**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)

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**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 designed8. 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 complex8, 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)**

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**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 designed8. 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 complex8, 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 primers8. 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 gene8. 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 organisms19, 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 BOLD21, 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#1that 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 products8.” **(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 justified13, 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)**