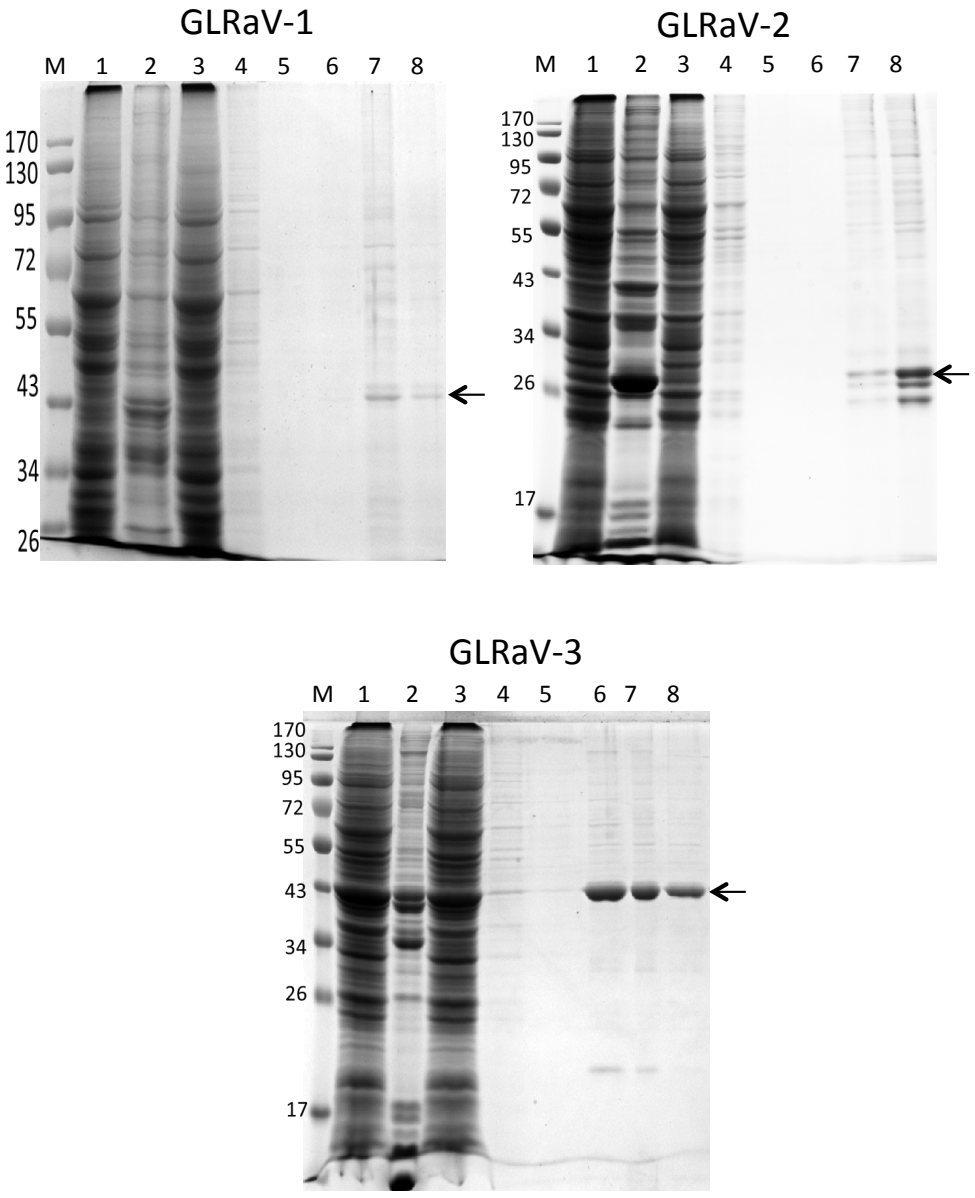

Figure 5 SDS-PAGE gels showing the purification of recombinant capsid proteins of GLRaV-1, GLRaV-2 and GLRaV-3 with Ni-NTA Affinity Chromatography. Lane M, protein ladder; lane 1, supernatant of infected insect cell lysate after centrifugation; lane 2, pellet of infected insect cell lysate after centrifugation; lane 3, flow-through from Ni-NTA column; lane 4, 1st washing with Tris Buffer A; lane 5, 2nd washing with Tris Buffer B; lane 6, 7 and 8, elution fraction 1, 2 and 3 (1.5 ml/ fraction) with elution buffer (Tris Buffer C). Recombinant capsid proteins are indicated with an arrow.

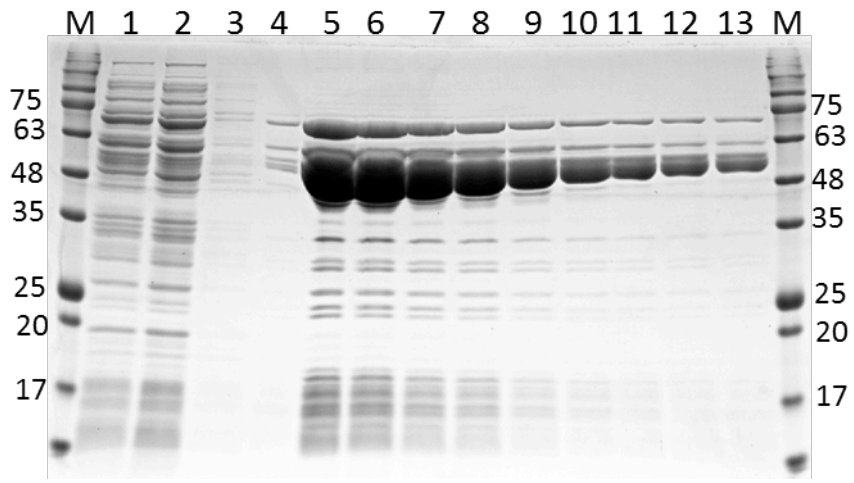

Figure 6 SDS-PAGE gels showing the purification of recombinant fusion protein of MBP:GRBaV-CP with Amylose Affinity Chromatography. Lane M, protein ladder; lane 1, supernatant of induced cell lysate after centrifugation; lane 2, flowthrough from amylose column; lane 3, washing with Column Buffer; lane 4-13, elution fractions 1-10 (1.5 ml/ fraction) with elution buffer (Column Buffer + 10 mM maltose).

Appendix 1. Nucleotide sequences of capsid protein gene of GLRaV-1, GLRaV-2, GLRaV-3 and GRBaV

>GLRaV-1 CP, 969 bp

ATGGCTAGCGTTATATCTCAAAATGATGATGACTATAATGTCGTTCGGGGCGGGAACATAGTTGTACCGC
GCTCGCCGAGTGTAAGTGGGTTCGGTACATCTGCTTTTACTATACCAGCCGGTGAGGCGACTGCTTACGT
GTTGAAGACACAGTACTCGAAGCCAGCAGCGGGTACTCCAGAGGCGGGCTACCCCGTGGTTGGAGTCGTG
CCGGAAGAGAGCGTGTTCGTGAAGGGACCCGGGGGTTATACACTCCGCCCATCTCGACCTTCGACGGGTC
AGCGTTCGGGGGATTCAGAGGTGGCTAGACGTGAAATGGGCGATAAGCTTAAACGAACTTTTAATATTGC
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GTACCAACCGGACCCGGCTTGGTCACGCCGGCGGTGGCAACTGCAATTTCCACAGAATTAAAAAATTTAT
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GTTGACGTTTAGCACGTCACCAGATACAGAGAGCAAAGAAGAGTATTTCGTGAATCTATATAGCAATGGC
GAGCGGAAATTGACTTATGAGAAGGTTAAGGGGGCCGTTGTCAAGGGAGCCGAAGGCTCGACTTTTGAAA
ATCCAATGCGTCAGTATGCTAGATTGTTCTCAGCGACAGCCGTTCATTTAATACTGAATGGAAAGTTGAA
GCCGAACGAGAAAGTTGCTATGCAGCACGGAGTACCTAAGAGGTTCCTCCCGTATACTTTTGATTTCTGC
AGACCATCTTACTCTCAGTTTAGCAACGACGCCATTAGGGCATGGCAGTTGGCGGCAGAATCAGCGTTCG
GGCGGAAAAGTAATGTGACAAGCTCGGTTCTGAGGAACACCAGCGAGCTTAAGGTGTAA

>GLRaV-2 CP, 597 bp

ATGGAGTTGATGTCTGACGACAACCTTAGCAACCTGGTGATAACTGACGCCTCTAGTCTAAATGGTGTCG
ATAAGAAACTTTTATCTGCAGAAATCATAAAAATGTTGGTGCAGAAAGGCGCTCCTAACGACGGTATAGA
AGTGGTGTTCGGTCTACTCCTTTACGCGCTTGCAGCGAGAACCACGTCTCCTAAAGTTCAACGCGCCGAT
TCAGACGTGATTTTTTCTAACCGTTTTGGAGAGAGTAACGTGGTAGTGACGGAGGGTGACCTTAAGAAGG
TGCTCGACGGGTGCGCACCTCTCACTAGGTTCACTAATAAACTTAGAACCTTCGGTCGCACTTTCACTGA
GGCTTACGTTGATTTTTGTATCGCGTATAAGCACAAATTACCGCAACTCAACGCCGCAGCGGAATTGGGA
ATTCCGGCTGAAGATTCATACTTGGCTGCAGATTTTCTGGGTACTTGCCCGAAGCTCTCTGAACTGCAGC
AAAGCAGAAAGATGTTCGCGAGCATGTACGCTCTAAAAACTGAAGGTGGAGTGGTAAATACACCAGTGAG
CAATCTGCGTCAGCTAGGTAGAAGGGAAGTTATGTAA

>GLRaV-3 CP, 942 bp

ATGGCATTTGAACTGAAATTAGGGCAGATATATGAAGTTGTCCCCGAAAACAATCTGAGAGTTAGAGTAG
GGGATGCGGCACAAGGAAAGTTTAGTAAGGCGAGTTTCTTAAAATACGTTAAGGACGGGACGCAGGCGGA
GTTAACGGGAATCGCTGTAGTGCCGGAAAAGTACGTATTCGCCACAGCAGCTTTAGCTACAGCGGCACAG
GAGCCACCTAAGCAGCCGACAACGCAAGTGGTGGAACCACCAGAAGCCGATATAGGGGTGGTGCCGGAAT
CTGAGACTCTTACACCAAATAAGCTGGTTTTTGAGAAAGATCCAGACAAGTTCTTAAAGACTATGGGTAA
GGGGATAGCTTTGGACTTAACGGGGGTCACCCATAAACCGAAAGTTATTAACGAGCCGGGAAAAGTATCA
GTAGAGGTAGCAATGAAGATTAATACCGCATTGGTAGAGCTGTGTAAGAAGGTTATGGGCTCCGATGACA
CAACAACCAAGACAAAATTCTTCTTGTACGTGATGCAGATCGCTTGCACGTTCTTTACATCGTCTTCGAC
GGAGTTCAAAGAGTTCGACTACATAGAAACGGACGATGGAAAGAAGATATATGCGGTGTGGGTATACGAT
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CAACCTTGATCACGGCGACCCTGAATGGTAAACTGGTGATGAATGAAAAGGTCATGGCACAGCATGGAGT
ACCACCGAAATTCTTTCCGTACGCGATTGACTGCGTTCGTCCGACGTACGATCTGTTCAATAACGACGCA
ATACTAGCATGGAATTTAGCTAGACAGCAGGCGTTTAGAAATAAGACGGTGACGGCCGATAACACCTTAC
ACAACGTCTTCCAACTATTGCAAAAGAACTAG

>GRBaV CP, 672 bp

ATGGTAATGAAAAAAAGGAGCCGCCAACGGAAGCAGAGAAGAAGGCGCAAAGCGACTGGGAGGAGTTCTG
CAGGAAGGCGGCGTGCTCGGCCACGTAGTAGGCCTTGTCAGTTTGCATTTCATGGGAACTCTTTTGGGGG
TACCCCTTCCCTTTTTTTTTTAACTCCCATTGCTTTAGGGACTGGTGCGGAAGACAGAACTGGTCCAGTA
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ATATTTATTTTTGGATTATTTTAGATCGGTTTCCTACTGGAACCGATCCTTCTGTATCTGACATATTCAC
TGGTAGTGATAATAGCGGAAGCATGATTGAGACATTGACGAGGAATCGTTTGAATCGTAAGAGATTTCGT
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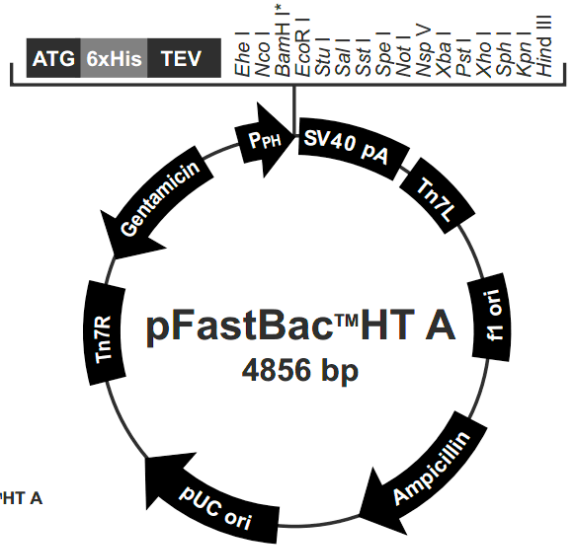
Appendix 2. Map and features of pFastBac HT A

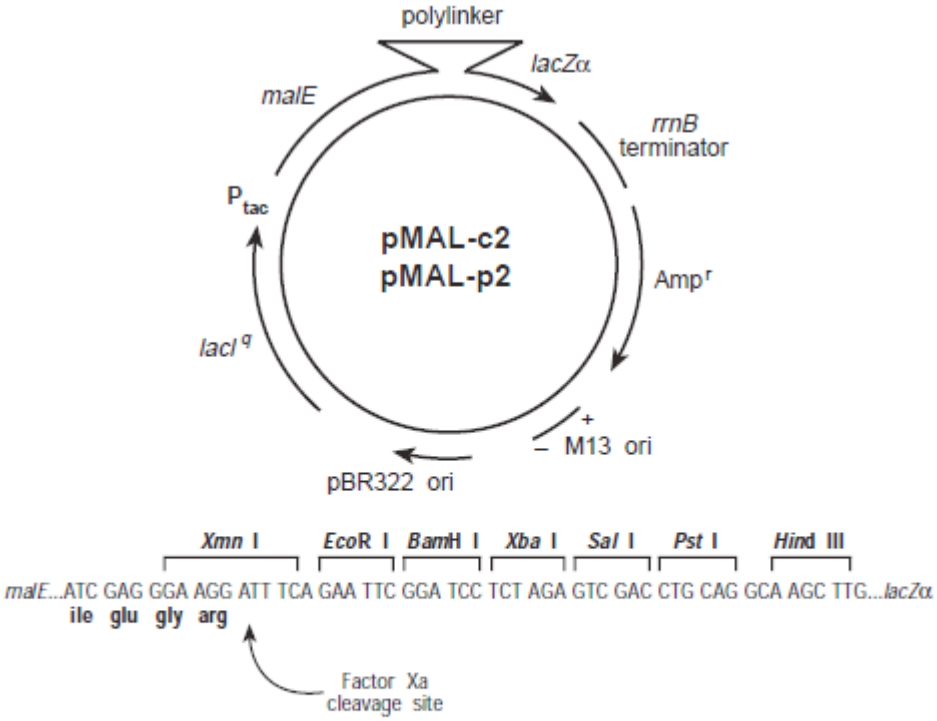


Comments for pFastBac™HT A
4856 nucleotides

f1 origin: bases 2-457
Ampicillin resistance gene: bases 589-1449
pUC origin: bases 1594-2267
Tn7R: bases 2511-2735
Gentamicin resistance gene: bases 2802-3335 (complementary strand)
Polyhedrin promoter (P_{PH}): bases 3904-4032
Initiation ATG: bases 4050-4052
6xHis tag: bases 4062-4079
TEV recognition site: bases 4101-4121
Multiple cloning site: bases 4119-4222
SV40 polyadenylation signal: bases 4240-4480
Tn7L: bases 4509-4674

*Frameshift occurs at the *Bam*H I site in each vector

Appendix 4. Map and features of pMAL-c2



pMAL™-2 Vectors. pMAL™-c2 (6646 base pairs) has an exact deletion of the *malE* signal sequence. pMAL™-p2 (6721 base pairs) includes the *malE* signal sequence. Arrows indicate the direction of transcription. Unique restriction sites are indicated.

Sites of insertion of GRBaV capsid protein gene: BamH I and Hind III.

Appendix 5. Procedures for purifying the recombinant GRBaV capsid protein with MBP's affinity for maltose

1. Subclone the GRBaV capsid protein gene into the pMAL-C2 vector and transform into Escherichia coli Rosetta-gami B (DE3)pLysS.

2. Grow cells containing the pMAL-C2 fusion plasmid in LB (containing 12.5 µg/ml tetracycline, 15 µg/ml kanamycin, 34 µg/ml chloramphenicol, and 50 µg/ml ampicillin and 0.2% glucose) to an A600 of around 0.5.

3. Induce by adding IPTG to a final concentration of 0.5 mM.

4. Grow for an additional 4 h at 30°C.

5. Harvest the cells and store at -20°C.

6. Resuspend in 25 ml column buffer per liter of culture (CB: 20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA, 10 mM β-ME).

7. Lyse the cells by French Press.

8. Clarify the lysed cells by centrifugation at 20,000 x g for 20 min.

9. Dilute the supernatant (crude extract) by adding 125 ml cold CB for every 25 ml crude extract.

9. Load the diluted crude extract on a 15 ml amylose column.

10. Wash the column with ≥ 12 column volumes of CB.

11. Elute the fusion protein with CB + 10 mM maltose.

Appendix 6. The procedure of generating polyclonal antiserum against recombinant capsid proteins of target viruses

Rats: Strain-Sprague Dawley, Sex-Female, Age-~8 weeks **2 rats per protein**	
Days	**Procedure**
0	**Pre-bleed for control serum**
0	**Primary (1^0) immunization:** 1:1 Emulsion of 50 μg of recombinant protein in *sterile* PBS pH 7.2 (10 mM) **and** TitreMax Adjuvant. Inject **IM** (intramuscularly) into **2 sites** on either hind leg muscles with max of 50 μl / site.
14	**First booster injection:** 1:1 Emulsion of 25 μg of protein in sterile PBS pH 7.2(10mM) + Freud's incomplete adjuvant. Inject **IM** (intramuscularly) into **2 sites** on either hind leg muscles with max of 50 μl/site.
17-19	**1^{st} test bleed to screen Ab-response** Bleed no more than 10% of total blood volume
28	**Second booster injection:** 1:1 Emulsion of 25 μg of protein in sterile PBS pH 7.2 (10mM) + Freud's **i**ncomplete adjuvant. Inject **IM** (intramuscularly) into **2 sites** on either hind leg muscles with max of 50 μl/site.
31-33	**2^{nd} test bleed to screen Ab-response** Bleed no more than 10% of total blood volume
40	**Third booster injection (optional, depending on titer of second test bleed):** 1:1 Emulsion of 25 μg of protein in sterile PBS pH 7.2 (10mM) + Freud's incomplete adjuvant. Inject **IM** (intramuscularly) into **2 sites** on either hind leg muscles with max of 50 μl/site.
43-45	**Final total blood collection**

Appendix 7. Indirect-ELISA Protocol

1. Dilute antigen to a final concentration of 1-20 μg/ml using PBS or Bicarbonate/carbonate coating buffer. Coat the wells of a PVC microtiter plate with the antigen by pipeting 50μl of the antigen dilution in the top wells of the plate. Dilute down the plate as required. Seal the plate and incubate overnight at 4°C.

2. Wash plate 3 times with PBS.

3. Block the remaining protein-binding sites in the coated wells by adding 200 μl blocking buffer, 3% nonfat dry milk/PBS, per well.

4. Cover the plate with an adhesive plastic and incubate for at least 2 h at room temperature or, if more convenient, overnight at 4°C.

5. Wash the plate 3 times with PBST.

6. Add 100 μl of diluted primary antibody to each well.

7. Cover the plate with an adhesive plastic and incubate for 2 h at room temperature.

8. Wash the plate 3 times with PBST.

9. Add 100 μl of conjugated secondary antibody (anti-rat IgG peroxide conjugate), diluted at 1:5000 in PBST immediately before use.

10. Cover the plate with an adhesive plastic and incubate for 1-2 h at room temperature.

11. Wash the plate 3 times with PBST.

12. Dispense 100 μl (or 50 μl) of the substrate (O-phenylenediamine dihydrochloride) solution per well with a multichannel pipet.

13. Record the absorbance at 450 nm on a plate reader within 30 minutes of stopping the reaction.

Appendix 8. Western blot protocol

Sample preparation

1. Lysis plant tissues in extraction buffer and clarify the extracts via centrifugation.
2. Remove a small volume of lysate to perform a protein quantification assay. Determine the protein concentration for each cell lysate.
3. Determine how much protein to load and add an equal volume 2X Laemmli sample buffer.
4. To reduce and denature your samples, boil each cell lysate in sample buffer at 100°C for 5 min. Lysates can be aliquoted and stored at -20°C for future use.

Loading and running the gel

1. Load equal amounts of protein into the wells of the SDS-PAGE gel, along with molecular weight marker. Load 20–30 μg of total protein from cell lysate or tissue homogenate, or 10–100 ng of purified protein.
2. Run the gel for 1–2 h at 100 V.

Transferring the protein from the gel to the membrane

The membrane can be either nitrocellulose or PVDF. Activate PVDF with methanol for 1 min and rinse with transfer buffer before preparing the stack. The time and voltage of transfer may require some optimization. We recommend following the manufacturer's instructions.

Antibody staining

1. Block the membrane for 1 h at room temperature or overnight at 4°C using blocking buffer.
2. Incubate the membrane with appropriate dilutions of primary antibody in blocking buffer. We recommend overnight incubation at 4°C; other conditions can be optimized.
3. Wash the membrane in three washes of TBST, 5 min each.
4. Incubate the membrane with the recommended dilution of conjugated secondary antibody in blocking buffer at room temperature for 1 h.
5. Wash the membrane in three washes of TBST, 5 min each.
6. For signal development, follow the kit manufacturer's recommendations. Remove excess reagent and cover the membrane in transparent plastic wrap.
7. Acquire image using darkroom development techniques for chemiluminescence, or normal image scanning methods for colorimetric detection.

Appendix 9. Dot-ELISA Protocol

1. Preparation of sample extract: 0.1 g of leaf tissue is ground with mortar and pestle in 1 mL of extraction buffers. The debris is removed by centrifugation (10 min, 12000×g, 4 °C).

For the following procedures, all steps are performed at room temperature in a shaker.

2. 8 μl of the sample extract or its dilutions are applied directly onto PVDF nylon membrane or nitrocellulose membrane in squares marked with pencil.

3. Membranes are blocked for 1 hour with 3% skim milk solution in blocking solution (PBST).

4. Wash the membranes 3 times with PBST.

5. Incubate the membranes in a diluted primary antibody solution for 1 hour.

6. Wash the membranes 3 times with PBST.

7. Incubate the membranes in a diluted alkaline phosphatase (AP) or horseradish peroxide (HRP)-conjugate secondary antibody for 1 hour.

8. Wash the membranes 3 times with PBST.

9. For signal development, the membranes are then applied with a chromogenic substrate and the appearance of the color dots is observed with the naked eye. The membranes can also be applied with a chemiluminescent substrate and then acquire image using darkroom development techniques for chemiluminescence.

Appendix 10. Double Antibody Sandwich ELISA (DAS-ELISA)

1. Dilute specific antibody in coating buffer (recommended dilution see delivery note and tube); i.e. 20µl in 20 ml buffer at a recommended dilution of 1:1000 or 40µl in 20 ml buffer at a recommended dilution of 1:500. Add 200µl to each well of the microtiter plate.

2. Cover the plates and incubate at 37 °C for 2- 4 h.

3. Wash plate with PBS-Tween using wash bottle, soak for a few minutes and repeat washing two times. Blot plates by tapping upside down on tissue paper.

4. Extract samples 1:20 (w/v) in extraction buffer. Add 200 µl aliquots of the test sample to duplicate wells.

5. Cover the plates and incubate overnight at 4 °C.

6. Wash three times as in step 3.

7. Add 200 µl enzyme conjugate, recommended dilution is given in the delivery note, in conjugate buffer.

8. Cover the plates and incubate at 37 °C for 2- 4 hours.

9. Wash three times as in step 3.

10. Add 200 µl aliquots of freshly prepared substrate (1 mg /ml para- nitrophenyl-phosphate in substrate buffer) to each well.

11. Cover the plate and incubate at 37°C for 30-60 min, or as long as necessary to obtain clear reactions.

12. Assess results by: a) Visual observation b) Spectrophotometric measurement of absorbance at 405 nm

Appendix 11. Single RT-PCR established in this project

The protocol for first-strand cDNA synthesis

First-strand cDNA synthesis is performed using High-capacity cDNA Reverse Transcription Kit (Life technologies, Cat# 4368814). The reaction mix (20 µl) includes 500 ng of total RNA, 2.0 µl 10X RT Buffer, 0.8 µl 25X dNTP Mix (100 mM), 2.0 µl 10X RT Random Primers, 1.0 µl MultiscribeTM Reverse Transcriptase (50U/ µl). The mix is incubated for 10 min at 25 ºC, then 120 min at 37 ºC, then 5 min at 85 ºC, and then stored at 4 ºC for immediate use or -20 ºC for later use.

The protocol for single RT-PCR

The PCR reaction mix (25 µl) for single RT-PCR contains 1 µl of cDNA (5% of the first-strand reaction, corresponding to about 25 ng of total RNA), 1X PCR Buffer (containing 2.0 mM MgCl2), 0.2 mM dNTPs, 0.2 µM each primer, and 1.0 units of Taq DNA polymerase (GeneDireX). PCR conditions for all primer pairs include an initial denaturation step at 94 ºC for 4 min, then 40 cycles at 94 ºC for 45 s, 50 ºC for 45 s and 72 ºC for 1 min, followed by a final extension at 72 ºC for 7 min. The PCR products are analyzed on 1.5% agarose gel, followed by staining with ethidium bromide.

Appendix 12. Multiplex RT-PCR established in this project

The protocol for first-strand cDNA synthesis

First-strand cDNA synthesis is performed using High-capacity cDNA Reverse Transcription Kit (Life technologies, Cat# 4368814). The reaction mix (20 µl) includes 500 ng of total RNA, 2.0 µl 10X RT Buffer, 0.8 µl 25X dNTP Mix (100 mM), 2.0 µl 10X RT Random Primers, 1.0 µl MultiscribeTM Reverse Transcriptase (50U/ µl). The mix is incubated for 10 min at 25 ºC, then 120 min at 37 ºC, then 5 min at 85 ºC, and then stored at 4 ºC for immediate use or -20 ºC for later use.

The protocol for multiplex PCR for Group A viruses (GRBaV, GLRaV-1, -2 and -3)

The multiplex PCR reaction mix (25 µl) contains 1 µl of cDNA (5% of the first-strand reaction, corresponding to about 25 ng of total RNA), 1.5 X PCR Buffer (containing 3.0 mM MgCl2), 0.2 mM dNTPs, and 2.0 units of Taq DNA polymerase (GeneDireX). The primers and their final concentrations are 0.3 µM GRBaV685F/GRBaV1403R for GRBaV, 0.4 µM LR1-502F/LR1-880R for GLRaV-1, 0.3 µM LR2-14568F/LR2-15092R for GLRaV-2, 0.3 µM LR3CP107F/LR3-CP407R for GLRaV-3, and 0.5 µM UBI-F/UBI-R for internal control-ubiquitin 60S-ribosome protein. Cycling conditions includes an initial denaturation step at 94 ºC for 4 min, then 40 cycles at 94 ºC for 45 s, 53 ºC for 45 s and 72 ºC for 1 min, followed by a final extension at 72 ºC for 7 min. The PCR products are analyzed on 1.5% agarose gel, followed by staining with ethidium bromide.

The protocol for multiplex PCR for Group B viruses (GRSPaV, GVA and GVB):

The multiplex RT- PCR reaction mix (25 µl) contains 1 µl of cDNA (5% of the first-strand reaction, corresponding to about 25 ng of total RNA), 1.5 X PCR Buffer (containing 3.0 mM MgCl2), 0.2 mM dNTPs, and 2.0 units of Taq DNA polymerase (GeneDireX). The primers and their final concentrations are 0.6 µM RSP35/RSP36 for GRSPaV, 0.2 µM GVA6538F/GVA6862R for GVA, 0.4 µM GVB6448F/ GVB7050R for GVB, and 0.5 µM UBI-F/UBI-R for internal control-ubiquitin 60S-ribosome protein. Cycling conditions includes an initial denaturation step at 94 ºC for 4 min, then 40 cycles at 94 ºC for 45 s, 50 ºC for 45 s and 72 ºC for 1 min, followed by a final extension at 72 ºC for 7 min. The PCR products are analyzed on 1.5% agarose gel, followed by staining with ethidium bromide.