

# Establishment and Application of a Reverse Transcription Loop-mediated Isothermal Amplification Assay for Detection of Grapevine Fanleaf Virus

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## Abstract

Grapevine degeneration in grapevines caused by Grapevine Fanleaf Virus (GFLV) has been documented in many viticulture regions worldwide. It is one of the major economically important virus diseases affecting the longevity of grapevines and reducing the fruit yield and fruit quality. To diminish the time required for some diagnostic assays, including reverse transcription polymerase chain reaction (RT-PCR), reverse transcription loop-mediated isothermal amplification (RT-LAMP) and also double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) into a minimum level, an innovative immunocapture RT-LAMP (IC-RT-LAMP) and immunocapture RT-PCR (IC-RT-PCR) protocol on the basis of GFLV genome were used and the reaction conditions were optimized for rapid detection of GFLV. In this regard, DAS-ELISA was employed first to validate the existence of the virus. All six RT-LAMP primers (i.e., F3, B3, FIP, BIP, LF and LB) together with RT-PCR primers (F and B) were designed on the basis of coat protein (CP) gene of GFLV. LAMP method, on the whole, had the following advantages over the other mentioned procedures: (I) fascinatingly, no need of RNA extraction; (II) no requirement of expensive and sophisticated tools for amplification and detection; (III) no post-amplification treatment of the amplicons; and (IV) a flexible and easy detection approach, which is visually detected by naked eyes using diverse visual dyes.

**Keywords:** DAS-ELISA assay; Grapevine fanleaf virus; Immunocapture assay; RT-LAMP assay; RT-PCR assay

## Introduction

Grapevine viruses occur in all grapevine-growing regions of the world, with significant economic impact and relevance in many countries. Among the damages caused by viruses, the reduction in productivity and quality of grapes, and the shortening of the productive life of the vineyard stand out. So far, at least 60 virus species are known to infect grapevines (*Vitis* spp.) [1]. Grapevine fanleaf virus (GFLV) is the oldest known virus of grapevine (*Vitis vinifera* L.) [2]. This member of the genus Nepovirus in the family Secoviridae is the most widely spread [3]. It causes a leaf degeneration disease and yield losses up to 80% can be incurred. Furthermore, the infection with GFLV reduces fruit quality and shortens the lifespan of vines [4]. GFLV is naturally transmitted by the dagger nematode *Xiphinema index* and experimentally by plant sap [5,6]. Having a worldwide distribution, it is nearly impossible to eradicate GFLV from vines that have been propagated with infected material and or infested with the nematode [7]. In Iran, the virus was first reported in 1989 in the northwestern region, based on symptoms and inoculations on indicator plants [6]. For the first time, the genetic diversity of the virus was studied based on the virus coat protein gene in the northwest of Iran [8].

The virus genome is composed of two single-stranded, linear RNA segments with positive polarity (RNA1 and RNA2), each containing one open reading frame (ORF) and coding for a polyprotein. The RNA1-encoded polyprotein (P1) is processed into five proteins and RNA2 codes for the P2 polyprotein which is proteolytically processed into three proteins; the Movement protein (MP) is one of the proteins coded by RNA2. Both RNA1 and RNA2 of the GFLV strain F13 have been sequenced, which are 7342 and 3774 nucleotides (NTs) in length, respectively [9-14]. The most important strategy to control viral diseases in grapes is preventive and consists of planting virus-free vines during vineyard establishment. Propagation material with optimal sanitary status is produced through certification schemes, which use, reliable techniques for detecting viruses associated with most common and harmful diseases. Grapevine virus detection is based on bioassays, enzyme-linked immunosorbent assay (ELISA) [5,15], and more recently, reverse transcription polymerase chain reaction (RT-PCR) [16,17], Multiplex RT-PCR (mRT-PCR) [18] and real-time RT-PCR [19,20].

Among various isothermal amplification systems developed over the recent years, the most frequently applied approach seems to be a loop-mediated isothermal amplification (LAMP), developed by Notomi et al., [21]. Due to its enormous rate of amplification paired with a very high specificity, sensitivity, rapidity and low artifact susceptibility, the method together with its modifications have been strongly recommended for detection of a great number of strains of bacteria as well as viruses worldwide [22]. The synthesis of DNA is done by a DNA polymerase such as *Bst* that only contains 5→3 polymerase activity, but lacks 5→3 exonuclease activity. Therefore a strand displacement process can be ensured. As these polymerases are not thermostable they cannot be used in a PCR reaction, but the LAMP is an isothermal cycling technique that is held under optimal temperature conditions. Briefly, each reaction is carried out by the use a set of four different primers: F3 (forward outer primer), B3 (backward outer primer), FIP (forward inner primer), BIP (backward inner primer), designed specifically to recognize six distinct regions on the target sequence in conjunction with two loop primers: LF (loop forward) and LB (loop backward) to accelerate the reaction [21,23]. The reaction produces a mixture of stem-loop and multi-loop cauliflower-like structures that are all constructed from multiple repeats of only the targeted genomic sequence [24-26]. Particularly LAMP can be used for detection of any genomic sequence under isothermal condition in the range of 60 to 65°C. RNA can be easily identified by just adding an initial reverse transcriptase (RT) step. Another improvement of the method includes the development of additional primer pair (to a total

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Received November 28, 2015; Accepted December 18, 2015; Published January 06, 2016

Citation: Almasi M (2016) Establishment and Application of a Reverse Transcription Loop-mediated Isothermal Amplification Assay for Detection of Grapevine Fanleaf Virus. Mol Biol 5: 149. doi:10.4172/2168-9547.1000149

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of six oligonucleotides) with loops already synthesized in the designed primers. Thus, the cycling stage starts earlier and both the sensitivity and specificity of the reaction are improved even further [21,27,28]. LAMP is highly specific as six separate genomic regions in the initial stage and four in the later steps, need to be recognized in order that the reaction is carried out. Therefore, a successful reaction would indicate the presence of the targeted sequence [29]. Magnesium pyrophosphate, a byproduct of the amplification process, is produced in proportional amounts to the amplified product. LAMP is highly efficient and large amounts of end products are synthesized, altogether with large amounts of pyrophosphate [21]. The white turbidity attributed to it may be visually observed. Therefore, the presence of turbidity indicates the formation of the targeted genomic region. Visual detection can also be achieved by adding an intercalating dye to the final reaction mixture. If an agarose gel electrophoresis of the LAMP product is performed, bands with various sizes will be visualized at regular intervals. Additionally, restriction analysis alone or followed by sequencing reactions can be performed for verification of new protocols that are tested for the first time [20,30-34].

Notably, despite a few numbers of studies about immunocapture reverse transcription loop-mediated isothermal amplification (IC-RT-LAMP) [29,35], because the technique has not been yet introduced for detection of GFLV, an attempt was accordingly made to optimize a new protocol of it to save time, particularly remove RNA extraction and finally improve detection sensitivity of GFLV. Here, two different visualization systems were consequently employed to assess the colour stability as well as safety feature of each one in a viral detection procedure of GFLV.

## Materials and Method

### Plant samples

A total of 100 grapevine leaves (2 leaves per plant from 50 grapevine) showing GFLV-incited symptoms such as mosaic, vein banding, open petiole leaf, mottling, leaf deformation and fanleaf were collected from vineyards in the three northwestern provinces of Iran including East and West Azerbaijan and Ardebil during the spring and summer 2013. Leave samples had been sliced and rapidly desiccated in anhydrous calcium chloride and were kept at -80°C at the laboratory.

### DAS-ELISA assay

The samples were first subjected to screening by the double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) in order to identify the infected samples. This was carried out because similar symptoms can be caused by different virus species. DAS-ELISA was carried out as described by Clark and Adams [36], with some minor modifications, using a commercially available polyclonal GFLV-specific antibody (coating antibody V215-C1, detecting conjugate AP V215-D1, positive control V215-P2, negative control V215-N1, ACD,

Inc.). Polystyrene microtiter plates were coated for 3 h at 37°C, with 100 µL per well of IgG coating, in 50 mM carbonate buffer, pH 9.6. The plates were then incubated for 1 h at 37°C with phosphate buffer saline (PBS) (10 mM phosphate buffer, pH 7.2, 0.8% NaCl and 0.02% KCl). After that, the plates were washed three times using washing buffer (0.8% NaCl, pH 7.2 and 0.05% Tween 20). The leaf extracts of each fresh sample were ground in ten volumes (w/v) of PBS buffer pH 7.2, containing 0.2% polyvinylpyrrolidone (PVP) and 2% of egg albumin (Sigma A5253). The infected preparations were serially diluted (five-fold dilution) at the same buffer. Aliquots of 100 µL of prepared samples and controls were added to each well (with two duplicate), and the plates were incubated overnight at 4°C. Plates were then washed three times with washing buffer, incubated for 4 h at 37°C, with 100 µL per well of alkaline phosphatase-conjugated IgG diluted in sample buffer, washed again, and incubated lastly for 1 h at 37°C, with p-nitrophenylphosphate (1 mg/mL), in 10% diethanolamine, pH 9.8.

### RNA extraction

RNA was purified from infection-free and infected leaves according to former protocol [37]. 2 mL of extraction buffer (21.7 g K<sub>2</sub>HPO<sub>4</sub>·3H<sub>2</sub>O, 1.4 g KH<sub>2</sub>PO<sub>4</sub>, pH 7.4, 100 g sucrose, 1.5 g BSA, 20 g PVP, and 5.3 g ascorbic acid) was transferred into a mortar containing 0.2 g of leave samples. Next, two successive centrifugations were performed, 10 min (1100 g) and 20 min (16800 g) at 4°C, respectively. Then, the resultant precipitate was mixed with 0.2 mL TE buffer (10 mM EDTA, 50 mM Tris-HCl, pH 8, 0.1% mercaptoethanol) and 10% Sodium Dodecyl Sulphate (SDS). Afterwards, 80 µL of 5 M acetate potassium was added and the solution incubated for 10 min at 60°C and for overnight at 4°C, respectively. Then, the tubes were centrifuged at 4°C for 15 min (16800 g). The aqueous phase was harvested and 30 µL acetate sodium 3 M was added followed by isopropanol. After incubation at -20°C for 2 h, the last centrifugation was performed at 4°C for 20 min (16800 g). The resultant pellet was washed with 70% ethanol, dried under a vacuum, and dissolved in a total volume of 15 µL of double distilled water.

### Design of primers for RT-PCR and RT-LAMP

RT-PCR and RT-LAMP specific primers were designed based on coat protein (CP) gene of GFLV (GFLV-F13, RNA2 encoding capsid protein, GenBank accession X16907.1) by using the oligo7 and Primer Explorer V4 software (specific for LAMP), respectively (Table 1). The sequence of CP gene with all available sequences of GFLV from the database was compared. The conserved segment within the CP gene of GFLV-F13 was selected as the target region for primer design. RT-PCR and IC-RT-PCR assays were carried out using forward (F) and backward (B) primers. At RT-LAMP and IC-RT-LAMP assays, a set of four primers recognizing six distinct regions in the target sequence were used, including F3, B3, FIP and BIP. Two of the oligonucleotides (FIP and BIP) each possess two sequences. Unlike primers that are used by other techniques, such as the PCR oligonucleotides, FIP and BIP are complementary to sequences from both strands of the targeted

Reaction	Primer	Type	Length of Primer	Sequence(5-3)	Length of Product
RT-PCR and IC-RT-PCR	F	Forward	20 nt	ACTTTCAAGATAGTTATTAG	424 bp
	B	Backward	20 nt	CAAAGAATATGGGAGGGCAA	
RT-LAMP and IC-RT-LAMP	F3	Forward outer	18 nt	ACTGGATTGACATGGGTG	Fragment with different sizes
	B3	Backward outer	22 nt	CTAACTCTTTGTTGTTGCCCGT	
	FIB	Forward inner	45 nt	GATAAGCCAATGTGGGACTGATTTTATGCTTATAACCGGATAACT	
	BIP	Backward inner	45 nt	ACGTTTCATGTGAGATAGATTTTATGCAACCTTGAGATTCAAAT	
	LF	Loop forward	22 nt	ATACAGGATCCGCACTAGCAGT	
	LB	Loop backward	22 nt	TGTGTGGTCATGCTATGTGGTT	

Table 1: Oligonucleotide primers used for different assays to detect GFLV.

DNA that have close location to each other (designated as F1 and F2c for FIP and B1 and B2c for BIP, respectively) [38,39]. The other two oligonucleotides (F3 and B3) are like ordinary PCR primers. As a first step, a stem-loop DNA structure, in which the sequences of both DNA ends are derived from the inner primers, is constructed as starting material [40]. Subsequently, one inner primer hybridizes to the loop on the product in the LAMP cycle and initiates strand displacement DNA synthesis, yielding the original stem-loop DNA and a new stem-loop DNA with a stem twice as long [41,42]. The final products are a mixture of stem-loop DNAs with various stem lengths and cauliflower-like structures with multiple loops formed by annealing between alternately inverted repeats of the target sequence in the same strand. The structures of the cycling inter-mediate and final products are schematically illustrated in ladder like DNA form [43,44]. Figure 1 shows the schematic position of RT-PCR and RT-LAMP primers within the CP gene.

### cDNA preparation and RT-PCR assay

Initially, cDNA was synthesized as described previously by Rowhani and Stace-Smith [37]. Extracted RNA (5 µL) of virus infected leaves (positive samples), a free virus sample (negative sample), positive control and negative control were incubated at 75°C for 3 min and chilled on ice for 3 min. The concentration of dNTPs, primers and annealing temperature for the amplification of gene was optimized under standard RT-PCR conditions. After optimization of the reaction conditions, 20 pmol B primer, 50 mM Tris-HCL, pH 8.3, 10 mM dithiothreitol (DTT), 2.5 mM MgCl<sub>2</sub>, 10 mM of each dNTP, 5 U of RNasin Ribonuclease Inhibitor (Fermentas Co, Cat. No EO0381), and 1.25 U of Avian myeloblastosis virus (AMV) reverse transcriptase (Fermentas Co, cat. no. EP0641) were added in a 25 µL volume to tube. Afterwards, mixtures were incubated at 60°C for 1 h. lastly; PCR reaction was performed on a Thermal Cycler (iCycler, BIO RAD, CA, USA) in a 25 µL volume containing 1X PCR buffer (10 mM Tris-HCL, pH 8.3 and 50 mM KCl), 1.5 mM MgCl<sub>2</sub>, 0.2 mM of each dNTP, 20 pmol of each F and B primers, 0.625 U of Taq DNA polymerase (Cinagen Co, Cat. No TA7505C) and 2 µL cDNA. Subsequently, master-mix were amplified at

95°C for 5 min, for 34 cycles followed by 1 min at 95°C, 1 min at 54°C and 1 min at 72°C. A final extension was accomplished for 10 min at 72°C. Finally, Amplified products (5 µL) were electrophoresed on 1.5% agarose gel, stained with ethidium bromide and photographed under UV light using Gel Documentation System (GELDOC 2000, Bio-Rad, USA).

### RT-LAMP assay

RT-LAMP was carried out by using obtained cDNA which was previously described. The amplification reaction was performed in a thermal block between 55°C to 65°C within 20 to 60 min, to ascertain the optimal incubation temperature and time. All of the experiments were repeated three times. After optimization of the reaction conditions, the cDNA (2 µL) was incubated at 95°C for 5 min and chilled on ice and it was served as a template in RT-LAMP reaction. The LAMP mixture containing 20 mM Tris-HCL, pH 8.8, 10 mM KCl, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.1% Triton X-100, 2 mM Betaine (Sigma-Aldrich, Saint Luis, MO, USA), 1 mM MgSO<sub>4</sub>, 10 mM each of dNTP, 0.2 µM each of F3 and B3, 0.8 µM each of FIP and BIP, 0.6 µM each of LF and LB, and 8 U of Bst DNA polymerase (New England Biolabs, Hertfordshire, England, UK). The mixture was incubated at 60°C for 30 min in a water bath. Finally, RT-LAMP products (5 µL) were visualized by naked eye and also analyzed by electrophoresis as described earlier.

### IC-RT-PCR assay

The same as DAS-ELISA method, here, tubes were first coated with GFLV specific IgG diluted in coating buffer and incubated for 3 h in 37°C. Plate, in the following, was washed with washing buffer (see "DAS-ELISA Assay" section). The extractions of positive leave samples (i.e. previously detected by DAS-ELISA assay) and a free virus leave sample (as negative sample) and also positive control and negative control were added to IgG-coated tubes and kept overnight at 4°C. Plate, the next day, was washed using washing buffer, dried and employed for next section. In section 2, 20 pmol B primer, 50 mM Tris-HCL, pH 8.3, 10 mM dithiothreitol (DTT), 2.5 mM MgCl<sub>2</sub>, 10 mM of

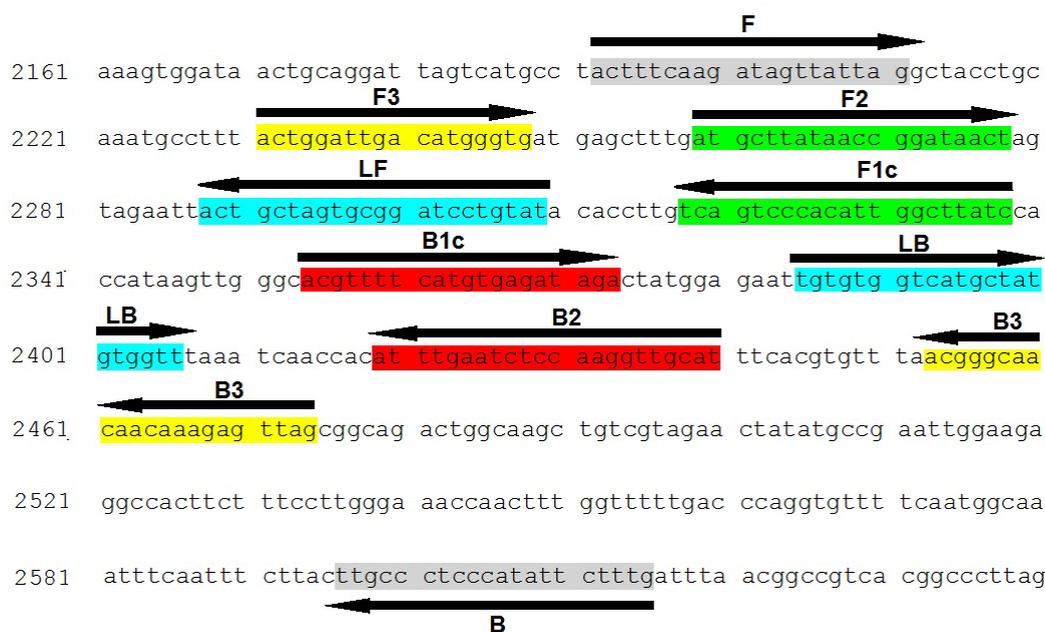


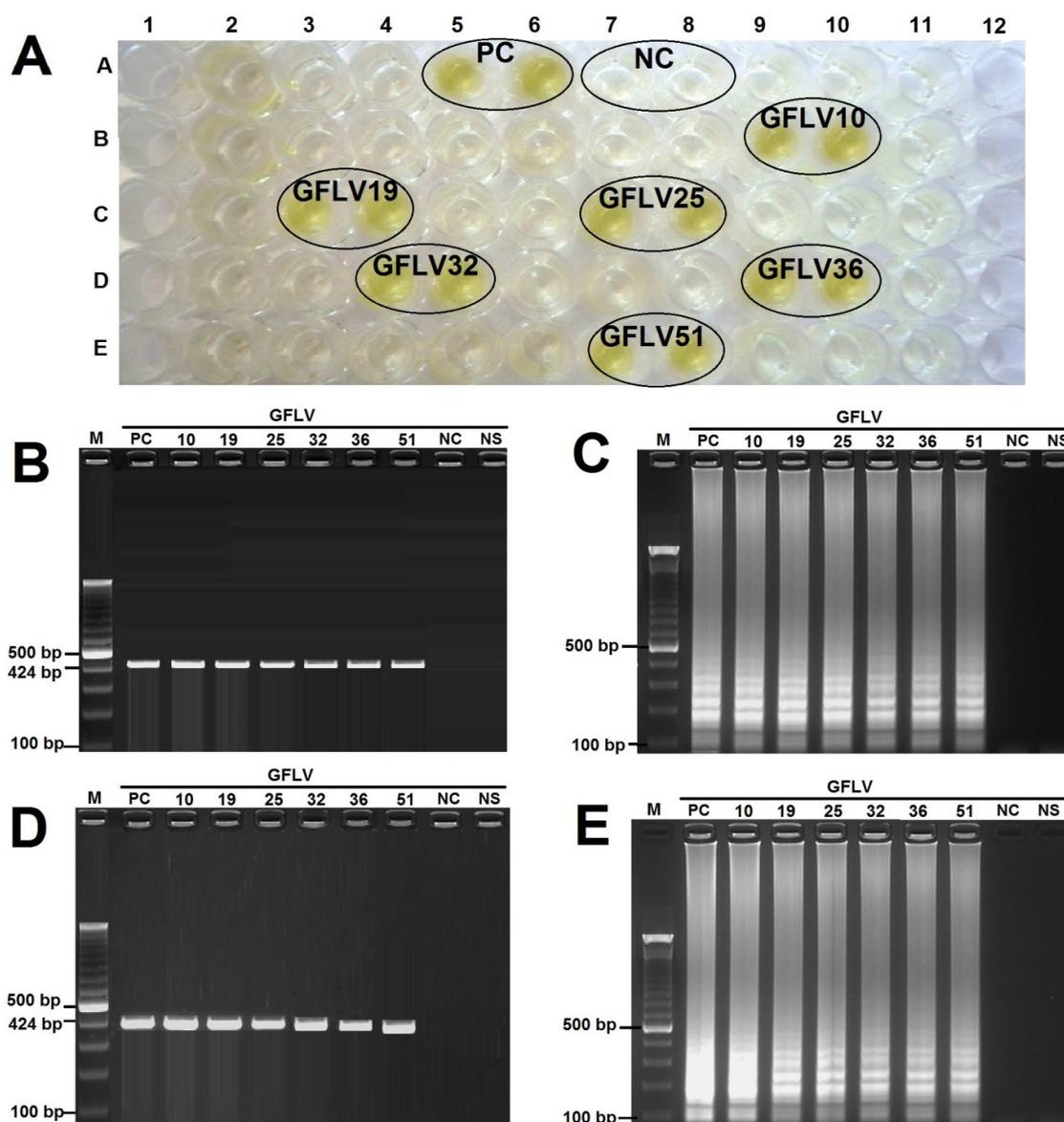
Figure 1: Schematic representation of position primers used in different assays. FIP and BIP primers contain two distinct sequences F1c plus F2 and B1c plus B2, respectively.

each dNTP, 5 U of RNasin Ribonuclease Inhibitor (Fermentas Co, Cat. No EO0381), and 1.25 U of AMV reverse transcriptase (Fermentas Co, cat. no. EP0641) were added to plate and incubated at 60°C for 1 h in water bath. 2 µL of product was harvested and transported to tube, then amplification was performed in a 50 µL volume containing 1X PCR buffer (10 mM Tris-HCl, pH 8.3 and 50 mM KCl), 1.5 mM MgCl<sub>2</sub>, 0.2 mM of each dNTP, 20 pmol of each F and B primers, 0.625 U of Taq DNA polymerase (Cinagen Co, Cat. No TA7505C) on a Thermal Cycler. Master-mix were amplified at 95°C for 5 min, for 34 cycles followed by 1 min at 95°C, 1 min at 54°C and 1 min at 72°C. A final extension was accomplished for 10 min at 72°C. The products were lastly analyzed by gel electrophoresis and visualized under UV light.

### IC-RT-LAMP assay

Even though the principles of the first section of IC-RT-LAMP

assay exactly followed the IC-RT-PCR with no RNA extraction step, in the second part, a different methodology was employed, leading to a significant reduction in the time as well as the cost. Section 1 same as the section 1 of IC-RT-PCR procedure (see above). In section 2, master-mix in total volume of 100 µL containing 10 mM DTT, 5 U of RNase Ribonuclease Inhibitor (Fermentas Co., cat. no. EO0381), 20 mM Tris-HCl, pH 8.8, 10 mM KCl, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.1% Triton X-100, 2 mM Betaine (Sigma-Aldrich, Oakville, ON, Canada), 10 mM each dNTP, 0.2 µM each of F3 and B3, 0.8 µM each of primer FIP and BIP, 1.25 U of AMV reverse transcriptase (Fermentas Co., cat. no.EP0641) and 8 U of Bst DNA polymerase (New England Biolabs Inc.) were added to plate and incubated at 60°C for 90 min in water bath. An agarose gel electrophoresis system under UV illumination and colorimetric assay could be also employed to visualize positive reactions as described above.



**Figure 2:** Result of analysis of samples to detect GFLV. (A) Verification of DAS-ELISA assay results as a green colour-based method. (B) Electrophoresis of the RT-PCR products. (C) Electrophoresis of the IC-RT-PCR products. (D) Electrophoresis of the RT-LAMP products. (E) Electrophoresis of the IC-RT-LAMP products. Line M, DNA size marker (100 bp); line and cells PC, positive control; line and cells NC, negative control; line and cells NS, negative sample.

### Visual detection and confirmation of RT-LAMP products

Here, the validations of positive RT-LAMP and IC-RT-LAMP reactions were justified by means of five staining approaches. To visually detect the LAMP products, two dyes were added to the RT-LAMP and IC-RT-LAMP reactions as described [31,42,44,45]. Prior to amplification, 1  $\mu$ L of GeneFinder™ (diluted to 1:10 with 6X loading buffer) (Biov. Bio. Xiamen, China) and 1  $\mu$ L of phenol red (diluted to 1:10 with 6X loading buffer) was separately added to the master mix. The tubes were easily monitored for colour change by simply looking at them in daylight.

### Sensitivity assay

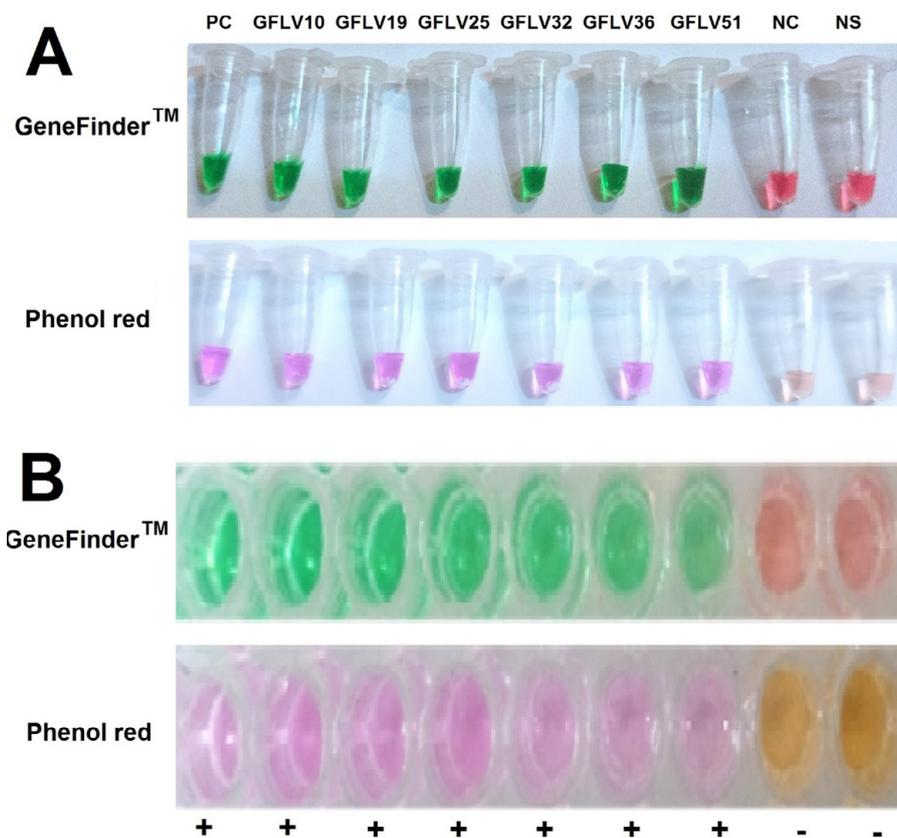
LAMP is comparable to PCR in terms of sensitivity, but is less affected by presence of non-targeted DNA and inhibitory molecules. Some researchers even report specific amplification with LAMP without prior extraction procedure, by directly adding reaction mixture to swab specimens or sera [23,26,33,45]. To evaluate sensitivity of the DAS-ELISA, RT-PCR, IC-RT-PCR, RT-LAMP and IC-RT-LAMP, an eight dilution series of a positive sample was prepared from  $10^1$  to  $10^8$  CFU/mL. The DAS-ELISA results carried out both visual detection and a DYNEX MRX microplate reader. Similarly, the detection limit of the other reactions was approved by electrophoresis on 1.5% agarose gel and visualization methods.

### Results

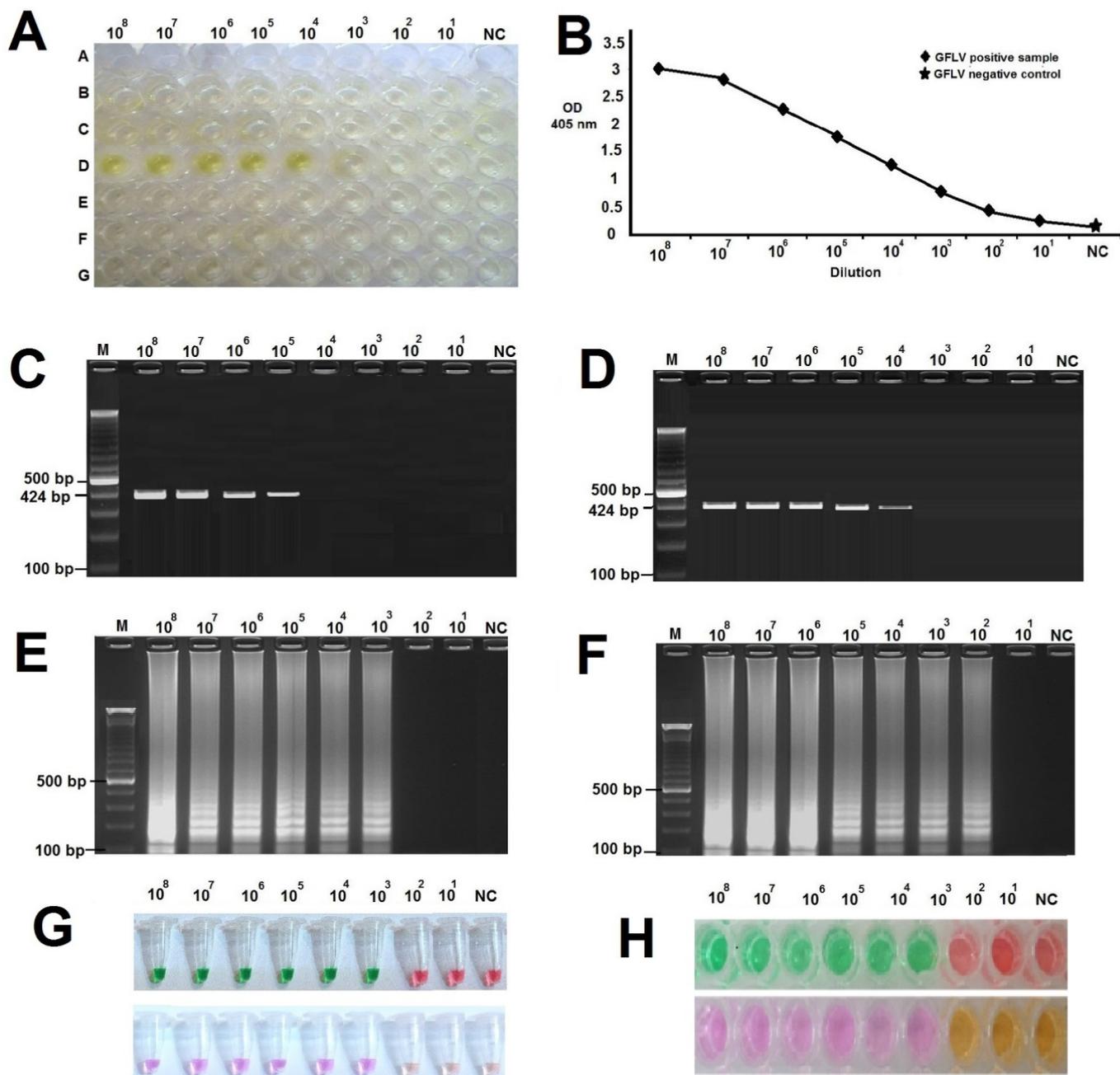
On the whole, 6 out of 100 leave samples suspicious of having infection with GFLV (6%) showed positive responses against DAS-

ELISA assay. Interestingly, due to observing a clear colour change in the wells containing positive reactions (green colour as an indicator), no attempt was accordingly made to employ an ELISA reader (Figure 2A). All six samples, subsequent to nomination as GFLV10, GFLV19, GFLV25, GFLV32, GFLV36 and GFLV51 were utilized lastly for further analyses.

As regards RT-PCR, following provide RNA template and cDNA (see “Materials and Methods” section for details), the amplification occurred via both backward and forward primers to generate ultimate products. The method, overall, could successfully identify six aforementioned positive samples. As expected, a fragment with the size band of 424 bp was detected when the RT-PCR products were run on 1.5% agarose gel and stained with ethidium bromide (Figure 2B). The same as RT-PCR, RT-LAMP protocol could successfully identify positive samples, interestingly with use of cDNA in a water bath. RT-LAMP amplicons were finally electrophoresed on a 1.5% agarose gel (as an optional system), and a large number of fragments (a ladder-like pattern) were eventually visualized. The presence of a smear or a pattern of multiple bands with different molecular weights indicated a positive result (Figure 2C). The same as RT-PCR and RT-LAMP, our new IC-RT-PCR and IC-RT-LAMP protocols could successfully identify positive samples, interestingly with no use of RNA extraction (Figure 2D and 2E). RT-LAMP and IC-RT-LAMP amplicons were able to be detected with the naked eye by adding different visual dyes followed by color changing in the solutions. In this regard, all used visual components could successfully make a clear distinction between positive infected samples and negative ones (Figure 3).



**Figure 3:** Details of two different visualization methods to analyze RT-LAMP and IC-RT-LAMP products. (A) RT-LAMP reaction. (B) IC-RT-LAMP reaction. Tube and cell PC, positive control; tube and cell NC, negative control; tube and cell NS, negative sample.



**Figure 4:** Comparative analysis of the sensitivity of DAS-ELISA, RT-PCR, IC-RT-PCR, RT-LAMP and IC-RT-LAMP using an eight dilution series of positive sample as template. (A) Verification of DAS-ELISA assay results as a green colour-based method. (B) DAS-ELISA assay results in a DYNEX MRX microplate reader. (C) Electrophoresis of the RT-PCR products. (D) Electrophoresis of the IC-RT-PCR products. (E) Electrophoresis of the RT-LAMP products. (F) Electrophoresis of the IC-RT-LAMP products. (G) Visualization methods to analyze RT-LAMP products. (H) Visualization methods to analyze IC-RT-LAMP products. Line M, DNA size marker (100 bp); line, cell and tube NC, negative control.

Our results, interestingly, indicated that IC-RT-LAMP and RT-LAMP can produce reliable products even under lower DNA concentration (10<sup>2</sup> CFU/mL or more), whilst DAS-ELISA, RT-PCR and IC-RT-PCR require higher level of DNA (10<sup>4</sup> CFU/mL or more) (Figure 4). The results showed that IC-RT-LAMP higher sensitivity for detection of GFLV in comparison with RT-LAMP, IC-RT-PCR, DAS-ELISA and RT-PCR (10-fold, 100-fold, 100-fold and 1000-fold, respectively) (Table 2).

## Discussion

Genetic analysis is preferable in testing a patient for infectious diseases because it can yield detailed genetic information about the virulence and antibiotic resistance of a particular microbial. However, the results of genetic analysis take more than 1 h to obtain. Nucleic amplification is one of the most important tools for many investigators, including molecular biologists. In particular, in application oriented

	10 <sup>8</sup>	10 <sup>7</sup>	10 <sup>6</sup>	10 <sup>5</sup>	10 <sup>4</sup>	10 <sup>3</sup>	10 <sup>2</sup>	10 <sup>1</sup>	NC
DAS-ELISA with visual detection	+	+	+	+	+	-	-	-	-
DAS-ELISA with elisa-reader	+	+	+	+	+	-	-	-	-
RT-PCR with electrophoresis	+	+	+	+	-	-	-	-	-
IC-RT-PCR with electrophoresis	+	+	+	+	+	-	-	-	-
RT-LAMP with electrophoresis	+	+	+	+	+	+	-	-	-
IC-RT-LAMP with electrophoresis	+	+	+	+	+	+	+	-	-

**Table 2:** Comparison of sensitivity of different assays at an eight dilution series.

Assay	amplification time	Detection method	Safety	Need to UV ray	Need to detect instruments	Cost	User Friendly	Need to RNA extraction
DAS-ELISA	2-3 day	Visual + ELISA reader	Yes	No	Yes	High	Low	No
RT-PCR	3 hours	Gel electrophoresis	No	Yes	Yes	High	High	Yes
IC-RT-PCR	6 hours	Gel electrophoresis	No	Yes	Yes	High	High	No
RT-LAMP	30 minute	Visual+ Gel electrophoresis	Yes	No and Yes	No and Yes	Low	High	Yes
IC-RT-LAMP	90 minute	Visual+ Gel electrophoresis	Yes	No and Yes	No and Yes	Low	High	No

**Table 3:** Comparison of DAS-ELISA, RT-PCR, IC-RT-PCR, RT-LAMP, IC-RT-LAMP and colorimetric IC-RT-LAMP assays.

fields such as clinical medicine, genetic diagnosis is used for monitoring infectious diseases, genetic disorders and genetic traits [38,46,47]. LAMP is a gene amplification method with a variety of characteristics and applications in a wide range of fields. In particular, LAMP is considered to be effective as a gene amplification method for use in gene point-of-care testing (g-POCT) devices, which are used for simple genetic testing whenever and wherever necessary. First, since LAMP can amplify genes isothermally, the amplification reaction can be carried out with a simple heater. There is no need for the special device used for polymerase chain reaction (PCR) to rapidly control the temperature. Next, a large amount of DNA (10-30 µg/25 µL) can be synthesized in a short time (15-60 min) while maintaining high specificity. This characteristic greatly facilitates detection of the LAMP reaction [27,47]. In this study, as a result, several detection methods including DAS-ELISA, RT-PCR, IC-RT-PCR, RT-LAMP and IC-RT-LAMP were assessed to explore positive and negative aspects of each one, followed by introducing the best one regarding CP gene detection in GFLV. Even though all techniques had enough potential to make differentiation and detect infected samples accurately, IC-RT-LAMP proved to be much more useful as some factors including time, safety, simplicity, cost and being user friendly are taken into account (Table 3). IC-RT-LAMP and RT-LAMP overall requires just 30 min to accomplish only amplification (as the least demanding detection method), while regarding IC-RT-PCR, RT-PCR and DAS-ELISA, 6 h, 3 h and 2-3 day should be served, respectively. One may conclude that IC-RT-LAMP faster for detecting of transgenic plants compared with other assays. This, in turn, would simplify the detection procedure and result in saving of significant time needing for separating of the amplified products on the gel and the analyzing of the data which are commonly used in the other RT-PCR-based methods. Safety regarding as a routine approach with enough potential to observe related amplicons. Just the same, such visual methods not only involve some expensive instruments but also during a period of time, exposure to the UV light (because it is harmful to the eyes, even watching for a short period would irritate the eyes and cause symptoms similar to conjunctivitis) as well as ethidium bromide could accompany a number of serious negative effects on researchers who use these methods [38]. More surprisingly, in IC-RT-LAMP and RT-LAMP amplified products can be easily visualized by means of different in-tube colour indicators whit no essential requirement of additional staining systems; thus, toxic staining materials would be significantly avoided.

Simplicity, cost and user friendly equipped labs with some molecular instruments as well as trained personnel are prerequisites to perform RT-PCR assays, all of which are undoubtedly costly. On the contrary, RT-LAMP can be easily accomplished just in a water bath or temperature block with no need of thermocycler and gel electrophoresis as the same results were recorded by Fukuta et al., [29]. Likewise, exclusive of the primer designing process which is somehow complicated and sensitive, other phases are simply applicable.

On the other hand, the presence of RT-LAMP positive amplicons proved to be confirmed by adding a number of fluorescent or metal dyes to the reaction tubes, allowing observation with the naked eye [31,45]. In the current study, therefore, RT-LAMP amplified products were confirmed by adding all aforementioned visual systems (see "Materials and Methods" section), prior the reaction along with forming diverse color patterns depending upon the chemical characteristics of the applied chemical substances as dye. According to our results, despite the precise detection of positive RT-LAMP and IC-RT-LAMP products using all dyes, some were significantly superior when the time of stability, cost and the safety were taken into consideration. As the last drawback, the corresponding lab(s) should be equipped with such instruments are commonly costly. GeneFinder™ and phenol red dyes were proven to be more powerful since tubes do not have to be opened after amplification consequent to accompany by no cross-contamination, judging positive reactions can be visualized using the naked eye, no need of post-amplification treatment of the amplicons. The presented loop-mediated isothermal amplification method-LAMP is reported to be simplified and efficient technique for detection of viral pathogens. The advantages of LAMP make it a suitable field test tool for diagnosis of emerging and reemerging diseases. As it is relatively cheap and reliable most probably LAMP will be more widely integrated into routine use of molecular techniques.

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