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A Loop-mediated isothermal amplification (LAMP) Assay for the Rapid Identification of Bemisia tabaci

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May 22, 2018

Dear editorial team

We are pleased to submit the attached manuscript entitled „A Loop-mediated isothermal amplification (LAMP) Assay for the Rapid Identification of *Bemisia tabaci*" for consideration for publication in the Biology section of JoVE.

Controls of plant imports at points of entry are the first line of defense against pest invasions. In order to prevent pest introduction events, plant shipments infested with quarantine pest species have to be destroyed. To justify such measures requires rapid identification of the intercepted specimens.

Because the identification of pest species can be extremely difficult for plant health inspectors with limited taxonomic knowledge, this identification step is often performed in central laboratories and generally takes two or more days. Hence, many plant commodities are spoiled even before the diagnosis results are available. A solution for this problem could be to enable on-site identification.

Here, we present an on-site identification assay for the rapid identification of *Bemisia tabaci*, an insect plant pest causing important economic damages to many different crops around the world. The novel assay enables non-specialist plant health inspectors to perform a rapid identification directly at the point of entry within one hour and will be of particular interest for scientists involved in knowledge transfer, plant health inspection services, as well as policy makers.

The final manuscript has been approved by the co-authors, and all co-authors made contributions deserving of their authorship. They confirm that the manuscript has not been previously published and is not currently under consideration by any other journal.

Yours sincerely,

Simon Blaser

TITLE:

A Loop-mediated Isothermal Amplification (LAMP) Assay for Rapid Identification of *Bemisia tabaci*

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KEYWORDS:

Bemisia tabaci, LAMP, loop-mediated isothermal amplification, point of entry diagnostics, plant health, rapid diagnostics, quarantine organisms

SHORT ABSTRACT:

This paper reports the protocol for a rapid identification assay for *Bemisia tabaci* based on loop-mediated isothermal amplification (LAMP) technology. The protocol requires minimal laboratory training and can, therefore, be implemented on-site at points of entry for plant imports such as seaports and airports.

LONG ABSTRACT:

The whitefly *Bemisia tabaci* (Gennadius) is an invasive pest of considerable importance, affecting the production of vegetable and ornamental crops in many countries around the world. Severe yield losses are caused by direct feeding, and even more importantly, also by the transmission of more than 100 harmful plant pathogenic viruses. As for other invasive pests, increased international trade facilitates the dispersal of *B. tabaci* to areas beyond its native range. Inspections of plant import products at points of entry such as seaports and airports are, therefore, seen as an important prevention measure. However, this last line of defense against pest invasions is only effective if rapid identification methods for suspicious insect specimens are readily available. Because the morphological differentiation between the regulated *B. tabaci* and close relatives without quarantine status is difficult for non-taxonomists, a rapid molecular identification assay based on the loop-mediated isothermal amplification (LAMP) technology has been developed. This publication reports the detailed protocol of the novel assay describing rapid DNA extraction, set-up of the LAMP reaction, as well as interpretation of its read-out, which allows identifying *B. tabaci* specimens within one hour. Compared to existing protocols for the detection of specific *B. tabaci* biotypes, the developed method targets the whole *B. tabaci* species complex in one assay. Moreover the assay is designed to be applied on-site by plant health inspectors with minimal laboratory training directly at points of entry. Thorough validation performed under laboratory and on-site conditions demonstrates that the reported LAMP assay is a rapid and reliable identification tool, improving the management of *B. tabaci.*

INTRODUCTION:

The whitefly *Bemisia tabaci* (Gennadius) is an invasive insect pest affecting the yield of many economically important crops including ornamental plants, vegetables, grain legumes, and cotton[1, 2]. Beside damage caused through direct phloem-feeding, the homopteran species harms plants indirectly by the excretion of large amounts of honeydew onto the surfaces of leaves and fruits, as well as by the transmission of numerous plant pathogenic viruses[1, 3, 4]. Recent genetic studies comparing DNA sequences of the mitochondrial gene cytochrome *c* oxidase 1 (COI) revealed that *B. tabaci* is a species complex of at least 34 morphocryptic species[3, 4]. Two highly invasive and damaging members within this complex, biotype B originating from the Middle East and the Asian Minor region, as well as biotype Q originating from the Mediterranean region, have been dispersed globally through international trading activities with plant products, particularly by the transportation of ornamentals[1, 5, 6]. Due to its worldwide pest status, the International Union for the Conservation of Nature and Natural Resources (IUCN) listed *B. tabaci* as one of the "world's 100 worst invasive alien species" and members of the species complex are regulated organisms by many countries[1, 3, 4].

In the European Union (EU), *B. tabaci* is listed in the Plant Health Directive 2000/29/EC Annex 1AI as a quarantine organism whose introduction from non-EU countries and its dissemination within the EU are banned[4]. An essential prevention measure against the spread of quarantine organisms is the inspection of plant shipments at points of entry (POEs) such as airports and seaports[7, 8]. In the case a quarantine organism is found, the National Plant Protection Organization (NPPO) in charge takes action by either rejecting or treatment (including destruction) of the infested

shipment[9]. However, officers inspecting the imports often do not have the taxonomic expertise to accurately identify the vast range of pest species associated with global trade[9]. Especially the identification of immature life stages (*e.g.,* eggs and larvae) without distinct morphological keys is virtually impossible for non-taxonomists[8–10]. Consequently, to enable implementation of quarantine measures with minimal delay, there is a need for alternative, rapid on-site identification assays[9].

A candidate method is the loop-mediated isothermal DNA amplification (LAMP) technology that has recently been shown to be a suitable technology for the identification of plant pathogens[11–13]. LAMP is highly specific because the method uses at least two primer pairs recognizing six distinct DNA target sequences[14]. Due to the DNA strand displacement activity of the *Bst* DNA polymerase, LAMP reactions are performed under isothermal conditions[14]. Hence, in contrast to conventional polymerase chain reaction (PCR)-based assays there is no need for a thermal cycler[13, 14]. Another advantage over PCR-based assays is its resilience against potential inhibitors in the DNA extract, circumventing the need for a DNA purification step[13]. Due to the protocol's speed and simplicity, LAMP may even be performed under on-site conditions using a portable, battery driven real-time detection device[8, 15].

A LAMP assay was designed in response to the demand for a rapid on-site identification method for *B. tabaci*[8]. The overarching aim was to develop a protocol that can be performed by plant health inspectors with limited laboratory training. A strong focus was, therefore, set on optimizing speed and simplicity of the protocol. While existing diagnostic tests have generally been developed for the identification of one or several biotypes of *B. tabaci*, the novel LAMP assay covers the whole *B. tabaci* species complex[8, 16–18]. The problem of the pronounced genetic within-taxon diversity of the complex was solved by using combinations of different primer sets and the application of degenerate primers[8]. The novel *B. tabaci* LAMP assay is designed in such a way that the primers target a fragment at the 3' end of the mitochondrial COI gene[8]. This gene presents a suitable target for animal diagnostic assays because it harbors regions conserved enough to ensure diagnostic sensitivity for a specific species, while discriminating enough between closely related organisms[19, 20]. Furthermore, the COI gene is often used as a genetic marker in population genetic studies and as a signature sequence in DNA barcoding analyses, resulting in numerous DNA sequence entries in open source databases such as GenBank and BOLD[21, 22]. Beside the publicly available COI sequences from *B. tabaci*, COI sequences from closely related species (*Aleurocanthus* spp. [N = 2], *Aleurochiton aceris*, *Aleurodicus dugesii*, *Bemisia* spp. [N = 3], *Neomaskellia andropogonis*, *Tetraleurodes acaciae*, and *Trialeurodes* spp. [N = 4]) were included in the primer design of this study and used to assess diagnostic sensitivity and specificity *in silico*[8].

Due to the accuracy of the method, its speed (<1 h) and the simplicity of the protocol, the assay has been shown to be suitable for on-site application when implemented as part of the import control procedure at a Swiss POE[8].

PROTOCOL:

1. Preparations

1.1. Preparing aliquots of alkaline DNA extraction solution.

1.1.1. Produce a stock of alkaline DNA extraction solution using molecular grade water supplemented with 600 µM potassium hydroxide (KOH) and 2 µM Cresol Red.

CAUTION: KOH is a strong base dissolved in water. Avoid spills, and skin and eye contact.

1.1.2. Dispense 30 µL of alkaline DNA extraction solution (prepared in step 1.1.1) into 0.5 mL microcentrifuge tubes and store the aliquots at 4 °C.

Note: Use the aliquoted DNA extraction solution within 1 year.

1.2. Preparing *B. tabaci* positive amplification control (PAC).

1.2.1. Generate PCR amplicons of the LAMP target DNA fragment.

Note: An introduction into general PCR principles and practices is given by Lorenz[23].

1.2.1.1. Synthesize or obtain the primers C1-J-2195 (5'-TTGATTTTTTGGTCATCCAGAAGT-3') and TL2-N-3014 (5'-TCCAATGCACTAATCTGCCATATTA-3') amplifying a fragment of the mitochondrial COI gene[24, 25].

1.2.1.2. Set up the PCR reaction as described in **Table 1**. Use DNA extract (see step 2.1) of a reference *B. tabaci* specimen as DNA template.

Note: Optionally, it is possible to extract the *B. tabaci* DNA for the PAC using a commercial kit according to the manufacturer's instructions.

1.2.1.3. Program a thermal cycler using the following conditions: 15 min at 95 °C; 45 cycles of 40 s at 95 °C, 15 s at 45 °C, ramping over 60 s to 60 °C, 2 min at 72 °C; 7 min at 72 °C; hold at 4 °C.

1.2.1.4. Clean the PCR amplification product using a commercial PCR clean-up kit according to the manufacturer's protocol and elute the final product in molecular grade water.

1.2.1.5. Use a commercial kit with DNA-intercalating dye to measure the DNA concentration of the PCR amplification product according to the manufacturer's instructions and dilute with molecular grade water to a concentration of 1 ng/µL. Store the diluted PCR amplification product as PAC stock solution at -20 °C.

Note: Use the PAC stock solution within 1 year.

1.2.1.6. Supplement the PAC stock solution (prepared in step 1.2.1.5) with 0.6 µM KOH and dilute with molecular grade water to a concentration of 5 x 10^{-3} ng/µL. Store the product at 4 °C.

Note: Use the PAC within 5 h for the preparation of the ready-to-use *B. tabaci* LAMP kits described in the next step.

1.3. Preparing ready-to-use *B. tabaci* LAMP kit (protocol for 20 units)

1.3.1. Use scissors to cut 8-tube LAMP strips into two 4-tube LAMP strips.

1.3.2. Label the tubes of the 4-tube LAMP strips according to the scheme shown in **Figure 1**.

1.3.3. Prepare *B. tabaci* LAMP reaction mastermix (protocol for 80 reactions).

1.3.3.1. Add 1195.1 µL of ready-to-use GspSSD isothermal master mix (containing GspSSD polymerase, pyrophosphatase, magnesium sulfate, deoxynucleotides, double strand binding DNA binding dye) and 717.4 µL of *B. tabaci* LAMP primer mix to a 2 mL microcentrifuge tube. Briefly vortex and pulse centrifuge.

1.3.3.2. Dispense 22.5 µL of *B. tabaci* LAMP reaction mastermix (prepared in step 1.3.3.1) into each tube of the 4-tube LAMP strips (prepared in step 1.3.1) and pulse centrifuge.

1.3.4. Add 2.5 µL of *B. tabaci* LAMP PAC (prepared in step 1.2) into the tube labelled with "PAC" of each 4-tube LAMP strip (**Figure 1**).

1.3.5. Close lids and store the ready-to-use *B. tabaci* LAMP kit units at -20 °C.

Note: Use them within 1 year.

2. On-site LAMP Analysis

2.1. DNA extraction

2.1.1. Use sterile toothpicks to transfer the insect specimens into 0.5 mL microcentrifuge tubes containing 30 µL of DNA extraction solution (prepared in step 1.1.2).

Note: Make sure that the insects are immersed in the extraction solution.

2.1.2. Incubate the samples for 5 min at 95 °C in a thermomixer (300 rpm). Briefly vortex and pulse centrifuge.

2.2. *B. tabaci* LAMP assay

2.2.1. Thaw a ready-to-use *B. tabaci* LAMP kit prepared in step 1.3. Vortex quickly and pulse centrifuge.

Note: With each kit, it is either possible to test two different specimens or to analyze the DNA extract of one specimen in duplicate.

2.2.2. Add 2.5 µL of sample DNA extract (prepared in step 2.1) into the tubes labeled "S1" and "S2" of the ready-to-use *B. tabaci* LAMP kit (**Figure 1**).

2.2.3. Add 2.5 µL of pure alkaline DNA extraction solution (prepared in section 1.1) into the tube labeled "NAC" for the negative amplification control (**Figure 1**).

2.2.4. Vortex the ready-to-use *B. tabaci* LAMP kit quickly and pulse centrifuge.

2.2.5. Insert the ready-to-use *B. tabaci* LAMP kits into the LAMP analysis device (with real-time fluorescence measurement) or a real-time PCR platform and perform an isothermal DNA amplification analysis at 65 °C for 60 min.

2.2.6. Measure the melting temperatures of DNA amplification products by heating up to 98 °C with a subsequent cooling step (ramp rate of 0.05 °C/s) to 75 °C, while measuring fluorescence in real-time.

2.3. LAMP assay read-out

2.3.1. Validate the LAMP read-out manually as follows.

2.3.1.1. If DNA amplifications were measured for the sample and the PAC, no DNA amplification was measured for the NAC, and the annealing temperature of the amplification products were between 80.0 and 85.5 °C, consider the LAMP results as POSITIVE (**Figure 2**).

2.3.1.2. If there is no DNA amplification for the samples (*i.e.,* tubes labeled S1 and S2) but for PAC and NAC then consider the LAMP result as NEGATIVE (**Figure 2**).

2.3.1.3. If DNA amplification was measured for the samples, but the annealing temperatures of corresponding amplification products were outside the range 80.0 – 85.5 °C, and/or PAC gave no DNA amplification, and/or NAC gave a DNA amplification, consider the LAMP result as INVALID (**Figure 2**).

2.3.2. Optionally, validate the LAMP read-out using the LAMP validation application (**Supplemental file 1**).

2.3.2.1. Define target species and define the number of tested samples. Click the **Generate Report** button.

2.3.2.2. Transfer the read-out (DNA amplification yes/no, annealing temperature amplification product, results of PAC and NAC) from the on-site LAMP analysis device or real-time PCR platform to the corresponding input fields of the validation application. The result of the validation is immediately displayed after entering the data.

REPRESENTATIVE RESULTS:

During the validation of the *B. tabaci* LAMP assay, insect specimens intercepted in the course of the regular Swiss import control process were analyzed[8]. The specimens originated from eight different countries (Canary Islands, Dominican Republic, Israel, Malaysia, Morocco, Singapore, Thailand, and Vietnam) and reflect the genetic diversity of *B. tabaci* found at European POEs[8]. All LAMP results were cross-validated by DNA barcoding[8].

From a total of 80 specimens analyzed by LAMP, 75 specimens (93.8%) were correctly identified as *B. tabaci* (true-positives), two specimens (2.5%) were correctly identified as not being *B. tabaci* (true-negatives), and three specimens (3.8%) were wrongly identified as not being *B. tabaci* (false-negatives)[8]. The correct-negative results originated from two *Trialeurodes vaporariorum* specimens, a non-regulated species at high risk to be confused with *B. tabaci* at points of entry for plant products[8]. Based on these results, the following measurements of diagnostic accuracy were calculated: test specificity (true-negative rate), 100%; test sensitivity (true-positive rate), 96.2%; test efficiency (percentage of correct test results), 96.3%[8]. When assessing the analytical sensitivity (detection limit), the *B. tabaci* LAMP assay successfully amplified sample DNA diluted to 100 fg/µL across three technical replicates (**Table 2**).

A subset of the assays (N = 13) was performed under on-site conditions at the Swiss POE Zurich Airport by plant health inspectors using the ready-to-use *B. tabaci* LAMP kits[8]. When cross-validated in the reference laboratory, all results from on-site testing were found to be correct (test efficiency = 100%)[8]. Assessing the on-site LAMP assay performance, the average time to positive (time until a positive results was available) was 38.4 ± 10.3 min (mean ± standard deviation)[8]. A representative DNA amplification plot and the corresponding annealing derivative from a *B. tabaci* LAMP analysis performed under on-site conditions are shown in **Figure 3A and B.** In this example, sample one and two were correctly identified as *B. tabaci* indicated by DNA amplification after approximately 30 min (**Figure 3A**) together with the expected annealing temperatures at approximately 82 °C (**Figure 3B**).

FIGURE & TABLE LEGENDS:

Figure 1: Visualization of the experimental set-up of a ready-to-use *B. tabaci* LAMP kit described in the protocol. S1, sample 1; S2, sample 2; PAC, positive amplification control; NAC, negative amplification control.

Figure 2: LAMP read-out validation schema. PAC, positive amplification control; NAC, negative amplification control.

Figure 3: DNA amplification plot (A) and annealing derivative (B) of a *B. tabaci* LAMP analysis performed under on-site conditions. Fluorescence was measured in relative intensity units. Green line, sample 1; orange line, sample 2; blue line, negative amplification control (NAC); red line, positive amplification control (PAC); F, fluorescence; T, temperature.

Table 1: Preparation of PCR reaction mastermix for the *B. tabaci* positive amplification control. Components and concentrations needed to set up one PCR reaction. The final reaction volume is 20 µL. Primer sequences are shown in 1.2.1.1.

Table 2: Analytical sensitivity (detection limit) of the *B. tabaci* LAMP assay. Each dilution was tested in triplicates. C_{DNA}, DNA concentration per reaction; N_{PR}, number of positive replicates; T_P, time until a positive result was available; T_A, annealing temperature; SD, standard deviation.

DISCUSSION:

The ability to accurately identify potentially harmful organisms without time delay represents a critical aspect for the management of pest species[9, 10, 26]. Besides being rapid, for plant import products, an ideal pest identification method should be simple to perform on-site at POEs[8, 26]. This paper reports the protocol of a novel LAMP assay for the rapid identification of *B. tabaci*, a quarantine insect organism frequently intercepted at European borders (https://ec.europa.eu/food/sites/food/files/plant/docs/ph_biosec_europhyt_annual-report _2016.pdf).

The rationale behind the development of the diagnostic test was to design an easy-to-follow protocol which can be performed during the plant import control procedure by plant health inspectors with minimal laboratory training. In order to make on-site testing as rapid and simple as possible, the protocol is divided into two parts, the preparation of a ready-to-use kit and the actual performance of the LAMP assay. The first part may be done in an external laboratory so that the plant health inspector can perform the DNA extraction and LAMP assay on-site with only one pipetting step.

Though only one step, pipetting small amounts of liquid may be challenging for users with little or no laboratory experience. To address this problem, a dye (cresol red) is added to the extraction solution so that the operator can visually confirm the small amount (*i.e.,* 2.5 µL) of DNA is correctly transferred to the respective tube. Another important simplification of the protocol is the validation application as it facilitates a reliable interpretation of the LAMP read-out (**Supplemental file 1**).

The novel *B. tabaci* LAMP assay has been validated under laboratory and on-site conditions by testing insect specimens intercepted during the regular import control process of Switzerland[8]. In total, 80 specimens from three continents, Africa, Eurasia, and North America, were analyzed by LAMP. Of the 80 specimens, only three (3.8%) were wrongly identified (false-negatives)[8]. When analyzing the primer target DNA sequences of the false-negative specimens, it was found that they were new *B. tabaci* haplotypes that have so far not been described[8]. Based on these results, the *B. tabaci* LAMP primer set has been modified and successfully re-validated[8].

One major limitation of any DNA amplification-based method including LAMP is that they only identify pre-defined target DNA sequences[8, 27]. A comprehensive knowledge of the genetic variation found in the primer target sequence is therefore crucial to ensure diagnostic accuracy[8, 27]. However, such information is often very limited, especially in the case of newly emerging pest species[8]. Though rare, false-negative results caused by mutations in the target sequence are expected[8]. In the case of the present *B. tabaci* LAMP assay, a solution for this problem is the combination with a DNA barcoding-based technology, a strategy realized in the course of the implementation of this diagnostic test at the POE Zurich Airport[8]. Here, all LAMP-negative results were re-analyzed by DNA barcoding in an external laboratory[8]. In case a novel pest haplotype not yet described is encountered, the LAMP primers can be modified using the DNA sequence generated in the barcoding process[8]. Thereby, the resulting loss of speed in case of a negative LAMP result is compensated for the maximum diagnostic accuracy ensured in this two-stage process[8].

The set-up costs for the current LAMP assay at a POE are approximately USD 25,000. With the increasing number of LAMP tests developed for plant pests (*e.g., Erwinia amylovora*, Flavescence dorée, *Guignardia citricarpa*), such a one-time investment appears justified[13, 15, 28]. However, the protocol could potentially be modified to reduce these costs even further. For example, for the DNA extraction step at 95 °C the thermo mixer used here could be replaced by a less expensive water bath, or by performing this step directly in the real time LAMP device. Furthermore, the mixing steps on the vortex could probably be replaced by manually flicking the tubes, and in the DNA transfer step the pipettor might be replaced by sterile inoculation loops.

Future improvements for a rapid identification of *B. tabaci* and pest species in general could be an implementation of an on-site sequencing approach that would allow to perform DNA barcoding analyses at POEs. A promising candidate system for such an implementation is the nanopore sequencing technology. Indeed, the technology has recently been successfully implemented in an on-site DNA barcoding effort to assess the biodiversity of a rainforest[8, 29, 30]. An on-site DNA barcoding identification system can completely replace the need for the development of targeted diagnostic tests and their validation. Also it allows collecting additional information about pest characteristics such as pesticide resistance genes[8]. Nevertheless, until novel sequencing technologies will be implemented routinely, the *B. tabaci* LAMP assay represents a rapid (<1 h) and accurate identification method.

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DISCLOSURES:

The author Michael Andreou is a shareholder of OptiGene Limited that produces reagents and instruments used in this article. The other authors have nothing to disclose.

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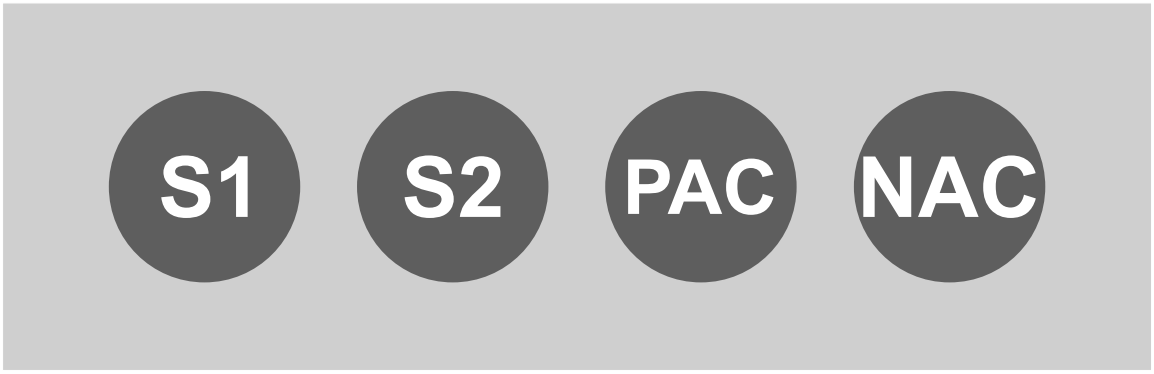

Component	Stock conc.	Final reaction conc.	Volume per reaction
Taq Polymerase Master Mix	2x	1x	10 µL
Primer C1-J-2195	20 µM	0.4 µM	0.4 µL
Primer TL2-N-3014	20 µM	0.4 µM	0.4 µL
Molecular Grade Water	-	-	8.2 µL
DNA Template	-	-	1 µL

C_{DNA} (fg/µL)	N_{PR}	T_P (min) (mean ± SD)	T_A (°C) (mean ± SD)
1×10^5	3	33.5 ± 2.9	81.3 ± 0.1
1×10^4	3	30.7 ± 1.1	81 ± 0.0
1×10^3	3	40.4 ± 3.9	81.1 ± 0.1
1×10^2	3	50.7 ± 1.6	81.1 ±0.1
1×10^1	0	-	-
1×10^0	0	-	-

Table of Materials

Click here to download Table of Materials Table_of_Materials.xls

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
B. tabaci LAMP primer mix	OptiGene Ltd.	on request	For preparation of read-to-use *B. tabaci* LAMP kit
Centrifuge MiniSpin	Eppendorf AG	5452000010	For several centrifugation steps
Cresol red (red dye)	Sigma-Aldrich Corp.	114472	Component of DNA extraction solution
Eppendorf ThermoMixer	Eppendorf AG	5382000015	For DNA extraction
Genie II (on-site LAMP analysis device)	OptiGene Ltd.	Genie® II	For LAMP analysis
Genie Strips (8-tube LAMP strips)	OptiGene Ltd.	OP-0008-50	For preparation of read-to-use *B. tabaci* LAMP kit
HotStarTaq Master Mix	Qiagen AG	203443	For generation of positive amplification control
Labcycler (Thermocycler)	SensoQuest GmbH, distributed by Witec AG	011-103	For DNA extraction
GspSSD Lyse n' Lamp Isothermal Mastermix	OptiGene Ltd.	ISO-001LNL	For preparation of read-to-use *B. tabaci* LAMP kit
Mini centrifuge Labnet Prism	Labnet International Inc.	C1801	For several centrifugation steps
NucleoFast 96 PCR	Marcherey-Nagel GmbH	743500.4	For clean-up of positive amplification control
Potassium hydroxide solution	Sigma-Aldrich Corp.	319376	Component of DNA extraction solution
Qbit Fluorometer 3	Thermo Fisher Scientific Corp.	Q33226	For measuring DNA conentration of positive control
Qubit dsDNA HS Assay Kit	Thermo Fisher Scientific	Q32854	For clean-up of positive amplification control
Safe-Lock Tubes 0.5 mL (microcentrifuge tube)	Eppendorf AG	0030 121.023	For DNA extraction
Safe-Lock Tubes 2.0 mL (microcentrifuge tube)	Eppendorf AG	0030 120.094	For preparation of read-to-use B. tabaci LAMP kit
Wood Toothpicks	VWR International LLC	470226-594	For DNA extraction
Vortex-Genie 2 (Vortex)	Scientific Industries Inc.	SI-0236	For several mixing steps

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Title of Article: A Loop-mediated isothermal amplification (LAMP) assay for the rapid identification of Bemisia tabaci

Author(s): Simon Blaser, Hanspeter Diem, Andreas von Felten, Morgan Gueuning, Michael Andreou, Neil Boonham, Jennifer Tomlinson, Pia Marthe, Jörg Lange, Beatrice Frey, Jörg E. Frey, Andreas Kiefer

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Name: Simon Blaser

Department: Department of Method Development and Analytics

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Article Title: A loop-mediated isothermal amplification (LAMP) assay for the rapid identification of Bacillus spp.

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MS # (internal use):

Blaser *et al*. "A Loop-mediated isothermal amplification (LAMP) Assay for the Rapid Identification of Bemisia tabaci" (your reference no. JoVE58502) – Point-by-point response

Wädenswil, 17 July 2018

Dear Dr. DSouza

We refer to your E-mail dated 26 June 2018 in connection with the aforementioned manuscript. We thank you and the four external reviewers very much indeed for having refereed our manuscript so carefully and in such a timely and constructive manner. In the meantime, we have revised our manuscript in light of the reviewers' feedbacks. We would like to point out that we added an additional Figure (**Figure 2**) as well as an additional Table (**Table 2**) to the manuscript during the revision process. Below, please find our point-by-point response, clearly indicating how and where in the manuscript (line numbers) changes have been made.

We look forward to your further disposition and would be delighted if our revised manuscript would be accepted for publication in JoVE.

Yours sincerely,

Simon Blaser & Jürg E. Frey
(on behalf of all the co-authors)

****

Corrections to the editorial comments

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
Response: This point has been addressed by proofreading the manuscript thoroughly.

2. Figure 3: Please use exponential notation (instead of E notation) for numbers on the y-axis; i.e., change 6.0E+4 to 6 × 104.
Response: This point has been addressed by changing notation of Figure 3 from "E" notation to exponential notation. During this revision step, we realized that there was a disagreement in the color scheme of sample 2 and the positive amplification control (PAC), which has been corrected in the revised Figure 3. **(See revised Figure 3)**

3. Table 1: Please change "µl" to "µL".
Response: This point has addressed by changing "µl" to "µL". **(See revised Table 1)**

4. Please provide an email address for each author.
Response: This point has been addressed by modifying the section INSTITUTIONS & AFFILIATIONS FOR EACH AUTHOR in accordance with the JoVE template manuscript "Alternative Cultures for Human Pluripotent Stem Cell Production, Maintenance, and Genetic Analysis". **(See revised manuscript, lines 10-79)**

5. Please rephrase the Long Abstract to more clearly state the goal of the protocol.
Response: This point has been addressed by modifying the LONG ABSTRACT. **(See LONG ABSTRACT of revised manuscript, lines 94-113)**

6. Please rephrase the Introduction to include a clear statement of the overall goal of this method.
Response: This point has been addressed by modifying the INTRODUCTION section and adding the paragraphs: "In response to the demand for a rapid on-site identification method of *B. tabaci*, a LAMP assay was designed[8]. The overarching aim was to develop a protocol that can be performed by plant health inspectors with limited laboratory training. A strong focus was, therefore, set on optimizing speed and simplicity of the protocol. While existing diagnostic tests have generally been developed for the identification of one or several biotypes of *B. tabaci*, the novel LAMP assay covers the whole *B. tabaci* species complex[8, 16–18]." **(See INTRODUCTION section of revised manuscript, lines 154-159)**

7. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.
Response: This point has been addressed by checking the PROTOCOL section for units not corresponding to the SI abbreviation system. Thereby, "sec" was replaced at several positions by "s". **(See PROTOCOL section or revised manuscript, lines 178 293)**

8. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: OptiGene Ltd, Microsoft Excel, etc.
Response: This point has been addressed by removing all commercial language from the manuscript.

9. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
Response: This point has been addressed by revising the protocol to avoid the use personal pronouns. Thereby, two points of the protocol were changed as follows to remove the pronoun "you":

Point 1.2.1.2: "Set up the PCR reaction as described in **Table 1**. Use DNA extract (see step 2. 1) of a reference *B. tabaci* specimen as DNA template. NOTE: Optionally, it is possible to extract the *B. tabaci* DNA for the PAC using a commercial kit according to the manufacturer's instructions." **(See revised manuscript, lines 198-200)**

Point 2.2.1: "Thaw a ready-to-use *B. tabaci* LAMP kit prepared in step 1.3. Vortex quickly and pulse centrifuge. NOTE: With each kit, it is either possible to test two different specimens or to analyze the DNA extract of one individual in duplicate". **(See revised manuscript, lines 250-252)**

10. 1.2.1.5: Please mention how to measure the DNA concentration.
Response: Point 1.2.1.5 has been changed as follows: "Use a commercial kit with DNA-intercalating dye to measure the DNA concentration of the PCR amplification product according to the manufacturer's instructions and dilute with molecular grade water to a concentration of 1 ng/µL." **(See revised manuscript, lines 209-213)**

11. References: Please do not abbreviate journal titles. Please include volume and issue numbers for all references.
Response: This point has been addressed by changing journal titles of REFERENCE section from "abbreviated" notation to "full journal titles". References were furthermore checked for missing issue numbers. For references no. 1, 3, 14, 17, 22, and 23, no issue numbers were available. **(See revised manuscript, REFERENCE section, lines 422-546)**

12. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.
Response: This point has been addressed by revising all trademark and registered symbols from the Table of Equipment and Materials. **(See revised Table of Equipment and Materials)**

****

Corrections to Review #1

Manuscript Summary
The manuscript JoVE58502- A Loop-mediated isothermal amplification (LAMP) Assay for the Rapid Identification of Bemisia tabaci, reported the protocol of a rapid identification assay for Bemisia tabaci based on Loop mediated isothermal amplification (LAMP) technology.
This is an interesting but incomplete study. The text also needs a careful revision in several parts before it can be accepted for publication. I have several comments and suggestions to the authors before the final acceptance and publication. An extensive revision is then required.

Major concerns
1. The target gene and primers sequences were not present in the paper. The reason why chose the COI gene was not mentioned for the study and the region of the target gene was used to design the LAMP primers was also not mentioned. The gene are highly conserved and specific to *Bemisia tabaci*? If genes were not selected wisely, it may lead to unspecific amplifications and results.
Response: First, we would like to thank Reviewer#1 for his revision of our manuscript and for his conclusive comments. Regarding the above concerns about the *B. tabaci* LAMP primer design, we have addressed it by revising the INTRODUCTION part as follows: "In response to the demand for a rapid on-site identification method of *B. tabaci*, a LAMP assay was designed[8]. The overarching aim was to develop a protocol that can be performed by plant health inspectors with limited laboratory training. A strong focus was, therefore, set on optimizing speed and simplicity of the protocol. While existing diagnostic tests have generally been developed for the identification of one or several biotypes of *B. tabaci*, the novel LAMP assay covers the whole *B. tabaci* species complex[8, 16–18]. The problem of the pronounced genetic within-taxon diversity of the complex was solved by using combinations of different primer sets and the application of degenerate primers[8]. The novel *B. tabaci* LAMP assay is designed in such a way that the primers target a fragment at the 3' end of the mitochondrial COI gene[8]. This gene presents a suitable target for animal diagnostic assays because it harbors regions conserved enough to ensure diagnostic sensitivity for a specific species, while discriminating enough between closely relative organisms[19, 20]. Furthermore, the COI gene is often used as a genetic marker in population genetic studies and as a signature sequence in DNA barcoding analyses, resulting in numerous DNA sequence entries in open source databases such as GenBank and BOLD[21, 22]. Beside the publicly available COI sequences from *B. tabaci*, COI sequences from closely related species (*Aleurocanthus* spp. [N=2], *Aleurochiton aceris*, *Aleurodicus dugesii*, *Bemisia* spp. [N=3], *Neomaskellia andropogonis*, *Tetraleurodes acaciae*, and *Trialeurodes* spp. [N=4]) were included in the primer design of this study and used to assess diagnostic sensitivity and specificity *in silico*[8]." **(See INTRODUCTION section of revised manuscript, lines 154-172)**

We developed the LAMP assay with the intention that it can be performed by plant health inspectors with minimal laboratory training directly at the point of entry. In order to reduce the risk of any contamination during the on-site LAMP process, it was our aim to reduce the number of pipetting steps of the protocol as much as possible. However, this can only be ensured if ready to use reagents (e.g. primer mixes) are readily available. For the long term supply of plant health inspectors at points of entry, it is thus crucial that a company produces

those reagents. Our partner for supplying all necessary primers and enzymes, disposables and hardware for the LAMP assays, OptiGene (represented by co-author Michael Andreou) guarantees availability for plant health inspectors as well as for the general public and thereby ensures the reproducibility of the results. We feel that it is therefore not necessary to reveal the sequences of these primers, especially as they are not patented.

2. The specificity and sensitivity (detection threshold) of the LAMP assay is very important. Please add the specificity and sensitivity (detection threshold) assay. To determine assay specificity, several related species (such as: *Alegrodes proletella*, *Aleurodicus disperses*, *Dialerodes citri*, and so on) may be used.
Response: We agree with Reviewer#1 that specificity is indeed very important for genetic tests. As reported above, we included COI target sequences of the following closely related species of *B. tabaci* into the initial primer design: *Aleurocanthus spiniferus, Aleurocanthus camelliae, Aleurochiton aceris, Aleurodicus dugesii, Bemisia afer, Bemisia berbericola, Bemisia tuberculate, Neomaskellia andropogonis, Tetraleurodes acaciae, Trialeurodes ricini, Trialeurodes lauri, Trialeurodes vaporariorum and Trialeurodes abutiloneus*. **(See INTRODUCTION section of reviewed manuscript, lines 168-172)**

The primer target sequences (for LAMP three primer sets recognizing eight different DNA sites) were chosen to maximize the number of differences to related species. In a first step, primers were tested for specificity *in silico* using the primer testing function implemented in the software Geneious version 10.0.9. In the Swiss import control procedure of plant products from non-European countries, *Trialeurodes vaporariorum*, the non-regulated greenhouse whitefly represents the insect species with the highest risk to be confused with *Bemisia tabaci* at points of entry. In a second specificity validation step, two specimens of *T. vaporariorum* were therefore tested using the *B. tabaci* LAMP assay giving correct-negative LAMP results. This information was added to the manuscript as follows: “The correct-negative results originated from two *Trialeurodes vaporariorum* specimens, a non-regulated species at high risk to be confused with *B. tabaci* at points of entry for plant products[8].” **(See REPRESENTATIVE RESULTS section of revised manuscript, lines 306-308)**

In order to address the comment of Reviewer#1 regarding the analytical sensitivity of the *B. tabaci* LAMP assay, we performed an additional experiment to assess the detection limit. Using three technical replicates, sample DNA was tested in a 1:10 dilution series and the minimal concentration needed for a DNA amplification was found to be 100 fg/µL. The additional data has been incorporated in the REPRESENTATIVE RESULTS section of the revised manuscript as follows: “When assessing the analytical sensitivity (detection limit), the *B. tabaci* LAMP assay successfully amplified sample DNA diluted to 100 fg/µL across three technical replicates (**Table 2**).” **(See revised REPRESENTATIVE RESULTS section of revised manuscript, lines 310-312 and Table 2)**

3. Line 159-160: the sum of 1.275 mL and 0.765 mL is 2.04 mL. Thus, 2 mL microcentrifuge tube is enough?
Response: We are thankful to Reviewer#1 for highlighting the point concerning the total volume of the *B. tabaci* LAMP Reaction Mastermix. We never faced a problem with the 2 mL tubes while preparing the LAMP kits for the validation of the method at the point of entry.

However, as correctly identified by Reviewer#1, the protocol in the manuscript can be used for approximately 88 reactions instead of 80 as declared in the manuscript.
In order to address the issue with the total volume, we adjusted the protocol as follows: “Add 1195.1 µL of ready-to-use GspSSD Lyse n’ Lamp Isothermal Master Mix (containing GspSSD polymerase, pyrophosphatase, magnesium sulfate, deoxynucleotides, double strand binding DNA binding dye) and 717.4 µL of *B. tabaci* LAMP primer mix to a 2 mL microcentrifuge tube.

Briefly vortex and pulse centrifuge" **(See PROTOCOL section of revised manuscript, lines 226-229)**

Thereby, the prepared Mastermix is calculated for 85 reactions (total volume *B. tabaci* LAMP Reaction Mastermix=1912.5 µL). However, to compensate for small losses during the dispensing step (GspSSD Lyse n' Lamp Master Mix is a slightly viscous liquid), we recommend to use the prepared Mastermix for 80 reactions.

4. Line 163: The B. tabaci LAMP reaction mastermix (2.04 mL) of 1.275 mL of Lamp Isothermal Master Mix and 0.765 mL of B. tabaci LAMP Primer Mix is for 80 reactions. Why dispense 22.5 µL, rather than 25.5 µL?
Response: During the validations, we always used a volume of 22.5 µL of the *B. tabaci* LAMP Reaction Mastermix per reaction. As discussed above, the discrepancy between reaction numbers and total volume was due to a mistake in the total Mastermix volume and not due to a wrong volume of Mastermix declared for each reaction.

5. Line 254: Sample one and two were not labelled in Figure 2A or Figure2B.
Response: In the FIGURE & TABLE LEGENDS section, the color code of Figure 2A and B is declared as follows: "Green line, sample 1; orange line, sample 2; blue line, negative amplification control (NAC); red line, positive amplification control (PAC)." **(See FIGURE & TABLE LEGENDS section of revised manuscript, lines 334-337)**

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Corrections to Review #2

Manuscript Summary
This work describes a LAMP protocol for the identification of Bemisia tabaci sensu stricto. It is well conceived, implemented, and written and appears quite successful. I am, however, no longer in a facility where I could test the protocol myself. The authors have validated on-site and specified concrete actions taken to improve the primers in the face of false negatives.
Response: We thank Reviewer #2 for the overall positive appraisal of our research.

Major Concerns

My major concern expands on my answer to "Is there sufficient introduction for the protocol? Is there an unbiased discussion of the protocol". The concern regards the author's notion of the low-tech point-of-entry laboratory. Such a low-tech laboratory at the Zurich airport evidently contains (or would purchase in order to implement this protocol): pipettes and associated disposables (lines 174,175), a thermomixer (line 178), a centrifuge (line 179), a vortex (line 178), LAMP kit associated consumables (lines 118-124,182), rtLAMP analysis device (lines 195-197). Are such sites in developed countries thus provisioned, generally speaking? I ask out of ignorance but also some skepticism that this is indeed the case. I recommend you report this information in the ms. If a site lacked this technology, what would be the cost to the laboratory for these items (minus the LAMP kit and DNA extraction solution if not yet commercially available). I recommend reporting this cost estimate information as well. the LAMP technology very nicely overcomes the need for thermocycling....at minimum is any of this instrumentation of comparable cost (or higher) to a thermocycler? LAMP may be 'lower tech', I'm just not quite convinced that it is 'low tech'. Just semantics? maybe, but I'm curious about the Editor's perspective on this.
Response: We are grateful to Reviewer#2 for highlighting the important issue concerning the use of the expression "low-tech". We go along with Reviewer#2 that the application of vortex,

centrifuge, thermoblock, and real-time LAMP device does not correspond to “low-tech” and rephrased the sentences as following: “Moreover the assay is designed to be applied on-site by plant health inspectors with minimal laboratory training directly at points of entry.” **(See LONG ABSTRACT section of revised manuscript, lines 110-111)**

Our aim was to point out, that the protocol needs only a few, easy to handle laboratory devices using limited space, which however should not imply that those devices have a low technology. The rationale behind our project was the development of a genetic identification protocol for *B. tabaci* with as few and easy handling steps as possible (to minimize performance time and risk for any contamination) while being as reliable as possible. To ensure this, we used equipment with the following prices: Vortex (450$), thermo mixer (~3800$), mini centrifuge (~160$), 1-10ul pipette (~400$), Genie II real-time LAMP device (18’000$). To our knowledge, such devices are available not only at the Airport Zurich, but for example also at the British Airport London-Heathrow. Of course, many plant protection organizations nowadays lack such equipment at points of entry because they did not perform on site LAMP analyses so far. However, with the increasing number of available tests for plant pathogens (e.g. *Erwinia amylovora*, Flavescence dorée, *Thrips palmi*, *Liriomyza* leaf miners, *Guignardia citricarpa*, *Dothistroma septosporum*), which all can be performed with the same equipment, the one-time investment is justifiable.

We are aware that there would be potential to reduce the price of the equipment of the protocol. For example, instead of using a heat block, the boiling step at 95°C for the DNA extraction can be performed in a simple water-bath or be performed directly in reaction tubes in the Genie II real time LAMP device. For the DNA transfer step, a diluting loop can be used instead of a pipette. The mixing step on the vortex can be replaced by flicking the tube with a finger. Many publications on LAMP reported also, that the isothermal LAMP reaction was successfully performed using a water bath, checking the read-out either by eye (turbidity, dye), using a turbidity meter, or on a lateral flow dipstick (see Zhang *et al.* 2010, Development of a Loop-Mediated Isothermal Amplification Assay for Rapid Detection of Subgroup J Avian Leukosis Virus; Njiru 2012, Loop-Mediated Isothermal Amplification Technology: Towards Point of Care Diagnostics). The costs for a real time LAMP device (18’000$) are still more expensive than the costs for a commercial PCR thermocycler. However, for the successful application of the technology the crucial factor is not the price, but rather the specificity of the LAMP method ensured by the usage of three primer pairs recognizing eight different sites on the target gene. Furthermore, the real time LAMP device has the possibility to generate a melting curve of the LAMP amplification product, which is used in our protocol as a “quality measure” for a correct positive result and helps therefore again to increase the specificity.

The point concerning the price of the equipment has been addressed as by adding the following paragraph to the DISCUSSION section: “The set-up costs for the current LAMP assay at a POE are approximately USD 25,000. With the increasing number of LAMP tests developed for plant pests (e.g. *Erwinia amylovora*, Flavescence dorée, *Guignardia citricarpa*), such a one-time investment appears justified[13, 15, 28]. However, the protocol could potentially be modified to reduce these costs even further. For example, for the DNA extraction step at 95 °C the thermo mixer used here could be replaced by a less expensive water bath, or by performing this step directly in the real time LAMP device. Furthermore, the mixing steps on the vortex could probably be replaced by manually flicking the tubes, and in the DNA transfer step the pipettor might be replaced by sterile inoculation loops.” **(See DISCUSSION section of revised manuscript, lines 393-400)**

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Corrections to Review #3

Manuscript Summary

After careful consideration, in the current format, the MS is not acceptable for publication after major revision. The suggestions as below.

1. Although the authors published the article "Blaser, S. et al. From laboratory to point of entry: development and implementation of a 366 loop-mediated isothermal amplification (LAMP)-based genetic identification system to 367 prevent introduction of quarantine insect species. Pest Manag. Sci. 74 (6), 1504-1512, 368 doi:10.1002/ps.4866 (2018).", howevr, It is recommended to refer to LAMP paper entitled "Hsieh, C.H., H.Y.Yang, Y.F.Chen, and C.C.Ko. 2012. Loop-mediated isothermal amplification for rapid detection of the globally invasive pest, Bemisia tabaci, biotypes B and Q and its application to insecticide selection. Pest Manage. Sci. 68: 1206-1213."

2. The main point is the real Bemisia tabaci is? It is highly recommended the requirements for providing the biotype level of economic importance.

Response: We thank Reviewer#3 for having reviewed our manuscript. We agree with Reviewer#3, that the differentiation between different *B. tabaci* biotypes is crucial for taking specific pest management actions in infested areas, especially considering the fact that they may respond in a different way to the application of pesticides.

However, the *B. tabaci* LAMP assay presented in this manuscript was developed for its specific use in the phytosanitary import control for European countries and Switzerland. As described in the INTRODUCTION section (**see lines 131-133**), in the European Union (EU), *B. tabaci* is listed in the Plant Health Directive 2000/29/EC Annex 1AI as a quarantine organism, for which introduction from non-EU countries and the dispersal within the EU countries are banned (https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:32000L0029). This regulation does not distinguish between different *B. tabaci* biotypes. Hence, there is a strong need for fast identification of *B. tabaci* specimens, independent on their biotype status. The procedure for the on-site identification of quarantine organisms at points of entry such as sea- and airports has to be fast. The responsible plant health inspectors are therefore dependent on fast diagnostic results in order to ensure the flow of commodities and to assure that the perishable goods such as fresh fruits and vegetables reach the consumers in time. Running multiple LAMP test for different *B. tabaci* biotypes at points of entry would therefore only delay the import process and would have no further impact on the final decision.

Thanks to Reviewer#3's comment regarding the publication of the LAMP assay for *B. tabaci* biotype B and Q, we realized that we indeed referenced only the real-time RT PCR assay and the antibody-based diagnostic method to identify individual biotypes in the initial manuscript. Therefore, the citation of the publication "Loop-mediated isothermal amplification for rapid detection of the globally invasive pest, *Bemisia tabaci*, biotypes B and Q and its application to insecticide selection" has been added to the revised manuscript. **(See INTRODUCTION section of revised manuscript, lines 157-159)**

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Corrections to Review #4

Manuscript Summary

The manuscript describes a LAMP-based detection method to identify *B tabaci* a worldwide invasive pest affecting agriculture. The protocol is well described and allow reproducibility. The results are consistent with high specificity and sensitivity.

Response: We are grateful to Reviever#4 for reviewing our manuscript and for the positive Manuscript Summary.

Major Concerns

The composition of the master mix is not given (OptiGene Ltd).

Response: This point has been addressed by modifying section 1.3.3.1. of the protocol as follows: "1.3.3.1. Add 1.275 mL of ready-to-use GspSSD Lyse n' Lamp Isothermal Master Mix (containing GspSSD polymerase, pyrophosphatase, magnesium sulfate, deoxynucleotides, double strand binding DNA binding dye) and 0.765 mL of *B. tabaci* LAMP primer mix to a 2 mL microcentrifuge tube. Briefly vortex and pulse centrifuge." **(See revised PROTOCOL section of revised manuscript, lines 226-229)**

Minor Concerns

There is no step called 1.3.2.1

Response: This point has been addressed by changing "(prepared in step 1.3.2.1)" to "(prepared in step 1.3.3.1)". **(See revised PROTOCOL section of revised manuscript, lines 231-232)**

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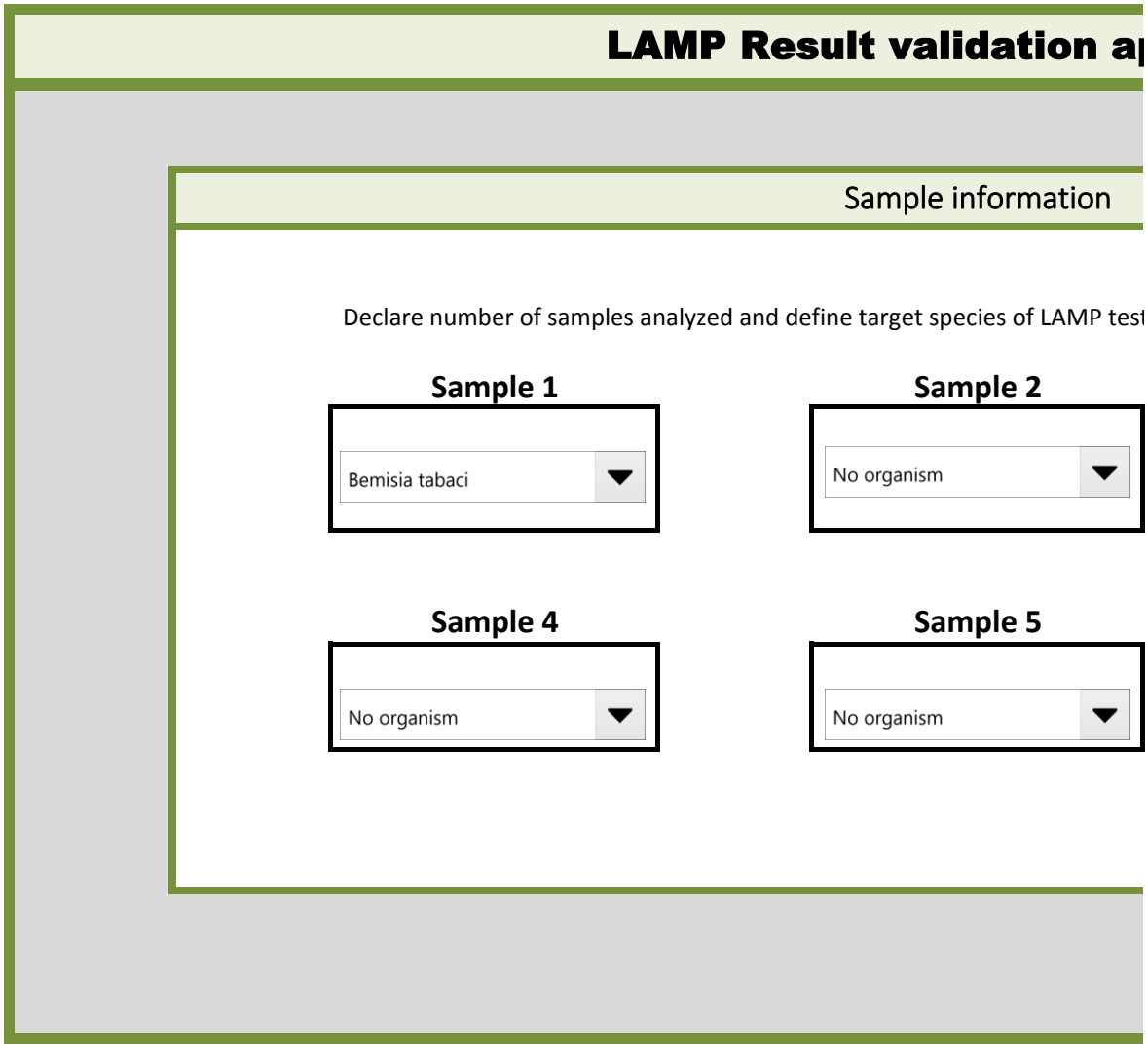

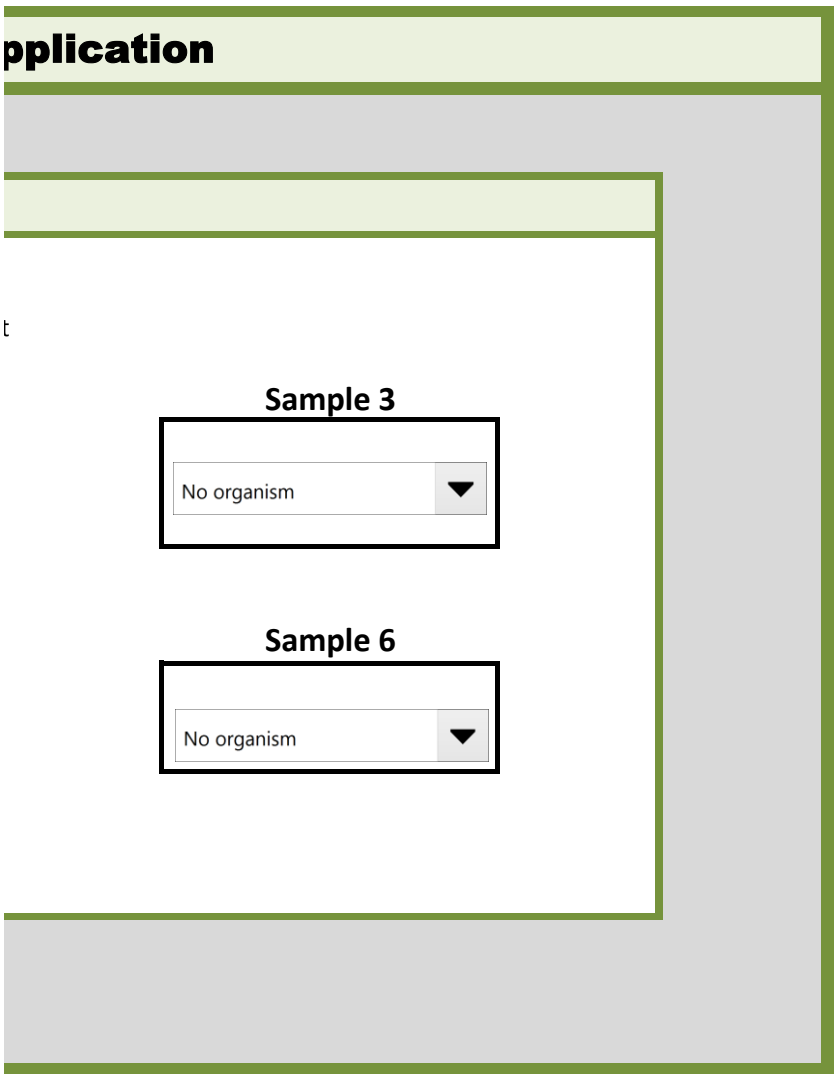
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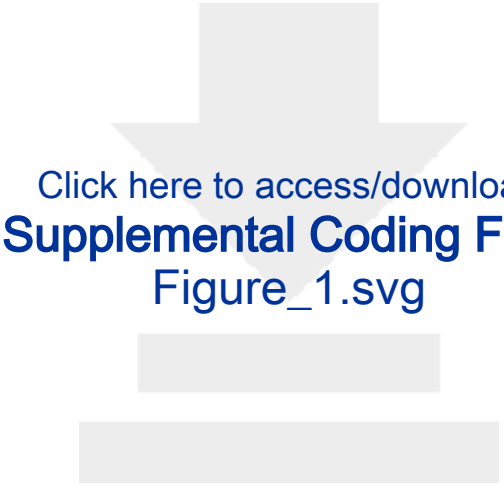
Sample 3
No organism
Sample 6
No organism

Figure_1



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Supplemental Coding Files

Figure_1.svg

Figure_2

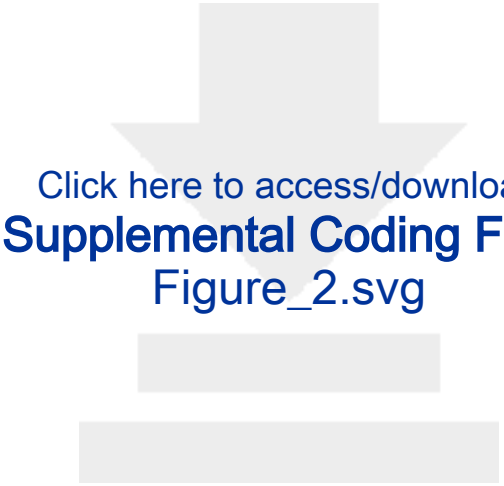

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Figure_2.svg

Figure_3

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