Benchmarks

Shining a light on LAMP assays— A comparison of LAMP visualization methods including the novel use of berberine

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The need for simple and effective assays for detecting nucleic acids by isothermal amplification reactions has led to a great variety of end point and real-time monitoring methods. Here we tested direct and indirect methods to visualize the amplification of potato spindle tuber viroid (PSTVd) by loop-mediated isothermal amplification (LAMP) and compared features important for one-pot in-field applications. We compared the performance of magnesium pyrophosphate, hydroxynaphthol blue (HNB), calcein, SYBR Green I, EvaGreen, and berberine. All assays could be used to distinguish between positive and negative samples in visible or UV light. Precipitation of magnesium-pyrophosphate resulted in a turbid reaction solution. The use of HNB resulted in a color change from violet to blue, whereas calcein induced a change from orange to yellow-green. We also investigated berberine as a nucleic acid-specific dye that emits a fluorescence signal under UV light after a positive LAMP reaction. It has a comparable sensitivity to SYBR Green I and EvaGreen. Based on our results, an optimal detection method can be chosen easily for isothermal real-time or end point screening applications.

Isothermal nucleic acid amplification methods such as loop-mediated isothermal amplification (LAMP) have become increasingly popular for the assay of a wide range of samples. Depending on the application, different detection formats for visualizing LAMP products may be chosen to meet specific require-

ments. We have performed a comparison of the most popular detection methods with respect to several parameters that are relevant to field assays but are also applicable to online monitoring in routine laboratory diagnostics. Our investigations were complemented by the use of berberine as a new detection alternative.

This report gives an overview of different visualization methods for isothermal amplification reactions, as exemplified by LAMP.

In contrast to isothermal amplification techniques, most of the mature PCR-based techniques such as PCR, reverse-transcription PCR (RT-PCR), quantitative PCR (qPCR), or multiplex PCR require precise instrumentation platforms and complex analysis procedures; they are also time-consuming and expensive (1). Routine applications for the detection of pathogenic microorganisms in the field are therefore hindered. The demand for rapid, simple, and cost-effective routine diagnostic assays is increasing, and novel isothermal amplification techniques, such as nucleic acid sequence-based amplification (NASBA), transcription-mediated amplification (TMA), strand displacement amplification (SDA), rolling circle amplification (RCA), helicase-dependent amplification (HDA), recombinase polymerase amplification (RPA), and LAMP, have been developed in the past few decades (2,3). Many of these rely on the superior strand-displacing activity of the thermostable *Bst* DNA Polymerase. LAMP was developed in 2000 and is characterized by high specificity, sensitivity, speed (<60 min), low reaction temperature (60°–65°C), and high robustness (4). The amplification efficiency is very high, allowing the development of simple assays without requiring dedicated devices (5,6). Typical applications for LAMP include the detection of pathogenic bacteria, viruses, fungi, and parasites (7).

Isothermally amplified DNA can be visualized by gel electrophoresis or using other post-amplification detection steps that require opening of the reaction tubes and therefore significantly increase the risk of carry-over contamination (1,3). To reduce this risk and to increase applicability for simple field assays, one-pot reactions are strongly recommended. Several detection methods suitable for such closed-tube amplification reactions are known today. These allow the detection of amplified DNA for both end point and real-time analysis.

METHOD SUMMARY

Here we present simple methods to visualize products of one-pot isothermal amplification reactions, exemplified by loop-mediated isothermal amplification (LAMP) for the detection of potato spindle tuber viroid (PSTVd), using turbidity, hydroxynaphthol blue (HNB), calcein, SYBR Green I, EvaGreen, and berberine.

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Indirect detection of reaction byproducts is based on the formation of pyrophosphate released by incorporation of deoxynucleotide triphosphates (dNTPs) into the DNA strand during polymerization (Figure 1A). The amount of pyrophosphate generated in LAMP reactions is very high and it forms a stable complex with bivalent metal ions such as magnesium, calcium, or manganese (8). Pyrophosphate precipitates with magnesium at concentrations above 0.5 mM and can be measured quantitatively in terms of turbidity (Figure 1B, upper diagram) or observed by the naked eye as a pellet after centrifugation (9). Another method, based on the decrease in the concentration of magnesium caused by its binding to pyrophosphate, uses hydroxynaphthol blue (HNB) (Figure 1B, middle diagram). HNB changes color from violet to sky blue upon depletion of free magnesium ions (10). Alternatively, calcein can be used in combination with free manganese ions that have a quenching effect on the chelating dye. As manganese binds strongly to newly formed pyrophosphate, calcein is released to complex free magnesium, resulting in bright green fluorescence after excitation with either visible or UV light (Figure 1B, lower diagram). In

visible light, a color change from orange to yellow-green can also be observed by the naked eye after successful amplification (11).

An alternative to these indirect detection techniques is the direct detection of isothermally amplified DNA by intercalating dyes. SYBR Green I and EvaGreen are widely used and well characterized for PCR (12). Both dyes emit a weak fluorescence signal in the presence of single-stranded DNA (ssDNA) but emit strongly upon binding to double-stranded DNA (dsDNA) and are therefore ideal candidates for the detection of DNA in LAMP reactions. Because of its inhibitory effect on DNA polymerization at higher concentrations, SYBR Green I is mainly used in LAMP for end point detection by adding it to the reaction mixture after the amplification reaction (13, 14) or by depositing the dye into the lid of the reaction vessel (15). EvaGreen is more applicable for the monitoring of LAMP reactions in real time because it does not affect DNA polymerization and therefore may be present throughout the entire reaction (16,17).

As a new alternative to the direct detection methods, we explored the applicability of the natural plant alkaloid berberine for the detection of DNA

Figure 1. Overview of indirect and direct detection methods used for monitoring loop-mediated isothermal amplification (LAMP). (A) Simplified chemical equation of a DNA polymerase reaction. Me^{2+} represents metal ions such as Mg^{2+} or Mn^{2+} . (B) Indirect detection via LAMP byproducts. Top: Magnesium pyrophosphate precipitates in the LAMP reaction, resulting in a turbid solution or a white pellet. Middle: The color change of hydroxynaphthol blue (HNB) is attributable to the decrease of magnesium. Below: Calcein is initially quenched by manganese. LAMP-generated pyrophosphate preferentially precipitates manganese, resulting in a calcein–magnesium complex visible as bright green fluorescence. All indirect methods are compatible with visual detection by the naked eye. (C) Direct detection of amplified DNA by DNA-intercalating dyes such as SYBR Green I, EvaGreen and berberine. All direct detection methods are suitable for real-time assays.

Figure 2. Detection sensitivity of end point potato spindle tuber viroid (PSTVd) loop-mediated isothermal amplification (LAMP) assays. Photographs of PSTVd-LAMP assays after incubation at 65°C for 60 min with serially diluted PSTVd DNA. All reactions were covered with mineral oil. Turbidity (A) and hydroxynaphthol blue (HNB) assays (B) in visible light only. Calcein/manganese in visible light (C) and ultraviolet (UV) light at 312 nm (D). Berberine in visible light (E), and UV light at 312 nm (F). Numbers 1 to 6 represent serial dilution of template PSTVd-DNA (Stock: 1.5×10^{10} copies/tube): 1:10⁰ (1), 1:10² (2), 1:10⁴ (3), 1:10⁶ (4), 1:10⁸ (5), 1:10¹⁰ (6). Tubes 7 and 8 are controls without template.

product formation in LAMP reactions. Berberine is not poisonous at typical assay concentrations and can also be used as fluorescent probe. The interaction of berberine with nucleic acids is known to be highly specific and sensitive (18,19). All of the above-mentioned detection methods were compared by using LAMP assays for the detection of PSTVd, which is listed as a quarantine organism in many countries and causes virus-like diseases in economically relevant crop plants (potato and tomato) as well as in some decorative plants (petunias) (20,21).

Dried PSTVd-positive material (PV0064) was purchased from the German strain culture collection (DSMZ, Braunschweig, Germany). RNA extraction was done with the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Oligonucleotides (Thermo Scientific, Schwerte, Germany) were HPLC purified. PCR primers were designed as previously described (22), and LAMP primers were designed according to

Lenarčič et al. (23). The isolated RNA was reverse transcribed into ssDNA using reverse primers and Maxima Reverse Transcriptase (Thermo Scientific). The DNA template for the LAMP reactions was generated by PCR as previously described (22). The primer pair generates a 360 bp product that was purified with the MSB Spin PCRapace Clean Up Kit (Stratec, Birkenfeld, Germany), quantified by Qubit dsDNA HS Assay (Life Technologies, Darmstadt, Germany), and used directly in LAMP. The LAMP assay was set up in a total volume of 25 µL containing 2.5 µL 10× supplied Thermopol reaction buffer (New England Biolabs, Frankfurt am Main, Germany), 1.4 mM of each dNTP (Thermo Scientific), 0.8 M betaine (Carl Roth, Karlsruhe, Germany), 8 U *Bst* 2.0 WarmStart DNA polymerase (New England Biolabs), 1.6 µM of FIP and BIP primer, 0.4 µM of LF and LR primer, 0.2 µM of F3 and B3 primer, 6 mM MgSO₄ (Carl Roth), and purified DNA template. Additional detection reagents were adjusted to reach a final

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concentration of 0.5 mM MnCl_2 (Sigma Aldrich, Taufkirchen, Germany) and 0.05 mM calcein (Sigma Aldrich), 120 μM HNB (VWR, Darmstadt, Germany), 1x EvaGreen (VWR), 1x SYBR Green I (Life Technologies), or 180 μM berberine sulfate (Sigma Aldrich). Negative samples were prepared by adding molecular grade water. All samples were covered with 15 μL mineral oil (Carl Roth) to prevent evaporation. LAMP reactions were monitored at 65°C for 60 min in the portable fluorometer ESE Quant Tube Scanner (Qiagen) using the FAM filter (470/520 nm). The sensitivity of all assays was determined by adding 2 μL serially diluted PSTVd DNA (100-fold) prepared with distilled water and the stock PSTVd DNA solution (3 ng; 0.75×10^{10} copies/ μL). The turbidity, HNB, calcein, and berberine assays were evaluated directly by the naked eye with respect to later in-field applicability. Assays with intercalating dyes were analyzed by real-time

measurement using standard curves and calculating regression coefficient values.

All LAMP assays showed clear differences after amplification between positive and negative reactions in end point analysis under visible and UV light (Figure 2). The evaluation of turbidity is more difficult with the naked eye because there is little contrast of the turbid solution relative to the background. Thus, turbidity as a means to evaluate a LAMP assay is not as simple as color-based assays because it requires additional equipment (e.g., use of cordless centrifuge to show precipitated pellet) to be applicable for field assays. The turbidity assay shows an adequate sensitivity (Figure 2A).

The use of colorimetric detection methods such as HNB largely improves the discrimination between positive and negative samples in the PSTVd LAMP assay. Typically, HNB results in a color change from violet to blue after 60 min in the presence of the isother-

mally amplified DNA (Figure 2B). HNB is a simple synthetic dye that is cost effective for use in LAMP because only minute amounts are needed in the assay. It can also be quantified by absorption measurements at 650 nm with a spectrophotometer (10). As shown in Figure 2B, it is as sensitive as detection by turbidity. The evaluation by naked eye is simple and therefore well suited for end point detection (24). A comparative multi-observer study showed that HNB was the best of the compared methods to guarantee a reliable interpretation of LAMP assays (25). However, for field conditions, we recommend individual optimization of the reaction volume and amount of HNB for best visibility of the results.

Calcein is a synthetic fluorescein derivative and emits bright fluorescence at 515 nm when excited at 495 nm. As shown in Figure 2, it can be used in LAMP for end point detection in visible and UV light.

Table 1. Summary of detection methods used in one-pot LAMP assays

	Turbidity	Hydroxynaphthol blue	Calcein	SYBR Green I	EvaGreen	Berberine
Substance	Mg-pyrophosphate (Mg-PPi)	Hydroxynaphthol blue (HNB)	Calcein AM + MnCl_2	SYBR Green I	EvaGreen	Berberine- SO_4
Origin	Amplification product	Synthetic	Synthetic	Synthetic	Synthetic	Natural
Toxicity	None	May cause eye irritation	May be harmful to skin and eyes	Mutation enhancer	Possible carcinogen	May be toxic in high concentrations
Detection mechanism	Insoluble complex; precipitation	Decrease of free Mg^{2+}	Decrease of free Mn^{2+}	dsDNA intercalation	dsDNA intercalation	Small groove intercalation
Readout	Turbidity Absorbance: 400 nm	Absorption Absorbance: 650 nm	Fluorescence Excitation: 495 nm Emission: 515 nm	Fluorescence Excitation: 494 nm Emission: 521 nm	Fluorescence Excitation: 500 nm Emission: 530 nm	Fluorescence Excitation: 450 nm Emission: 530 nm
Effect on amplification	None	None	Manganese may inhibit reaction	Not inhibiting when used 0.5–1x	Not inhibiting when used 0.5–1x	Not inhibiting ($\leq 180 \mu\text{M}$)
One-pot real-time assay	++	-	+++	++++	++++	++++
Equipment for real-time detection	Turbidometer	-	Fluorometer with FAM filter	Fluorometer with FAM filter	Fluorometer with FAM filter	Fluorometer with FAM filter
One-pot end point assay	+++	++++	++++	-	-	+++ (UV light with low background signal)
Equipment for end point detection	None (cordless centrifuge)	None	UV lamp (optional)	Not applicable	Not applicable	UV lamp
Evaluation of results	+ Turbid - Clear	+ Sky blue - Violet	+ Green - Orange	+ Fluorescence signal - No signal	+ Fluorescence signal - No signal	+ Fluorescence signal - No signal
Percentage of overall costs *	0	<0.1	<0.1	26.01	1.18	0.82
Relative sensitivity **	+++	+++	+++	+++	+++	+++
Field applicability ***	+++	++++	++++	++	++	++++

Summary of features relevant for in-the-field loop-mediated isothermal amplification (LAMP) assays tested for detection of potato spindle tuber viroid (PSTVd). Number of “+” describes the applicability/relevance of the feature.

* Overall costs represent the basic chemicals of common suppliers for one LAMP reaction, depending on reaction volume.

** In our assays. Analytical sensitivity may depend on LAMP setup and has to be optimized separately.

*** Depending on visualization.

Figure 3. Sensitivity of real-time potato spindle tuber viroid (PSTVd) loop-mediated isothermal amplification (LAMP) assays. Sensitivity of the real-time LAMP assays is shown as threshold time of the fluorescence signal above 0.2 RFU (y-axis) against the log copy number of the initial template DNA (x-axis). SYBR Green I (red), EvaGreen (black), and berberine (blue). Experiments were done in triplicate. Regression equation and coefficient of determination are shown in the upper right corner. Supplementary Figure S1 provides a more detailed depiction of the raw data.

In visible light, the positive calcein assay exhibits a yellow to green color, but a negative sample retains the orange color (Figure 2C). When excited with UV light (312 nm), the positive samples become visible by bright green fluorescence, but the negative control remains dark (Figure 2D). Calcein-based detection is applicable as a real-time assay as well (26). In contrast to SYBR Green I and EvaGreen, the use of calcein and manganese for real-time fluorescence detection increased the threshold time for a positive LAMP amplification by 10 min (data not shown). Manganese is known to inhibit the LAMP reaction and may decrease the overall sensitivity of the assay (10). Nevertheless the calcein-based end point assay is as sensitive as HNB (see Figure 2, B and D), and the evaluation can be done without special training and equipment (a UV lamp is optional).

Generally, methods based on DNA intercalation are more sensitive but also more expensive as well as toxic. The dsDNA-intercalating dyes SYBR Green I and EvaGreen resulted in increasing fluorescence signals in real-time measurements in less than 10 min (Supplementary Figure S1, A and B). Both have similar excitation and emission properties and can be used in LAMP, but EvaGreen has better DNA binding properties and is more compatible with polymerase

reactions than SYBR Green I. Depending on the concentration, the two dyes are well suited to real-time measurements. Both dyes require polymerase-inhibiting concentrations for end point visualization by the naked eye in the presence of UV light and are therefore only compatible with a spectrophotometer-free one-pot LAMP assay when added separately. The sensitivity of both dyes is similar (Figure 3) and no inhibition of the LAMP reaction was observed at working concentration (Supplementary Figure S1, A and B).

As a new alternative to these dyes, we discovered that berberine displayed favorable properties in our LAMP assays. Although there was no discernible difference between positive and negative samples in visible light (Figure 2E), in UV light (312 nm) we were able to distinguish between the amplified PSTVd DNA template and the negative control (Figure 2F). A slight green color in the negative sample corresponds to background fluorescence that could not be suppressed in our experiments and is likely caused by binding of berberine to the single-stranded primers. In the real-time assay, the fluorescence signal increased after 10 min and is thus comparable to SYBR Green I and EvaGreen (Supplementary Figure S1C), reaching a plateau without significant signal decay as observed for SYBR Green I.

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Berberine has a slightly different fluorescence spectrum than SYBR Green I and EvaGreen (see Table 1). Our approach was only investigated with the ESE Quant Tube scanner and the FAM filter. Other filter sets may fit the spectrum of berberine better. However, in contrast to the other dyes in our comparison, berberine is also suitable for in-field applications when using a UV lamp (312 nm). It is a nontoxic natural product and is considerably cheaper than the other intercalating dyes. It displays sufficient product sensitivity, is applicable to real-time detection, and is suitable for end point detection using UV light. We also did not observe inhibition of M-MuLV reverse transcriptase by berberine at default concentrations (data not shown) and or any inhibitory effect on the LAMP reaction at default concentrations (Supplementary Figure S2). However, we recommend prior testing if other reverse transcriptases such as that from avian myeloblastosis virus (AMV) or commercial variants are used in the assay with RNA as the template. All three dyes are similarly quantitative and sufficiently reproducible in real-time measurements (Figure 3 and S1). Quantification for one-pot in-field LAMP assays as investigated here is not necessary because merely positive or negative results are sufficient. To detect a lower amount of target DNA, further optimization for each real-time assay may be advantageous.

In conclusion, we have compiled a table listing the most important features relevant for in-field LAMP assays. It provides an overview of the different features of the assays we tested to facilitate the proper choice of the optimal LAMP detection format with respect to the desired application (end point or real time). Our experimental results indicate that the detection of the quarantine phytopathogen PSTVd with LAMP by using HNB, calcein, or—as a new approach—berberine is sensitive and allows for use in the field. Finally, this report highlights the main features of simple methods to visualize LAMP products for the detection of target sequences in both end point analysis using a simple water bath and a UV lamp and real-time-analysis using a portable fluorometer. It can therefore serve as a useful starting point for investigations of the many other isothermal amplification techniques based on the popular *Bst* DNA polymerase.

Author contributions

J.F. performed all LAMP assays, including analysis, and wrote the manuscript. N.C.X. established and performed preliminary berberine assays. M.F. supervised the project. J.F.G. conceptualized the experimental design and assisted in writing the manuscript.

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Competing interests

The authors declare no competing interests.

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