

# Colorimetric Immunocapture Reverse Transcription Loop-Mediated Isothermal Amplification Assay for Rapid Detection of the *Potato virus Y*

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## Abstract

Loop-Mediated Isothermal Amplification (LAMP) assay is a novel technique for amplifying DNA under constant temperature, with high specificity, sensitivity, rapidity and efficiency. To diminish the time required for some diagnostic assays including Reverse Transcription PCR (RT-PCR), Reverse Transcription Loop-Mediated Isothermal Amplification (RT-LAMP) and also DAS-ELISA into a minimum level, an innovative colorimetric IC-RT-LAMP and IC-RT-PCR protocol on the basis of Potato Virus Y (PVY) genome were used and optimized. Firstly, DAS-ELISA assay was performed to detect of the virus in a collection containing 95 suspicious samples. Lastly, five samples were detected as the positive samples. Then, the positive samples were verified by molecular methods. In this regard, all four RT-LAMP primers (i.e. F3, B3, FIP and BIP) together with RT-PCR primers (F and B) were selected on the basis of coat protein gene (CP) of PVY genome. Even though DAS-ELISA, RT-PCR and RT-LAMP assays could successfully detect positive infected plant samples, considering the time, safety, sensitivity, cost and simplicity, the last one was overall superior. Furthermore, the results demonstrated that the RT-LAMP assay was 100 times sensitive and 3 time faster compared to RT-PCR. LAMP assay was accomplished in the water bath either frees from any thermal cycler machine or sophisticated laboratories facility. Meanwhile, among six different visual dyes to accurately detect IC-RT-LAMP products, Hydroxynaphthol blue, GeneFinder™ and SYBR Green I could produce long stable colour change and brightness in a close tube-based approach to prevent cross-contamination risk, concluded eventually as the best ones. We accordingly propose this colorimetric assay as a highly reliable alternative viral recognition system regarding PVY recognition and probably other viral-based diseases.

**Keywords:** Colorimetric assay; DAS-ELISA assay; IC-RT-LAMP assay; IC-RT-PCR assay; *Potato virus Y*

## Introduction

Potato (*Solanum tuberosum L.*), as the world's most important non-grain food crop [1]. Potato plants, like other crops, are exposed frequently to plenty of infectious viruses, economically the most important of which are Potato leafroll virus (PLRV, genus *Polerovirus*), *Potato virus Y* (PVY, genus *Potyvirus*), *Potato virus X* (PVX, genus *Potexvirus*) and *Potato virus S* (PVS, genus *Carlavirus*) [2]. PVY, one species of the genus *Potyvirus* is a phloem-limited virus that infects potato leading to a significant reduction in potato annual yield as well as tuber quality worldwide [3,4]. As a result, fast, cost effective, reliable and sensitive indexing techniques are requisite tools to determine the virus. Some alternative approaches were gradually developed including DAS-ELISA [5,6], bioassay [7], and Polymerase Chain Reaction (PCR) [8], all of which were unfortunately time-consuming and require expensive or carcinogenic materials to visualize DNA amplification [6,9]. Meanwhile, extraction of RNA is another exhausting task, accomplished commonly under various protocols, all of which are typically accompanied by some drawbacks [10-12]. Among various isothermal amplification systems developed over the recent years, the most frequently applied approach seems to be loop-mediated isothermal amplification (LAMP), implemented first by Notomi et al. [13]. 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 [14-17]. Although a large number of studies have been accomplished using LAMP or RT-LAMP including *Potato virus Y* [4], *Potato Leafroll virus* [18], *Japanese yam mosaic potyvirus* [19], *Tomato Yellow Leaf Curl virus* [20], *Rabies virus* [21], *Macrobrachium rosenbergii nodavirus* [22] and *Cymbidium*

*mosaic virus* [23], all of which basically require more precise extraction protocol(s) about generating higher concentrations of DNA or RNA followed by the least amounts of probable contaminations which act as inhibitors in an amplification process. Notably, despite a few number of studies about immunocapture reverse transcription loop-mediated isothermal amplification (IC-RT-LAMP) [17,24] because the technique has not been yet introduced for detection of PVY, an attempt was accordingly made to optimize a new protocol of it to save time and remove RNA extraction. As the second purpose, since the existence of one-step IC-RT-LAMP positive amplicons has been proved to be confirmed by adding a number of fluorescent dsDNA intercalating dye including ethidium bromide [25], SYBR Green [26] and propidium iodide [27] after the reaction is completed or metal indicators such as calcein [28], Gene-Finder™ [29,30], hydroxyl naphthol blue [31-33] and magnesium pyrophosphate [17,18] prior to the reaction, allowing observation with the naked eye, here, six 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 PVY.

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## Materials and Methods

### Plant samples

A total of 95 fresh potato leaf samples infected dubiously with PVY on the basis of plantsymptoms containing yellowing and erect leaves as well as plant stunting were collected from ten separate potato fields in Provinces Zanjan, Hamadan, Kordestan and East Azarbaijan of Iran.

### Double antibody sandwich enzyme linked immune sorbent assay (DAS-ELISA)

DAS-ELISA was carried out as described by Clark and Adams [5] with some minor modifications, using a commercially available PVY IgG and the alkaline phosphatase-conjugated PVY IgG. Polystyrene microtiter plates were coated for 3 h at 34°C, with 200 µl per well of IgG coating, in 50 mM carbonate buffer, pH 9.6. The plates were then incubated for 1 h at 34°C with 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 infection-free (control) and PVY-infected potato leaf samples were ground in ten volumes (w/v) of PBS buffer pH 7.2, containing 0.2% polyvinyl pyrrolidone and 2% of egg albumin (Sigma A5253). The infected preparations were serially diluted (fivefold dilution) at the same buffer. Aliquots of 195 µl of prepared samples were added to each well, 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 190 µl per well of alkaline phosphatase-conjugated PVY IgG diluted in sample buffer, washed again, and incubated lastly for 90 min, with p-nitrophenylphosphate (1 mg/ml), in 10% diethanolamine, pH 9.8. Data were expressed and recorded using Multiskan at A<sub>405</sub> nm.

### RT-PCR assay

RNA was purified from PVY-infected potato leaves according to former protocol [34]. Extracted RNA (5 µl) was incubated at 75°C for 3 min and chilled on ice for 3 min. The RT-PCR was developed using a PVY-specific primer designed on the basis of virus coat protein (CP) gene (Table 1) [4]. First, 20 pmol backward (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 (dATP, dTTP, dCTP and dGTP), 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 to RNA. Afterwards, mixtures were incubated at 60°C for 60 min. lastly; the obtained cDNA was incubated (2 µl) was incubated at 95°C for 5 min and chilled on ice and it was served as a template in PCR reaction. PCR reaction was carried out on a Thermal Cycler (iCycler, BIO RAD, CA, USA) in a 25 µl volume containing 1× PCR buffer (10 mM Tris-HCL, pH 8.3, 50 mM KCl), 1.5 mM MgCl<sub>2</sub>, 0.2 mM of each dNTP, 20 pmol of each forward and backward primers, 0.625 U of Taq DNA polymerase (Cinagen Co, Cat. No TA7505C) and 2 µl cDNA. Subsequently, mastermix were amplified at 94°C for 3 min; for 35 cycles followed by for 1 min at 94°C, 1 min at 54°C, 1 min at 72°C. A final extension was accomplished for 10 min at

72°C. The products were lastly analyzed by gel electrophoresis in which 5 µl of the products (480 bp) was loaded on a 1.5% agarose gel and visualized by staining with ethidium bromide.

### RT-LAMP assay

In order to perform RT-LAMP, on the basis of CP gene of the PVY genome, four specific primers including outer primers (F3 and B3) and inner primers (FIP and BIP) were used (Table 1) [4]. RT-LAMP reaction was performed in a total volume of 50 µl: 5 µl of RNA, 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), 1 mM MgSO<sub>4</sub>, 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.). Tubes were then incubated at 60°C for 90 min in water bath. It is noticeable that the first 45 min is allocated only to synthesize cDNA, while in the second round, LAMP amplicons are amplified. An agarose gel electrophoresis system (optional; 1.5%) under UV illumination could be also employed to visualize positive reactions: 5 µl of each product is loaded on a 1.5% agarose gel.

### IC-RT-PCR assay

The IC-RT-PCR was developed using a PVY-specific primer designed on the basis of virus coat protein (CP) gene (Table 1) [4]. The protocol, to generate IC-RT-PCR products, was divided into two successive sections as below:

**Section 1:** The same as DAS-ELISA method, here, PCR tubes were first coated with PVY specific IgG diluted in coating buffer and incubated for 4 h in 37°C. Tubes, in the following, were washed with washing buffer (see “DAS-ELISA Assay” section). The extractions of positive potato samples (i.e. previously detected by DAS-ELISA assay as positive control) and a free virus plant sample (as negative control) were added to IgG-coated tubes and kept overnight at 4°C. Tubes, the next day, these were washed using washing buffer, dried and employed as RNA template in IC-RT-PCR reactions.

**Section 2:** In this part, the reaction was carried out in a Bio-Rad thermocycler. The amplification was performed in a 40 µl volume containing 20 pmol of each backward and forward primers (F and B), 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), 1.25 U of Avian myeloblastosis virus (AMV) reverse transcriptase (Fermentas Co, cat. no. EP0641), 1× PCR buffer (10 mM Tris-HCL, pH 8.3, 50 mM KCl) and 0.625 U of Taq DNA polymerase (Cinagen Co, cat. no.TA7505C). First, strand of cDNA was amplified at 60°C for 60 min from RNA extracted by immunocapture method using the backward primer. One set of primer (Table 1), that is backward and forward primers, was used for the PCR amplification of the cDNA. Amplification was performed with the following PCR profile: 94°C for 3 min; 35 cycles of 1 min at 94°C, 1 min at 54°C, 1 min at 72°C and 10

Primer	Type	Position on gene	Length	Sequence (5'-3')
F	Forward	8721–8740	20 mer	ACGTCCAAATGAGAATGCC
B	Backward	9181–9200	20 mer	TGGTGTTCGTGATGTGACCT
F3	Forward outer	8870–8890	21 mer	ATACGACATAGGAGAACTGA
B3	Backward outer	9059–9078	20 mer	ACGCTTCTGCAACATCTGAG
FIP	Forward inner	8931–8951 and 8900–8920	42 mer	GTTTGGCGAGTTCATTTTC-TGTGATGAATGGGCTTATGGT
BIP	Backward inner	9004–9024 and 9036–9056	42 mer	TGAAACCAATCGTTGAGAATG-ATGTGCCATGATTTGCCTAAG

**Table 1:** Oligonucleotide primers used for one-step colorimetric IC-RT-LAMP and one-step IC-RT-PCR for detection CP gene of PVY.

min at 72°C for final extension. The products were lastly analyzed by gel electrophoresis and visualized by staining with ethidium bromide.

### IC-RT-LAMP assay

In order to perform IC-RT-LAMP, on the basis of CP gene of the PVY genome, four specific primers including outer primers (F3 and B3) and inner primers (FIP and BIP) were used (Table 1) [4]. 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. The details are as follows:

**Section 1:** Just the same as the section 1 of IC-RT-PCR procedure (see above).

**Section 2:** Each reaction was performed in a total volume of 50  $\mu$ l: 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  $(\text{NH}_4)_2\text{SO}_4$ , 0.1% Triton X-100, 2 mM Betaine (Sigma-Aldrich, Oakville, ON, Canada), 1 mM  $\text{MgSO}_4$ , 10 mM each dNTP, 0.2  $\mu$ M each of F3 and B3, 0.8  $\mu$ M 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.). Tubes were then incubated at 60°C for 90 min in water bath. It is noticeable that the first 45 min is allocated only to synthesize cDNA, while in the second round, LAMP amplicons are amplified. An agarose gel electrophoresis system (optional; 1.5%) under UV illumination could be also employed to visualize positive reactions.

### Colorimetric IC-RT-LAMP assay

The details of colorimetric assay are described below:

**Magnesium pyrophosphate:** Like other metal indicators, magnesium pyrophosphate must be added before reaction. At the end of the amplification process, positive reactions were accompanied by a visible darker phase in the tubes in consequence of the formation of magnesium pyrophosphate [13,35], which can be easily visualized with the naked eye. It is noticeable that the turbidity of the positive samples is stable but just for a short time, which should be consequently judged soon after taking out of the samples either from the water bath or the thermal cyclor.

**SYBR® Premix Ex Taq™ II:** To conquer time-dependent instability of magnesium pyrophosphate-based detection method, an alternative visual system using SYBR® Premix Ex Taq™ II was employed [20,25,36]. Hence, 2  $\mu$ l SYBR® Premix Ex Taq™ II (Perfect Real Time, Takara Bio Co., Ltd., RR081A) was added into each completely finished IC-RT-LAMP reaction containing 25  $\mu$ l of products; all positive reactions were effectively identified. Under UV illumination (302 nm), a green color pattern is an identical characteristic of all positive reactions as the same was monitored in this study.

**Hydroxynaphthol blue (HNB) dye:** In this protocol, 1  $\mu$ l of the hydroxynaphthol blue dye (3 mM, Lemongreen, Shanghai, China) is mixed prior to amplification; all positive reactions can be easily identified using the naked eye, interestingly with no probable cross contaminations which usually arise from opened tubes after amplification [20,31-33]. In this context, a sky blue color pattern implies the existence of the reference virus, whereas a violet color change is observed when the control(s) are taken into consideration.

**GeneFinder™:** An obvious Green fluorescence pattern was observed to confirm positive IC-RT-LAMP products through visual observation with the naked eye when 1  $\mu$ l of GeneFinder™, diluted

to 1:10 with 6 $\times$  loading buffer (TaKara, Dalian, China), was added to each reaction as described previously [29,30]. Remarkably, concerning negative reaction, the original orange color could be observed.

**SYBR Green I:** 1  $\mu$ l of SYBR Green I (Invitrogen, Sydney, Australia) diluted to 1:10 with 6 $\times$  loading buffer was separately added to each reaction as described previously [36]. Remarkably, concerning negative reaction, the original orange color could be observed.

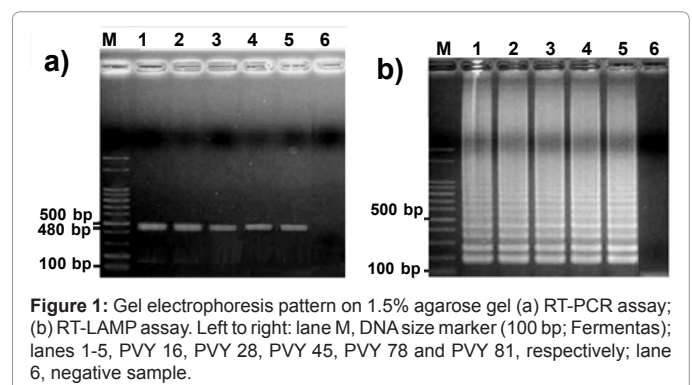
**Ethidium bromide:** About 0.5  $\mu$ g ethidium bromide/ml (Sigma) was added to each tube [18,25,35]. Under a UV transilluminator, positive products will be consequently marked if a detectable yellow color pattern is observed. As a result, the intensity of the fluorescent emissions moves up in positive tubes, while the reverse is true regarding negative tubes with no amplified fragments.

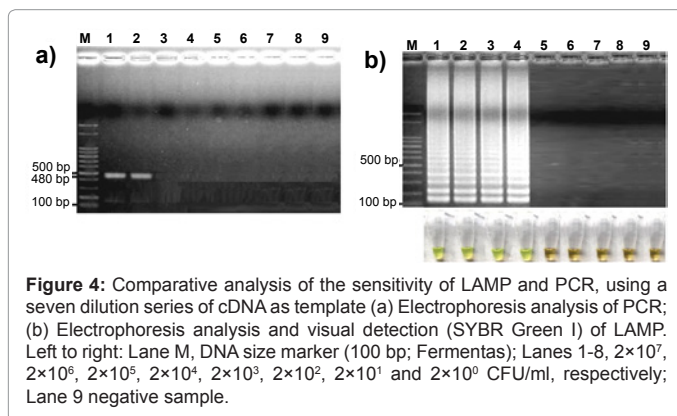
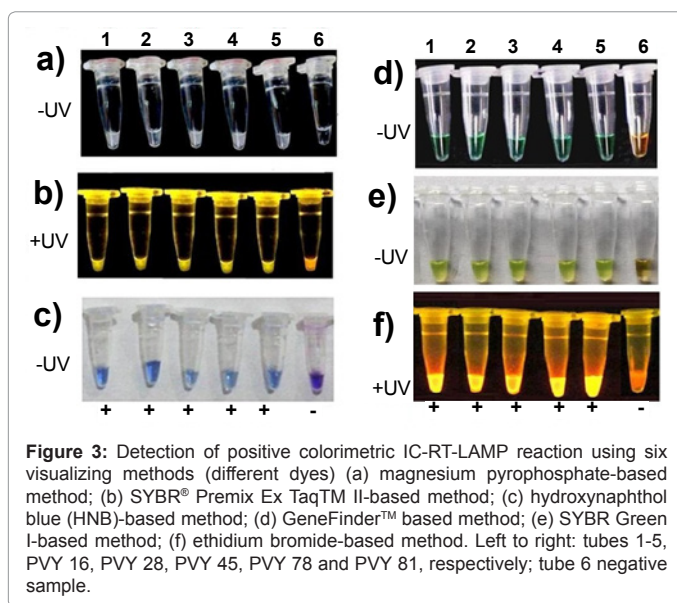
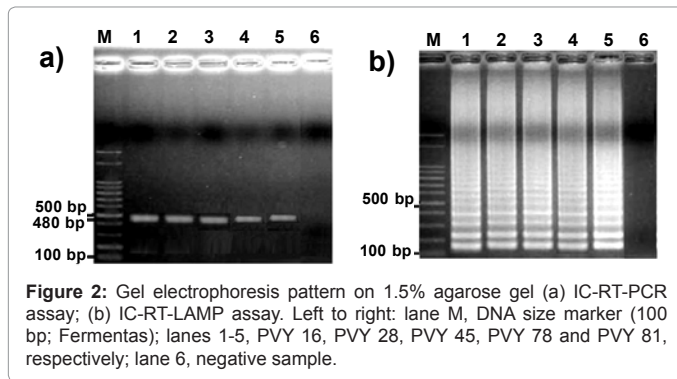
### Sensitivity and specificity of the LAMP assay

Both quality and quantity of DNA template may have a dramatic influence on the results of each PCR method. To determine limit of the LAMP assay was compared with that of PCR, a seven dilution series ( $2 \times 10^0$  to  $2 \times 10^7$  CFU/ml) of cDNA were prepared in water; 2  $\mu$ l of each dilution was used for LAMP and PCR reactions. To determine specificity of the primers, LAMP and PCR reactions were carried out to PLRV (*Potato Leafroll Virus*) and CMV (*Cucurbitta Mosaic Virus*) cDNAs. Furthermore, SYBR Green I dye was added to LAMP products and positive reactions were directly detected by visual inspection. Similarly, the detection limit of the LAMP and PCR was approved by electrophoresis on 1.5% agarose gel.

### Results

On the whole, 5 out of 95 leaf samples suspicious of having infection with PVY (5.3%) showed positive responses against DAS-ELISA assay. All five samples, subsequent to nomination as PVY 16, PVY 28, PVY 45, PVY 78 and PVY 81, 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 five aforementioned positive samples. As expected, a fragment with the size band of 480 bp was detected when the RT-PCR products were run on 1.5% agarose gel and stained with ethidium bromide (Figure 1a). The same as RT-PCR, RT-LAMP protocol could successfully identify five positive samples, interestingly with no use of cDNA but with use of RNA in a water bath directly. 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 (Figure 1b). 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 and cDNA synthesis (Figures 2a and 2b). 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 (Figures 3a-f). Our results, interestingly, indicated that LAMP can produce reliable products even under lower cDNA concentrations ( $2 \times 10^4$  CFU/ml or more), whilst PCR, requires higher level of cDNA (at

least  $2 \times 10^6$  CFU/ml) (Figures 4a and 4b). Surprisingly, no false positive amplification was detected in specificity test and a 100% predictive value was obtained for PCR and LAMP assays (Figures 5a and 5b).

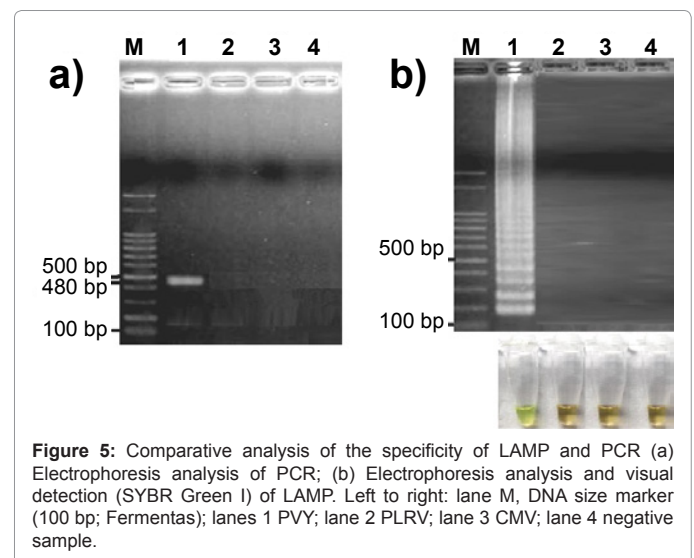
## Discussion

In this study, as a result, three detection methods including DAS-ELISA, IC-RT-PCR and IC-RT-LAMP were assessed to explore positive and negative aspects of each one, followed by introducing the best one regarding PVY detection (Table 2). Even though all three techniques had enough potential to make differentiation and detect infected plant samples accurately, colorimetric 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:

Time DAS-ELISA as compared with IC-RT-LAMP and IC-RT-PCR commonly needs a long time to identify positive infected samples (two or few additional days). In reality, with the exception of section one which takes equal time (see “Material and Methods” section), IC-RT-LAMP overall requires just 90 min to accomplish (as the least demanding detection method), while regarding IC-RT-PCR and DAS-ELISA, 3 h and at least 1 day should be served, respectively. 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 PCR-based methods.

Safety Regarding a number of detection methods, application of gel electrophoresis systems has emerged 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 ray (because it is harmful to the eyes, even watching for a short period would irritate 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 [17,25,30,33,36]. More surprisingly, in IC-RT-LAMP and other LAMP variants, amplified products can be easily visualized by means of different in-tube colour indicators with 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



Assay	Total time	Detection method	Safety	Need to UV ray	Need to detect instruments	Cost	User Friendly	Accuracy	Need to RNA extraction	Need to cDNA synthesis separately
DAS-ELISA	1-2 days	Visual + ELISA reader	Yes	No	Yes	High	Low	High	No	No
RT-PCR	5 hours	Gel								
Electrophoresis	No	Yes	Yes	High	Low	High	Yes	Yes		
RT-LAMP	120 minute	Visual+ Gel								
Electrophoresis	Yes	No and Yes	No and Yes	Low	High	High	Yes	No		
IC-RT-PCR	3 hours	Gel								
electrophoresis	No	Yes	Yes	High	High	High	No	Yes		
IC-RT-LAMP	90 minute	Visual+ Gel								
electrophoresis	Yes	No and Yes	No and Yes	Low	High	High	No	No		
Colorimetric IC-RT-LAMP	90 minute	Visual	Yes	No	No	Low	Very high	High	No	No

**Table 2:** Comparison of DAS-ELISA, RT-PCR, RT-LAMP, IC-RT-PCR and IC-RT-LAMP assays.

to perform DAS-ELISA and IC-RT-PCR assays, all of which are undoubtedly costly. On the contrary, colorimetric IC-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 Almasi et al. [17], Almasi et al. [18], Almasi et al. [25], Ahmadi et al. [33]. Likewise, exclusive of the primer designing process which is somehow complicated and sensitive, other phases are simply applicable. It is noticeable that in all RT-based methods, RNA extraction is an unavoidable step, needing different protocol(s) followed by optimization (mostly is a time-consuming process) to acquire purified RNA stock [12,37], whereas IC-RT-PCR and IC-RT-LAMP can be easily performed with no attempt for RNA isolation. The IC-RT-LAMP method would lastly simplify the detection procedure and would result in saving of significant time which is needed for separation of the amplified products on the gel. On the other hand, the presence of LAMP and 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 [18,30,33]. In the current study, therefore, IC-RT-LAMP- amplified products were confirmed by adding all aforementioned visual systems (see “Materials and Methods” section), either prior to or after the reaction along with forming diverse color patterns depending upon the chemical characteristics of the applied chemical substances as dye.

## Conclusion

In summary, a novel colorimetric IC-RT-LAMP assay for rapid and easy detection of PVY was developed here, its potential compared with DAS-ELISA RT-PCR, RT-LAMP and IC-RT-PCR assays. The method, on the whole, had the following advantages over the two mentioned procedures and also the methods including RT-LAMP and RT-PCR: (1) fascinatingly, no need of RNA extraction (2), no requirement of expensive and sophisticated tools for amplification and detection; (3) no post-amplification treatment of the amplicons; and (4) a flexible and easy detection approach, that is visually detected by naked eyes using diverse visual dyes. On the other hand, among different visual systems HNB, SYBR Green I and GeneFinder™ were proved to be more powerful. As the last point of view, the current diagnostic approach can be suitable not only for laboratory research but also regarding field diagnoses of many infectious diseases worldwide.

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