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Manual of Standard Operating Procedures for malaria LAMP

DNA extraction methods

Amplification and detection of results

Version one

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1. ABBREVIATIONS

LAMP	Loop-mediated isothermal DNA amplification
DNA	Deoxyribonucleic acid (genetic material)
PCR	Polymerase chain reaction

2. CONTACT PERSONS

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3. BACKGROUND

3.1. Introduction

Loop-mediated isothermal amplification (LAMP) of DNA is a novel molecular technology platform developed by laboratory scientists at Eiken Chemical Company of Japan (http://www.eiken.co.jp/en). The LAMP technology amplifies previously determined genes and can be used to detect any pathogen. LAMP may be considered as an alternative to polymerase chain reaction (PCR) for the detection of nucleic-acid sequences (DNA and/or RNA). Both methods amplify and detect DNA, but unlike traditional PCR, LAMP does not require a thermocycler or gel imaging system. Results can be recorded by real-time turbidimetry or through visual detection of fluorescence. Amplification and detection of the target nucleic-acid sequence is essentially completed in a single step, by incubating the mixture of sample, primers, DNA polymerase with strand displacement activity and substrates at a constant temperature. LAMP provides high efficiency, with DNA being amplified 10^9-10^{10} times in 15 - 60 minutes. Therefore LAMP can provide a result faster than traditional PCR, and can be performed in basic laboratories without the need for specialized infrastructure.

Like many other living organisms, malaria parasites store and reproduce their genetic information with DNA molecules. LAMP detects this DNA. If a person is infected with malaria parasites, the parasite DNA can be detected in the blood during the blood stage of the parasite lifecycle. Normally, the DNA is present in very small amounts. Therefore, it is necessary to amplify or increase the amount of DNA in order to detect and identify it.

3.2. LAMP technology

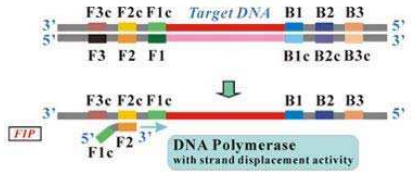
The LAMP technology is characterized by: i) the use of a single polymerase enzyme to catalyse DNA amplification under isothermal conditions; ii) very high specificity that results from the use of six primers recognizing eight distinct regions on the target DNA; and iii) high amplification efficiency capable of producing high concentrations of amplified product in a short time, allowing for visual or automated detection of results. Another advantage of the LAMP reaction is its robustness and tolerability to common PCR inhibitors. This allows the use of a simplified sample preparation that just requires boiling and centrifugation, or use of a 'PURE' device that rapidly removes impurities from the DNA sample. It is not necessary to understand the details of LAMP in order to perform it correctly. However, the basic principles are reviewed here for interest (Figure 1).

The LAMP reaction begins with short single stranded molecules called oligonucleotides or primers. These are designed to bind to the target DNA sequence – in our case, regions of the mitochondrial genome of malaria parasite. If parasite DNA is present, one of the specially designed LAMP primers can anneal (bind or stick) to the complementary or matching DNA from the parasite. This can occur because DNA is in dynamic equilibrium (constantly unfolding and refolding) when it reaches the reaction temperature. Primer binding initiates the process of DNA synthesis, whereby the *Bst* DNA polymerase enzyme generates new DNA that matches the parasite DNA. As the DNA synthesis continues, some of the new DNA folds back on itself to form a "stem-loop" structure that looks like a dumbbell. This structure is the starting point for the amplification cycle of LAMP. The loops on the dumbbell structure now act as additional primers for on-going DNA synthesis. As more loops are created, there are more starting points for DNA synthesis as the LAMP reaction continues. The creation of multiple loops of DNA, which provide a starting point for additional DNA synthesis, is one reason LAMP is much faster than traditional PCR.

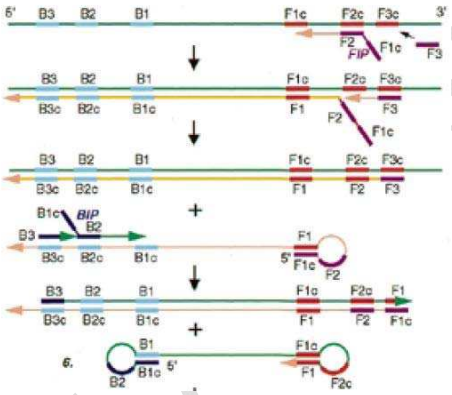
The automated detection of amplified products is based on turbidimetric measurement of magnesium pyrophosphate (a white precipitate produced as a by-product of DNA amplification). The visual detection under ultraviolet light is based on the presence of calcein. Before DNA amplification, calcein contained in the reagent is in its quenched state as it is bound to manganese ions. Upon the start of DNA amplification, pyrophosphate ions bind to manganese ions and calcein is released producing fluorescence. If we see fluorescence, the LAMP result is positive; if there is no fluorescence, the result is negative. More details about the LAMP reaction can be found on the website of Eiken Chemical Co., Ltd. (Japan):
http://loopamp.eiken.co.jp/e/lamp/index.html.

Figure 1: Loop-mediated isothermal amplification (LAMP) reaction.

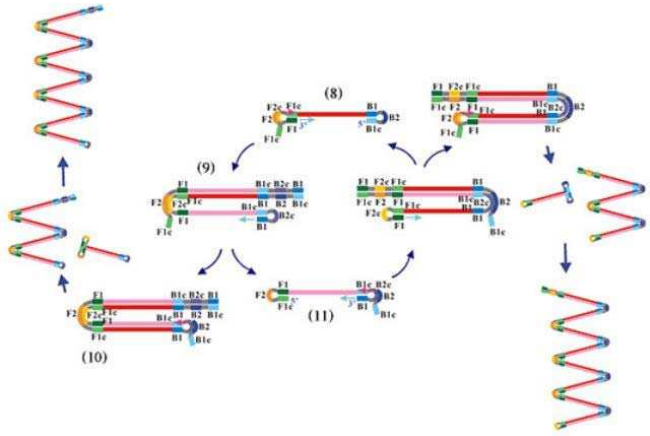
Annealing of LAMP primers and strand displacement



DNA synthesis and loop formation



Amplification products



3.3. LAMP kit for malaria diagnosis

Proof of principle for LAMP detection of malaria parasites was published by Poon, et al, in 2006. FIND has been working with Eiken Chemical Co. (Japan) and the Hospital for Tropical Diseases in London (UK) on the development of an optimized LAMP malaria diagnostic assay for the detection of *Plasmodium* DNA extracted from human blood. Two primer sets provided with the kit have been designed to detect the mitochondrial DNA of *Plasmodium* parasites. The Pan (genus) specific primers detect a target DNA sequence well conserved in all *Plasmodium* species and are able to detect a wide range of *Plasmodium* species including the four most common ones that cause human malaria (*P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*). The *P. falciparum* (Pf) specific primers have been confirmed by *in silico* alignment analysis and *in vitro* experimentation to be specific for *P. falciparum* parasites. The limit of detection of these primers is 5.0 DNA copies and 7.5 DNA copies per test for the Pan and Pf assays respectively. It has been demonstrated experimentally that both assays are able to detect parasitemias as low as 1 parasite/ul of blood in 40 minutes amplification time.

Prototypes of this malaria LAMP assay have been compared to nested PCR with 705 patient samples (56 positives by microscopy) collected at the HTD in London. Sensitivity and specificity of the Pf LAMP were 98.4% and 98.1% respectively. Sensitivity and specificity of the Pan LAMP were 97.0% and 99.2% respectively. The malaria LAMP kit was similar in sensitivity and specificity to nested PCR and was superior to microscopy in detecting infected samples. Similar results were obtained with samples collected in an endemic area on Uganda. The study in Uganda also demonstrated that LAMP can be applied in a simple laboratory space in a clinic setting by technicians without previous molecular diagnostic training, after a short training period. As a novel molecular diagnostic technique, LAMP promises to bring the sensitivity of PCR to field settings, without the need for sophisticated laboratory infrastructure, with automated or visual reading of results. Both studies have demonstrated that LAMP is equivalent to PCR in sensitivity and specificity with faster time-to-results.

This malaria LAMP kit is designed to address the need for a molecular test that achieves higher sensitivity and specificity than microscopy and rapid diagnostic tests (RDTs), but requires fewer infrastructure than PCR and can be adapted to field settings. Therefore, in addition to replacing microscopy in situations such as screening of returned travellers in non-endemic countries, the assay has potential as a reference standard in low-resource settings; and as a tool for surveillance and screening when a highly sensitive assay is needed.

4. PURPOSE

These Standard Operating Procedures describe the materials, equipment, and procedures required to correctly and safely use the LAMP kit to diagnose malaria using blood samples. This manual describes protocols to:

- Process blood samples for DNA extraction.
- Use the LAMP kit for malaria diagnosis.

5. SCOPE

These SOPs have been developed for the training of laboratory personnel using the LAMP kit for malaria diagnosis in clinical and research settings.

6. PERSONNEL QUALIFICATIONS

The LAMP kit for malaria diagnosis can be used by laboratory technicians without any previous training in molecular methods. Knowledge and skills provided in this manual can be acquired in a short training period of less than 3 days. However, strict adherence to the procedures below is necessary to achieve reliable results.

7. SAFETY CONSIDERATIONS

- Blood samples pose a potential risk of infection. Use universal precautions to minimize biohazard.
- Some of the components of the kit are toxic. Avoid any contact with eyes, mouth or skin. In case of accidental contact with any reagent, immediate rinse the affected area with running water and seek medical advice.
- Be careful when removing tubes from the incubators or water bath to avoid burns.
- When a UV lamp is used for visual fluorescence judgment, do not stare directly at the UV light. Since UV light is harmful to the eyes, even watching for a short period may irritate eyes and cause symptoms similar to conjunctivitis. Use a glass screen or wear protective goggles/glasses/face shield whenever looking directly toward the UV lamp.

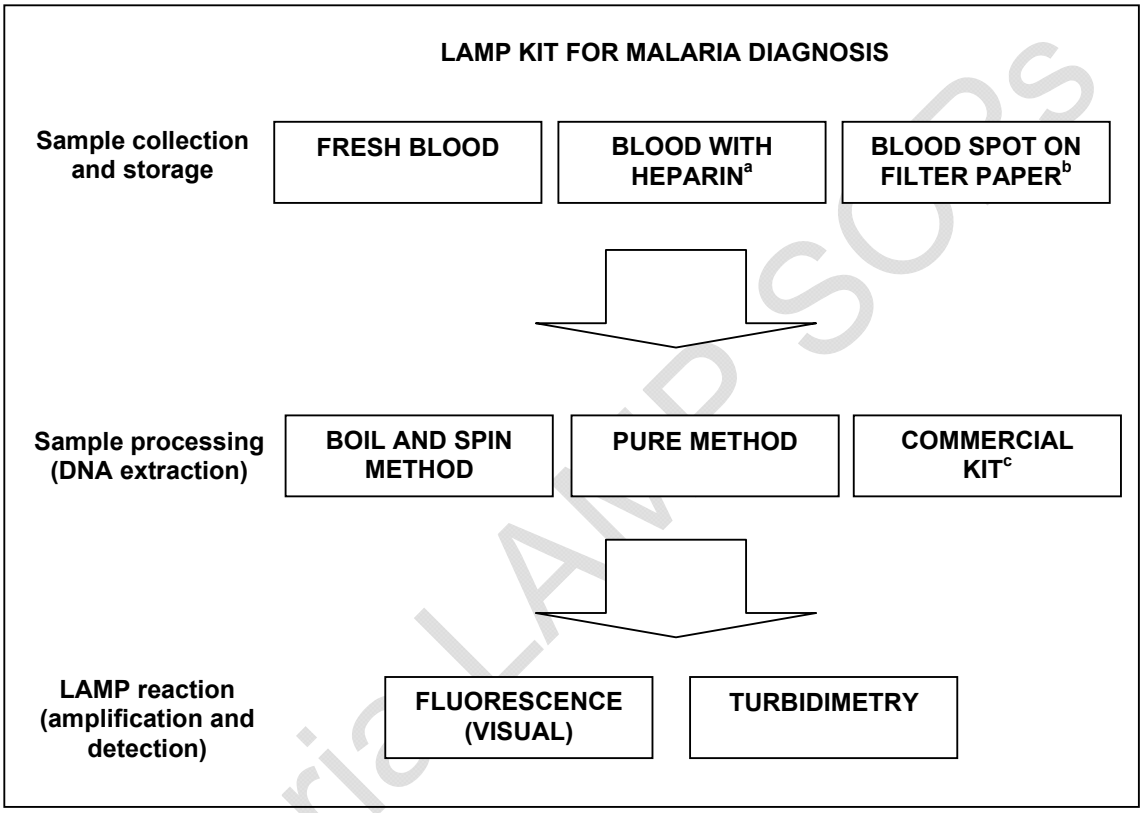
8. CAUTIONS

- Ensure that the required equipment is available and read the instruction manuals before commencing the procedures.
- The performance of the LAMP kit for malaria diagnosis is dependent on operator proficiency and adherence to these SOPs. Testing should be performed by properly trained personnel strictly according to the instructions provided.
- The LAMP kit for malaria has been developed for *in vitro* diagnostic use only and for the detection of *Plasmodium* parasites in blood samples of human origin. Do not use it for other purposes.
- The use of heparin as anticoagulant is essential if reading results by fluorescence. EDTA may produce false positive fluorescence results. Both heparin and EDTA and other anticoagulants can be used when reading results by turbidimetry.
- Similarly, avoid using any buffer containing EDTA for DNA elution to avoid interferences with fluorescence. The use of nuclease-free water is recommended.
- The LAMP reaction is very sensitive and contamination with small amounts of DNA or amplified product might lead to false positive results. Therefore, separate the sample preparation and amplification areas. Collect blood samples in a separate room from the LAMP amplification room. Avoid producing aerosols during sample preparation, as these may contain *Plasmodium* DNA and cause contamination.
- Exposure to heat, humidity, and light may cause deterioration of the LAMP kit for malaria. Remove only the required number of Reaction tubes and re-seal the aluminium pouch immediately.
- Do not remove the desiccant from the aluminium pouch.
- Do not touch the inside of the cap of the Reaction tubes. The cap of each Reaction tube contains reagents in dried form.
- Before using the Reaction tubes, check carefully to see if they have cracks or scratches. Damaged tubes might give false results and lead to DNA contamination of the incubator and work area.
- When handling this product, avoid microbial contamination and nuclease contamination. A small amount of contamination of the Reaction tubes from sweat or saliva may decompose DNA and cause a false result.
- Use the Positive Control (PC) only as described in this SOPs in order to avoid DNA contamination.
- Store the Positive Control (PC) and patient samples separately from other kit reagents.
- Reagent in the Reaction tubes should be fully dissolved. Any un-dissolved portion may influence performance, such as causing a decrease in sensitivity.

- Since bubbles in Reaction tubes may interfere with amplification and measurement, avoid forming any bubble when mixing reagents and sample solution. If bubbles occur, spin or flick down the Reaction tube.
- Do not expose Reaction tubes to UV light before the end of the LAMP reaction. Prolonged exposure to UV light might damage the tubes and lead to false results.
- Never open the Reaction tubes once the LAMP reaction has started or after completion. Be particularly careful when unloading the Reaction tubes from the incubator to avoid opening the tubes accidentally.
- Do not reuse any amplified product in the tubes for electrophoresis or other applications.
- Do not freeze the reagents.
- Do not use any expired reagents.
- Do not mix reagents from different lots.

9. FLOWCHART FOR USE OF SOPs FOR LAMP MALARIA

The LAMP malaria kit can be used with fresh blood or blood stored frozen or dried on filter paper and with DNA extracted with several available methods. The following SOPs describe the methods recommended and previously tested with clinical samples in field studies.



[a] EDTA and other anticoagulants can be used only if results are read by turbidimetry.

[b] The use of blood spots for LAMP has been demonstrated in the laboratory but not in field studies.

[c] The use of DNA extracted by available commercial kits has been demonstrated in the laboratory but not in field studies. Other DNA extraction methods could produce unreliable results and users should previous validate them to ensure the accuracy of results.

This document presents two different SOPs:

1. SOPs for LAMP malaria assay with DNA extracted by the “Boil and Spin” method.
2. SOPs for LAMP malaria assay with DNA extracted by the PURE method.

10. SOPs 1.1: LAMP MALARIA ASSAY WITH DNA EXTRACTED BY THE BOIL AND SPIN METHOD

The LAMP kit for malaria diagnosis can be used with DNA extracted from blood by different methods. These SOPs present the use of the 'Boil and Spin' method which has been evaluated with patient whole blood samples.

10.1. Sample collection and storage

- These SOPs apply to fresh blood samples collected by finger prick and blood samples collected in heparin.
- Finger-prick blood should be used immediately.
- Blood in heparin can be stored for up to two weeks at 4°C.
- These SOPs are **NOT** recommended for blood dried on filter paper (please refer to SOPs 1.2).
- For the Boil and Spin method, **60 µl** of whole blood are required.

10.2. DNA extraction by the Boil and Spin method

Equipment: Hot-block or water bath at 95°C (temperature accuracy ±0.5°C)
Vortex (optional)
Micro-centrifuge (10,000 g)
Timer

Materials: Marking pen
Disposable examination gloves (powder free)
0.5% sodium hypochlorite
10-µl to 100-µl micropipette with sterile disposable tips
Extraction Tube: 1.5-ml eppendorf tube with 60µl extraction buffer (400 mM NaCl, 40 mM Tris pH 6.5, 0.4% SDS)
Dilution Tube: 1.5-ml eppendorf tube with 345 µl sterile water

Procedures:

- Turn on the hot-block or water bath and verify that the temperature has reached **95°C**.
- Work with double pair of disposable examination gloves (discard outer ones when finishing Sample Processing before starting the LAMP amplification steps).

- Work in a sample preparation area separated from the amplification area.
- Clean the bench with 0.5% sodium hypochlorite.
- Place on this clean surface all required materials.
- Write the patient name or code on the cap of the Extraction and Dilution tubes.
- Transfer **60 µl** of whole blood to the Extraction Tube using a 60-µl blood transfer device for a finger prick or a micropipette for blood in heparin. Repeat this step for every sample being sure of using a single Extraction Tube per sample.
- Mix the sample and the extraction buffer by vortex for 10 seconds or pipetting 10 times.
- Place the Extraction Tube with the sample in the hot-block or water bath at **95°C** for **5** minutes.
- Remove the tube from the incubator or water bath immediately after the timer sounds. Over-heating may degrade DNA which lowers the sensitivity of the test.
- Centrifuge at **10,000 g** for 3 minutes.
- Transfer **30 µl** of clear supernatant to the Dilution Tube (the precipitated haemoglobin makes a browny-red pellet at the bottom of the tube). Mix by pipetting 10 times to dilute the DNA sample (S).
- Repeat these steps for every sample being sure of using a single Extraction Tube and a single Dilution Tube per sample. If not used immediately, the DNA sample (S) can be stored at -20°C.

10.3. LAMP amplification

Equipment: Incubator or hot-block at 65°C with hot bonnet over 70°C and below 90°C (temperature accuracy ±0.5°C)
UV lamp (wavelength = 240 nm to 260 nm and 350 nm to 370 nm)
Timer
OR
Loopamp LF-160 Homeothermal Equipment with UV lamp (incubator) (REF: MVKM17 - Eiken Chemical Co.)
OR
LA-500 Real Time turbidimeter (REF: MVL300 - Eiken Chemical Co.)

Materials: Marking pen
Disposable examination gloves (powder free)
0.5% sodium hypochlorite
10-µl to 100-µl micropipette with sterile disposable tips

Loopamp™ MALARIA Pan/Pf detection kit (REF: LMC562 - Eiken Chemical Co.):

Malaria Pan reaction tubes with detection reagents and/or

Malaria Pf reaction tubes with detection reagents

Pan/Pf Positive Control (PC)

Pan/Pf Negative Control (NC)

Zip-lock plastic bag (for waste disposal)

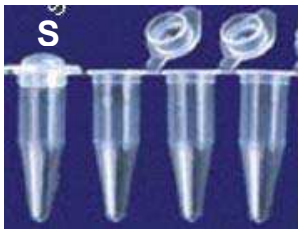
Protective goggles/glasses/face shield if using UV lamp or Loopamp LF-160

Procedures:

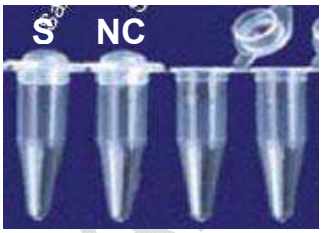
- Read the package insert of the Loopamp™ MALARIA Pan/Pf detection kit carefully.
- Read the LF-160 or LA-500 instruction manuals carefully before operating these devices.
- Turn on the incubator or hot-block **or** the Loopamp LF-160 **or** the LA-500 turbidimeter and verify that the temperature has reached **65°C**. Allow the LA-500 turbidimeter to warm up for 20 minutes before use (set the "MALARIA" programme in the respective amplification unit).
- Work in an amplification area separated from the sample preparation area.
- Clean the bench with 0.5% sodium hypochlorite.
- Discard outer gloves leaving a single glove in each hand.
- Place on this clean surface all required materials.
- Remove the required number of Reaction tubes from the aluminium pouch and put them in an appropriate rack (one reaction tube per sample, plus one for Positive Control (PC) and one for Negative Control (NC)). Pan and Pf reaction tubes can be run simultaneously or separately.

- After removing the necessary Reaction tubes, seal immediately (tightly closed) the aluminium pouch with unused tubes.

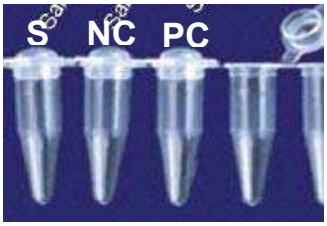
- Flick or spin down the tubes with the Positive (PC) and Negative (NC) controls before use to collect the content in the bottom of the tube.
- Write the patient name or code on the respective Reaction tube.
- Dispense **30 µl** of diluted DNA sample (sample=S) into the Reaction tube and close the cap. Repeat this step for all samples to be amplified in the same run.



- Dispense **30 µl** of Negative Control (NC) into a Reaction tube and close the cap.

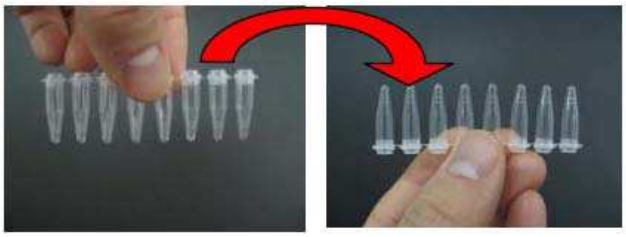


- Dispense **30 µl** of Positive Control (PC) into a Reaction tube and close the cap. Always dispense the Positive Control (PC) into the last tube as a final step. Dispensing earlier may cause contamination.



- Close the Positive Control (PC) tube immediately after dispensing the required volume in the Reaction tube.

- Pick up the Reaction tubes from the rack and turn the tubes upside down. Shake firmly so that the DNA solution moves into the inside of the tube cap.



- Place the Reaction tubes cap-side down on the bench for **2 minutes**. This allows the DNA solution to dissolve the dried LAMP reagents inside the tube cap.

- Invert (turn upside down) the reaction tubes **5 times**. Shake the tubes with each inversion to ensure proper mixing and dissolving of the LAMP reagents.

- Finally, shake the reaction tubes downwards to collect the solution into the bottom of the tubes (or pulse spin in a micro-centrifuge for a few seconds).

- Immediately place the reaction tubes into the incubator or hot-block **or** the Loopamp LF-160 **or** the LA-500 turbidimeter (at **65°C**) and close the bonnet.

Loopamp LF-160

OR

LA-500 turbidimeter

For incubator or hot-block:

- Start amplification reaction at **65°C** and set timer for **40 minutes**.
- At the end of amplification reaction, heat the tubes at **80°C for 5 minutes or 95°C for 2 minutes**, to terminate the reaction (enzyme inactivation).

For Loopamp LF-160:

- Press the green button to start the LAMP reaction (previously set at 65°C for 40 minutes).
- Confirm the completion of enzyme inactivation (automatically completed).

For LA-500 Real Time turbidimeter:

- Check that the temperature displayed is 65°C.
- Load the Reaction tubes and start measurement.
- Verify in the display the increase of turbidity for the Positive Control (PC).
- Confirm the completion of enzyme inactivation (automatically completed).

10.4. Detection and interpretation of results

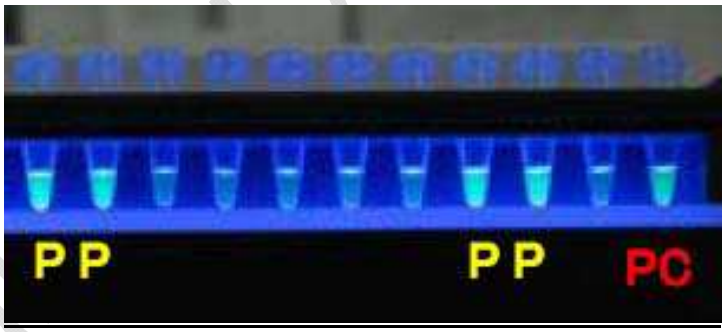
For UV lamp:

- Irradiate the bottom of each Reaction tube and observe from the side through goggles or other UV-protection eye shielding.
- For valid run the following results must be obtained:
    - Positive Control: Green light is emitted.
    - Negative Control: No light is emitted.

- If any control is invalid, all samples in the run should be reported as invalid and the test should be repeated.
- After confirming that the run is valid, evaluate samples as follows:
    - Positive Sample: Green light is emitted.
    - Negative Sample: No light is emitted.

For Loopamp LF-160:

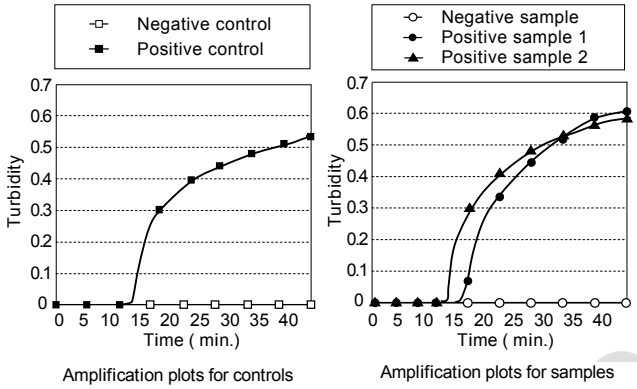
- Place the reaction tubes into the Fluorescence Visual Check Unit, turn on the lamp and observe the tubes from the side through goggles or other UV-protection eye shielding.
- For valid run the following results must be obtained:
    - Positive Control: Green light is emitted.
    - Negative Control: No light is emitted.
- If any control is invalid, all samples in the run should be reported as invalid and the test should be repeated.
- After confirming that the run is valid, evaluate samples as follows:
    - Positive Sample: Green light is emitted.
    - Negative Sample: No light is emitted.



For LA-500 Real Time turbidimeter:

- Watch the display of the turbidimeter to check the Positive and Negative controls for any increase in turbidity.
- If the turbidity increases in the Positive Control but does not in the Negative Control, amplification reaction is proceeding properly. If that is not the case, amplification reaction may be proceeding in a wrong way. In such a case, restart testing from transfer of DNA solution to reaction tubes.

- After confirming that controls are properly working, evaluate samples in accordance with the following criteria:
    - Positive Sample: Some increase is observed in turbidity.
    - Negative Sample: No increase is observed in turbidity.



10.5. Waste disposal

- **DO NOT** open the tubes after DNA amplification. Reaction tubes can contain very high concentrations of DNA after amplification. Opening the tube results in a very high risk of DNA contamination to work areas, posing a risk of subsequent false-positive LAMP and PCR results.Leave the cap closed and place the used Reaction tubes into a zip-lock plastic bag before discarding them as medical waste for incineration. Do not autoclave any used Reaction tubes.
- Dispose of any other reagent, container, or lab ware in accordance with local regulations.

10.6. CHANGE HISTORY

New version #/date	Old version #/date	No. of changes	Description of changes	Source of change request
1.0/AUG12				

11. SOPs 1.2: LAMP MALARIA ASSAY WITH DNA EXTRACTED BY THE PURE METHOD

The LAMP kit for malaria diagnosis can be used with DNA extracted from blood by different methods. These SOPs present the use of the PURE method which has been evaluated with patient whole blood samples. The **PURE device** is a specially designed series of interlocking plastic components which provide a closed system for sample processing and direct dispensing of extracted DNA to Reaction tubes.

11.1. Sample collection and storage

- These SOPs apply to fresh blood samples collected by finger prick, blood samples collected in heparin tubes, and whole blood dried on filter paper.
- Finger-prick blood should be used immediately.
- Blood in heparin can be stored for up to two weeks at 4°C.
- For the PURE method, **30 µl** of whole blood are required.

For whole blood dried on filter paper, it is recommended to:

- Use Whatman 3mm or printed filter mat A – Wallac (standard FTA cards DO NOT perform well in the LAMP assay and are not recommended).
- Let the blood spot air-dry for minimum 2 hours prior to storage in zip-lock plastic bags with desiccant at room temperature in a dry place.
- Cut a **6 mm punch** from the centre of the dried blood spot directly over the Heating Tube. Remember to clean the punch in 70% ethanol and then punch a clean filter paper three times before cutting a new sample to avoid cross-contamination.

11.2. DNA extraction by PURE method

Equipment: Water bath at 75°C (temperature accuracy ±0.5°C)
Timer
OR
Loopamp LF-160 Homeothermal Equipment with UV lamp (incubator) (REF: MVKM17 - Eiken Chemical Co.)

Materials: Marking pen
Disposable examination gloves (powder free)
0.5% sodium hypochlorite

10-µl to 100-µl micropipette with sterile disposable tips

Loopamp™ PURE DNA Extraction Kit (REF: LMC802):

Heating Tube with extraction buffer

334 mM NaCl solution

Absorbent Tube

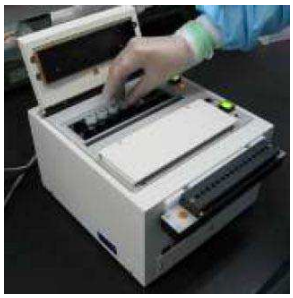
Injection Cap

Procedures:

- Read the package insert of the PURE kit carefully.
- Read the LF-160 instruction manual carefully before operating this device.
- Turn on the incubator or water bath **or** the Loopamp LF-160 and verify that the temperature has reached **75°C** (in the Heating block of the Loopamp LF-160).
- Work with double pair of disposable examination gloves (discard outer ones when finishing Sample Processing and starting the LAMP malaria assay).
- Work in a sample preparation area separated from the amplification area.
- Clean the bench with 0.5% sodium hypochlorite.
- Place on this clean surface all required materials.
- Write the patient name or code on the cap of the Heating tube.
- Transfer **30 µl** of whole blood **or** a **6-mm blood spot punch** to the Heating Tube using a 30-µl blood transfer device for a finger prick, a micropipette for blood in heparin, or a 6-mm punch for blood on filter paper. Repeat this step for every sample being sure of using a single Heating Tube per sample.
- Transfer **30µl** of the 334 mM NaCl solution to the Heating tube.

- Mix by inverting the tube **3 times**.

- Place the tube in the hot-block or water bath at **75°C for 5 minutes or** in the Heating Block of the Loopamp LF-160 (automatically completed).



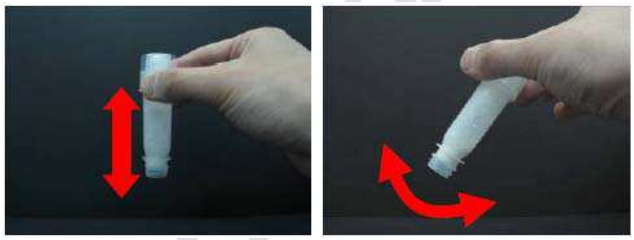
- Remove the tube immediately after the timer sounds. Over-heating may degrade DNA which lowers the sensitivity of the test.
- Let the tubes cool for **2 minutes**.

- Hook one finger into the cap-ring of the Absorbent Tube and pull the cap out slowly while twisting it slightly. Keep the cap-ring for later use.

- Mix again the Heating Tube with sample by inverting the tube **3 times**.

- Screw the Heating Tube firmly into the Absorbent Tube, using the opening that resulted when you removed the cap-ring. Notice that the sample drains from the Heating Tube onto the powder in the Absorbent Tube.

- Using a flicking or swinging motion of your wrist to shake the tube vertically 10 times, and then swing it horizontally 10 times to mix the sample with the absorbent powder (ensure complete mixing by repeating the shaking motions until there is no white powder visible clinging to the inside of the tube).



- Place the tube horizontally over a clean surface while processing additional samples.
- Repeat these steps for every sample being sure of using a single Absorbent tube per sample. DNA extracted from the PURE device should be transferred to the reaction tubes immediately.

11.3. LAMP amplification

Equipment: Incubator or hot-block at 65°C with hot bonnet over 70°C and below 90°C (temperature accuracy ±0.5°C)
UV lamp (wavelength = 240 nm to 260 nm and 350 nm to 370 nm)
Timer
OR
Loopamp LF-160 Homeothermal Equipment with UV lamp (incubator) (REF: MVKM17 - Eiken Chemical Co.)
OR
LA-500 Real Time turbidimeter (REF: MVL300 - Eiken Chemical Co.)

Materials: Marking pen
Disposable examination gloves (powder free)
0.5% sodium hypochlorite
10-µl to 100-µl micropipette with sterile disposable tips
Loopamp™ MALARIA Pan/Pf detection kit (REF: LMC562 - Eiken Chemical Co.):
Malaria Pan reaction tubes with detection reagents and/or
Malaria Pf reaction tubes with detection reagents
Pan/Pf Positive Control (PC)
Pan/Pf Negative Control (NC)
Zip-lock plastic bag (for waste disposal)
Protective goggles/glasses/face shield if using UV lamp or Loopamp LF-160

Procedures:

- Read the package insert of the Loopamp™ MALARIA Pan/Pf detection kit carefully.
- Read the LF-160 or LA-500 instruction manuals carefully before operating these devices.
- Turn on the incubator or hot-block **or** the Loopamp LF-160 **or** the LA-500 turbidimeter and verify that it has reached **65°C**. Allow the LA-500 turbidimeter to warm up for 20 minutes before use (set the "MALARIA" programme in the respective amplification unit).
- Work in an amplification area separated from the sample preparation area.
- Clean the bench with 0.5% sodium hypochlorite.
- Discard outer gloves leaving a single glove in each hand.
- Place on this clean surface all required materials.
- Remove the required number of Reaction tubes from the aluminium pouch and put them in an appropriate rack (one reaction tube per sample, plus one for Positive Control (PC) and one for Negative Control (NC)). Pan and Pf reaction tubes can be run simultaneously or separately.

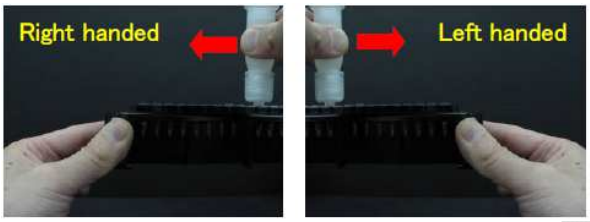
- After removing the necessary Reaction tubes, seal immediately (tightly closed) the aluminium pouch with unused tubes.
- Flick or spin down the tubes with the Positive (PC) and Negative (NC) controls before use to collect the content in the bottom of the tube.
- Write the patient name or code on the respective Reaction tube.
- Attach one cap-ring (previously removed from Absorbent tube) to the nozzle of one injection cap.

- Line up the wing of the injection cap with the wing of an inverted Absorbent Tube with sample. Push the injection cap onto the Absorbent Tube until hearing a click and then screw the cap until tight.

- Keeping the PURE device inverted, remove the cap-ring from the nozzle of the injection cap.

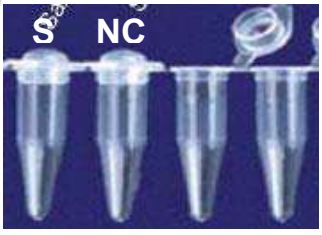
- Turn the PURE device over and hold it with the injection cap facing down.

- Using a swinging motion of your twist, shake/swing the mixture of sample and powder down into the injection cap.
- Hold the rack with the Reaction tubes up to your eye level and insert the nozzle of the PURE device fully into the Reaction tube.

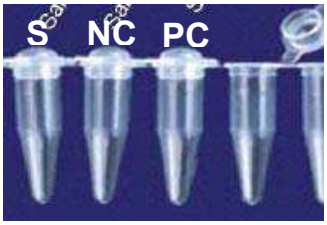


- Squeeze the PURE device to drip the DNA sample (S) into the Reaction tube.
- Fill in the Reaction tube up to the middle of the two lines on the Reaction tubes to ensure **30 µl** of volume and close the cap. For accurate test results it is important not to over-fill or under-fill the Reaction tube. Repeat this step for all samples to be amplified in the same run.

- Dispense **30 µl** of Negative Control (NC) into a Reaction tube and close the cap.

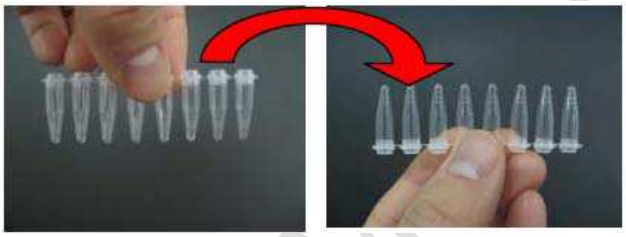


- Dispense **30 µl** of Positive Control (PC) into a Reaction tube and close the cap. Always dispense the Positive Control (PC) into the last tube as a final step. Dispensing earlier may cause contamination.



- Close the Positive Control (PC) tube immediately after dispensing the required volume in the Reaction tube.

- Pick up the Reaction tubes from the rack and turn the tubes upside down. Shake firmly so that the DNA solution moves into the inside of the tube cap.



- Place the Reaction tubes cap-side down on the bench for **2 minutes**. This allows the DNA solution to dissolve the dried LAMP reagents inside the tube cap.

- Invert (turn upside down) the reaction tubes **5 times**. Shake the tubes with each inversion to ensure proper mixing and dissolving of the LAMP reagents.

- Finally, shake the reaction tubes downwards to collect the solution into the bottom of the tubes (or pulse spin in a micro-centrifuge for a few seconds).

- Immediately place the reaction tubes into the incubator or hot-block **or** the Loopamp LF-160 **or** the LA-500 turbidimeter (at **65°C**) and close the bonnet.

Loopamp LF-160

OR

LA-500 turbidimeter

For incubator or hotblock:

- Start amplification reaction at **65°C** and set timer for **40 minutes**.
- At the end of amplification reaction, heat the tubes at **80°C for 5 minutes or 95°C for 2 minutes**, to terminate the reaction (enzyme inactivation).

For Loopamp LF-160:

- Press the green button to start the LAMP reaction (previously set at 65°C for 40 minutes).
- Confirm the completion of enzyme inactivation (automatically completed).

For LA-500 Real Time turbidimeter:

- Check that the temperature displayed is 65°C.
- Load the Reaction tubes and start measurement.
- Verify in the display the increase of turbidity of the Positive Control (PC).
- Confirm the completion of polymerase inactivation (automatically completed).

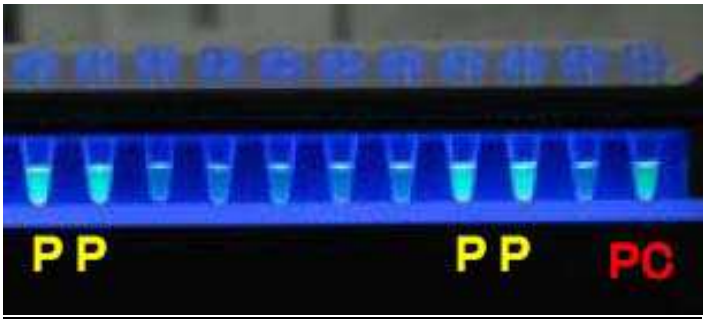
11.4. Detection and interpretation of results

For UV lamp:

- Irradiate the bottom of each Reaction tube and observe from the side through goggles or other UV-protection eye shielding.
- For valid run the following results must be obtained:
    - Positive Control: Green light is emitted.
    - Negative Control: No light is emitted.
- If any control is invalid, all samples in the run should be reported as invalid and the test should be repeated.
- After confirming that the run is valid, evaluate samples as follows:
    - Positive Sample: Green light is emitted.
    - Negative Sample: No light is emitted.

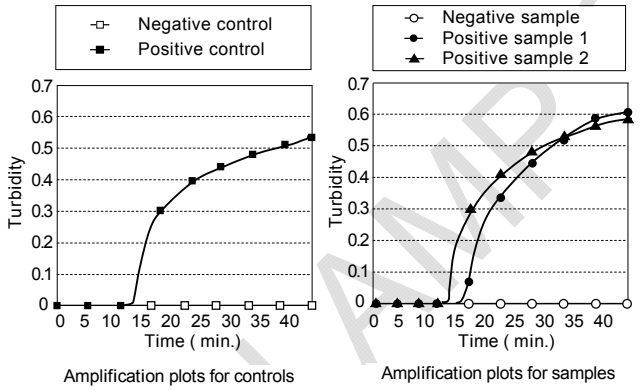
For Loopamp LF-160:

- Place the reaction tubes into the Fluorescence Visual Check Unit, turn on the lamp and observe the tubes from the side through goggles or other UV-protection eye shielding.
- For valid run the following results must be obtained:
    - Positive Control: Green light is emitted.
    - Negative Control: No light is emitted.
- If any control is invalid, all samples in the run should be reported as invalid and the test should be repeated.
- After confirming that the run is valid, evaluate samples as follows:
    - Positive Sample: Green light is emitted.
    - Negative Sample: No light is emitted.



For LA-500 Real Time turbidimeter:

- Watch the display of the turbidimeter to check the Positive and Negative controls for any increase in turbidity.
- If the turbidity increases in the Positive Control but does not in the Negative Control, amplification reaction is proceeding properly. If that is not the case, amplification reaction may be proceeding in a wrong way. In such a case, restart testing from transfer of DNA solution to reaction tubes.
- After confirming that controls are properly working, evaluate samples in accordance with the following criteria:
    - Positive Sample: Some increase is observed in turbidity.
    - Negative Sample: No increase is observed in turbidity.



11.5. Waste disposal

- **DO NOT** open the tubes after DNA amplification. Reaction tubes can contain very high concentrations of DNA after amplification. Opening the tube results in a very high risk of DNA contamination to work areas, posing a risk of subsequent false-positive LAMP and PCR results.Leave the cap closed and place the used Reaction tubes into a zip-lock plastic bag before discarding them as medical waste for incineration. Do not autoclave any used Reaction tubes.
- Dispose of any other reagent, container, or lab ware in accordance with local regulations.

11.6. CHANGE HISTORY

New version #/date	Old version #/date	No. of changes	Description of changes	Source of change request
1.0/AUG12				