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Manual of Standard Operating Procedures for LAMP from Blood

Method for DNA extraction and amplification, and result detection

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BACKGROUND

INTRODUCTION

Loop-mediated isothermal amplification (LAMP) of DNA is a novel molecular technology platform developed by Eiken Chemical (https://www.eiken.co.jp/en). The LAMP technology amplifies previously determined genes and can be used to detect any pathogen. LAMP may be considered an alternative to polymerase chain reaction (PCR) for the detection of nucleic-acid sequences (DNA and/or RNA). Both methods amplify and detect DNA; unlike conventional PCR, LAMP does not require a thermocycler or gel imaging system. Results can be recorded by real-time turbidimetry or 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 solution, primers, strand displacement DNA polymerase, and deoxynucleotide triphosphates at a constant temperature. LAMP provides high efficiency by amplifying DNA 10^9–10^{10} times for 15–60 min. Therefore, LAMP can provide a faster result than conventional PCR and can be performed in basic laboratories without the need for special equipment.

Like many other living organisms, malaria parasites store and replicate their genetic information with DNA molecules that is detected by LAMP. When infected with malaria parasites, the parasite DNA can be detected in the blood during the blood stage of the parasite lifecycle. Normally, DNA is present in very small amounts. Therefore, amplifying or increasing the amount of DNA is necessary to detect and identify them.

LAMP technology

The LAMP technology is characterized by (i) the use of a single polymerase enzyme to perform 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 a high concentration of amplified product in a short time, allowing for visual or automated detection of results. Additionally, the LAMP reaction is robust and tolerable to common PCR inhibitors. This makes it possible to prepare samples simply like “boil and spin” or the use of “PURE” device that rapidly removes impurities from the DNA sample. Although understanding the details of LAMP is not required to perform it properly, an overview of its basic principles is shown in Figure 1.

The LAMP reaction begins with short single-stranded molecules called oligonucleotides or primers. They are designed to bind to the target DNA sequence. If the target DNA is present, one of the specially designed LAMP primers can anneal (bind or stick) to the sense or antisense strand of the target DNA. This can occur because DNA is in a dynamic equilibrium (constantly unfolding and refolding) when it reaches a certain temperature. Primer binding initiates DNA synthesis, thereby the *Bst* DNA polymerase enzyme generates new DNA that is complementary to the target DNA. As the DNA synthesis proceeds, some of the new DNA folds back on itself to form a dumbbell-like “stem-loop” structure. This structure is the starting point for the amplification cycle of LAMP. The loops on the stem-loop structure act as additional primers for the on-going DNA synthesis. As more loops are created, more starting points are available for DNA synthesis. The multiple loops of DNA, which provide a starting point for additional DNA synthesis, are the reason why LAMP is much faster than conventional 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). Visual detection under ultraviolet light is based on the presence of calcein. Before DNA amplification, calcein in the reagent is in the quenched state because it is bound to manganese ions. At the start of DNA amplification, pyrophosphate ions bind to manganese ions, and calcein is released and fluoresces. If fluorescence is observed, the LAMP result is positive; if fluorescence is absent, the result is negative. However, if malaria acquires mutations in the target DNA region, its sensitivity and specificity might be reduced; thus, the clinical conditions should be considered when making a final diagnosis. More details about the LAMP reaction can be found on the website of Eiken Chemical Co., Ltd. (Japan): https://loopamp.eiken.co.jp/en/

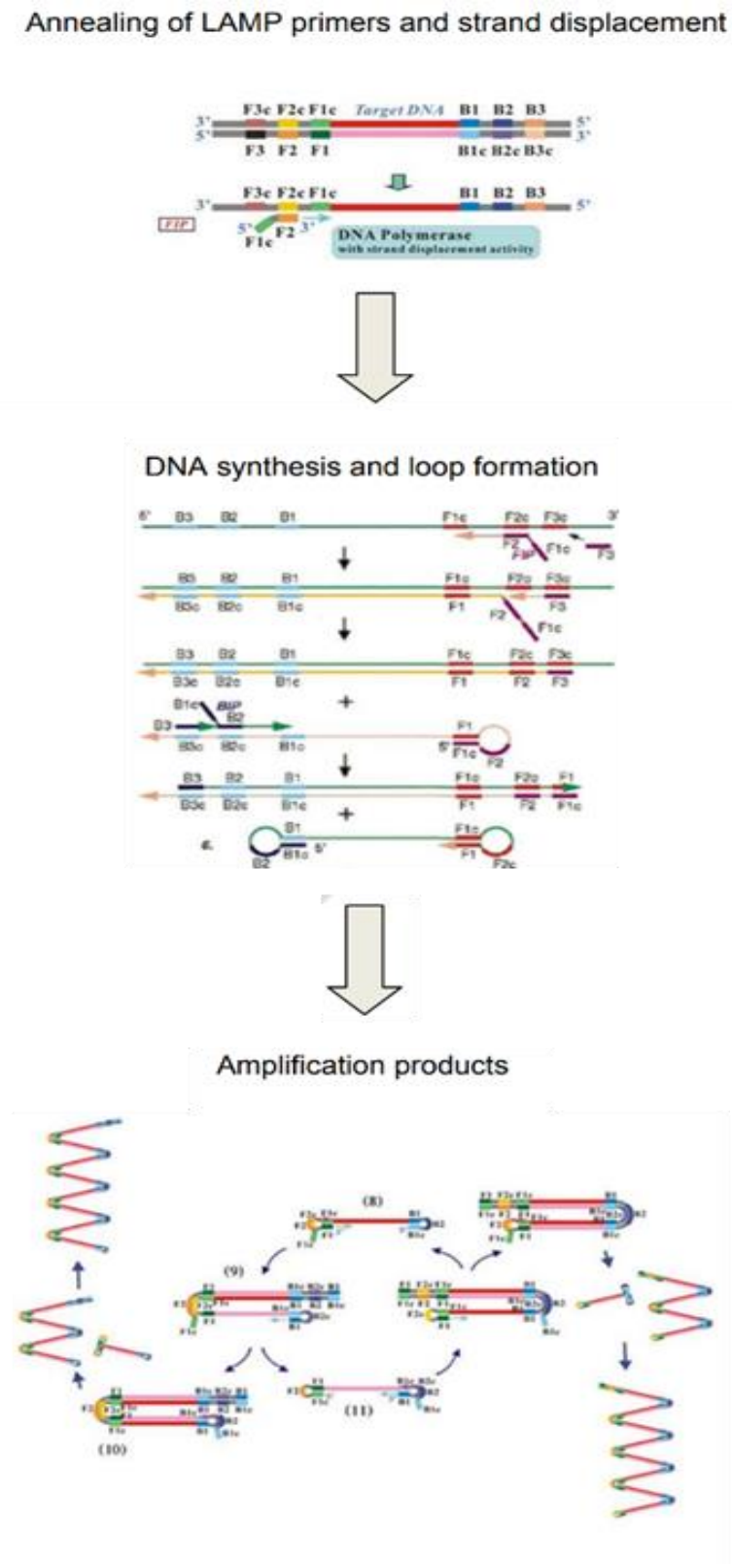


Figure 1. LAMP reaction

PURPOSE

These standard operating procedures (SOPs) describe the materials, equipment, and procedures required for appropriate and safe use of the LAMP kits to diagnose target microorganisms in blood samples.

This manual describes protocols for:

- Process blood samples for DNA extraction.
- Use of LAMP kits.

SCOPE

These SOPs have been developed for the training of laboratory personnel who use LAMP kits in clinical research.

PERSONNEL QUALIFICATIONS

The LAMP kit can be used by laboratory technicians without any prior training in molecular diagnosis. The information provided in this manual can be acquired in a short training period of <3 days; however, strict adherence to the procedures is necessary to achieve reliable results.

SAFETY CONSIDERATIONS

- Blood samples pose a potential risk for infection. Use universal precautions to minimize biohazard.
- Some of the kit components are toxic. Avoid any contact with eyes, mouth, or skin. In the case of accidental contact with any reagent, rinse the area immediately with running water and seek medical advice.
- Be careful when removing tubes from the incubators or water bath to avoid burns.
- UV light is harmful to the eyes; watching for even a short period may irritate the eyes and cause eye diseases such as conjunctivitis. Use a glass screen or wear protective goggles/glasses/face shield whenever looking directly at the UV light.

CAUTIONS

- Ensure that the required equipment is available, and the instruction manuals are read before proceeding with the procedures.
- The performance of the LAMP kits for diagnosis is dependent on operator proficiency and adherence to these SOPs. Testing should be performed by well-trained personnel strictly according to the “Instructions for Use.”

- The LAMP kit has been developed for in vitro diagnostic use only and designed to detect the DNA of *Plasmodium* parasites in human blood samples. Do not use it for other purposes.
- The use of heparin as anticoagulant is essential if results are interpreted by fluorescence. ethylenediaminetetraacetic acid (EDTA) may produce false-positive fluorescence results. Heparin, EDTA, and other anticoagulants can be used when interpretating 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 area between sample preparation and amplification. Collect blood in a room separate from the LAMP amplification room. DNA-containing aerosols can be generated during blood collection and can lead to contamination.
- Exposure to heat, humidity, and light may deteriorate the LAMP kit. Remove only the required number of reaction tubes and reseal the aluminum pouch immediately.
- Do not remove the desiccant from the aluminum pouch.
- Do not touch the inside of the cap of the reaction tubes. The cap of each reaction tube contains dried reagents.
- Before using the reaction tubes, check carefully for any cracks or scratches. Damaged tubes might give false results and lead to DNA contamination of the incubator and work area.
- When handling the LAMP kits avoid microbial and nuclease contamination. A small amount of contamination of the reaction tubes from sweat or saliva may degrade DNA and give a false result.
- Use the positive control (PC MAL, PC PV) only as described in these SOPs to avoid DNA contamination.
- Store the PC and any positive blood samples separately from other kit reagents.
- The dried reagent in the cap should be fully dissolved. Any undissolved portion may influence its performance, such as reduced sensitivity.
- Since bubbles may interfere with amplification and measurement, avoid making any bubbles when mixing the reagents and sample solution. If bubbles occur, spin or flick down the reaction tube.
- Do not expose the reaction tubes to the 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 after DNA simplification. Be 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.

LAMP kit for malaria

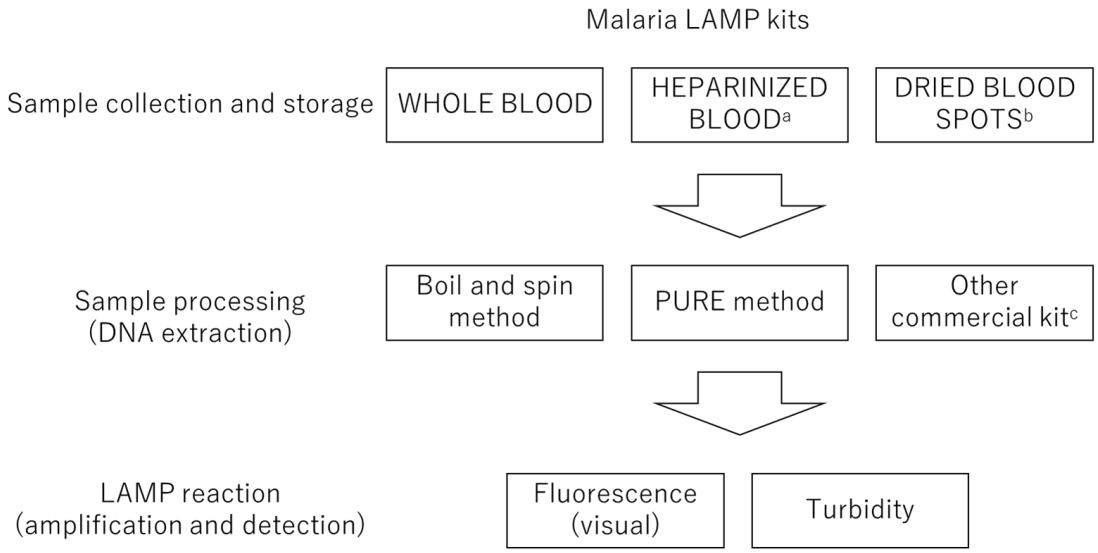
- The first malaria detection by LAMP was reported by Poon et al. in 2006. FIND has been working with Eiken Chemical Co. Ltd., (Japan) and the Hospital for Tropical Diseases (HTD) in London (UK) on the development of an optimized LAMP malaria diagnostic assay for the detection of *Plasmodium* DNA extracted from human blood. At present, three reagents are commercially available to diagnose malaria in general, falciparum malaria, and vivax malaria. They can be distinguished by the color of their caps. The caps of the general reagents (Loopamp™ Malaria Pan Detection Kit), falciparum malaria reagents (Loopamp™ Malaria Pf Detection Kit), and vivax malaria reagents (Loopamp™ Malaria Pv Detection Kit) are blue, red, and yellow, respectively.
- The Pan (genus)-specific primers detect a target DNA sequence that is well-conserved among all *Plasmodium* species and can detect a wide range of *Plasmodium* species, including the five most common species of human malaria (*P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale, and P. knowlesi*). The *P. falciparum* (Pf)-specific primers have been confirmed by in silico alignment analysis and in vitro experiments specific for *P. falciparum*. The limits of detection for Pan, Pf, and Pv assays are 7.5, 12.5, and 7.5 DNA copies per test, respectively. Experimentally, both assays can detect as low as 1 parasite/μL from human blood in 40 min of amplification time.
- Prototypes of this malaria LAMP assay have been compared to nested PCR with 705 patient samples (56 positives by microscopy) collected at HTD in London. The sensitivity and specificity of the Pf LAMP were 98.4% and 98.1%, and those of Pan LAMP were 97.0% and 99.2%, respectively. Both malaria LAMP kits were similar in sensitivity and specificity to those of nested PCR and was superior to microscopy in detecting infected samples. Similar results were obtained with samples collected from an endemic area in Uganda. The study in Uganda also demonstrated that LAMP can be applied in a simple laboratory space, such as a clinic, by technicians without previous molecular diagnostic training, with a short training period. As a novel molecular diagnostic technique, LAMP promises to bring the sensitivity of PCR to field environment, without the need for sophisticated laboratory equipment, with automated or visual judgment. Both studies have

demonstrated that LAMP is equivalent to PCR in sensitivity and specificity, but it is more rapidly than PCR.

- The study conducted in Peru by FIND and University Peruana Cayetano Heredia revealed that the sensitivity and specificity against of Loopamp™ Malaria Pv Detection Kit against nested PCR were 98.4% and 98.1%, respectively.
- Compared with PCR, these three malaria LAMP kits are designed to meet the requirement for a molecular test that achieves higher sensitivity and specificity than microscopy and rapid diagnostic tests, while requiring less equipment and adapting to field settings. Therefore, in addition to replacing microscopy in situations such as screening of returning travelers in nonendemic countries, the assay has the potential to be the reference standard in low-resource settings and surveillance and screening tool when a highly sensitive assay is needed.

FLOWCHART FOR THE USE OF SOPs FOR LAMP MALARIA

The malaria LAMP kits can be used with nonanticoagulated/heparinized whole blood or dried blood spots and DNA extracted via several available methods. The following SOPs describe the methods recommended and previously tested with clinical samples in field studies. In this SOP, Loopamp™ Malaria Pan Detection Kit is used as an amplification reagent; however, Loopamp™ Malaria Pf Detection Kit and Loopamp™ Malaria Pv Detection Kit can be used similar to the Loopamp™ Malaria Pan Detection Kit. Therefore, the information on how to use these two reagents is omitted.



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

B

The use of dried blood spots for LAMP was confirmed in the laboratory but not in field studies.

C

The use of other DNA extraction kits was demonstrated in the laboratory but not in field studies. Other DNA extraction methods could produce unreliable results; thus, the users should validate them to ensure the accuracy of the results.

BOIL AND SPIN METHOD

The Malaria LAMP kits can be used with DNA extracted from the blood using different methods. These SOPs present how to extract DNA with the “boil and spin” method, which has been evaluated with whole blood samples.

Sample collection and storage

The whole blood collected by finger prick and drawn with heparin tubes is available for this SOP.

The finger-pricked blood should be used immediately.

Heparinized blood can be stored for up to 2 weeks at 4°C.

This SOP is **not** recommended for dried blood spot. For dried blood spot, refer to the SOP for the “PURE METHOD.”

For the boil and spin method, 60 µL of whole blood is required.

DNA extraction by the Boil and Spin method

Equipment: Heat block at 95°C (acceptable temperature accuracy ± 0.5°C)

Vortex (optional)

Microcentrifuge (10,000 ×g)

Timer

Materials: Marking pen

Disposable gloves (powder free)

0.5% sodium hypochlorite

10–100-µL pipette

Sterile disposable tip for 10–100-µL pipette

Extraction tube: 1.5-mL tube with 60 µL of extraction buffer; 400 mM NaCl, 0.4% SDS)

Dilution tube: 1.5-mL tube with 345 µL of sterile water

Procedure:

- Turn on the heat block or water bath and confirm that the temperature has reached 95°C.
- Wear two pairs of disposable gloves (discard outer ones before starting the LAMP amplification steps).
- Work in the sample preparation area separate from the amplification area.
- Clean the bench with 0.5% sodium hypochlorite.
- Place all the required materials after cleaning the bench.
- Write the patient's names or code on the cap of each extraction and dilution tubes.
- Transfer **60 µL** of whole blood to the extraction tube using a 10–100-µL pipette or some device. Repeat this step for every sample, using one extraction tube per sample.
- Mix the sample and the extraction buffer using vortex for 10 s or pipetting 10 times.
- Place the extraction tube with the sample in the heat block or water bath at 95°C for 5 min.
- Remove the tube from the heat block or water bath immediately after the timer goes off. Overheating may degrade the DNA and reduce test sensitivity.
- Centrifuge at 10,000 ×g for 3 min.
- Transfer 30 µL of clear supernatant to the dilution tube (the precipitated hemoglobin makes a brownish red pellet at the bottom of the tube). Mix by pipetting 10 times to dilute the DNA sample [hereafter referred as (S)].
- Repeat these steps for every sample and negative control (NC Mal) ensuring to use a single extraction tube and a single dilution tube per sample. Hereafter, the extracted NC Mal will be referred as negative solution. If not used immediately, S can be stored at −20°C.

LAMP amplification

Equipment: incubator or heat block at 65°C with hot bonnet (acceptable temperature accuracy ± 0.5°C)

UV light or blue LED light (wavelength: 240–260 nm and 350–370 nm)

Timer

or

LF-160/HumaLoop M (REF: 962000 – Eiken Chemical Co./Human Gesellschaft fur Biochemica und Diagnostica mbH)

or

LA-500/HumaTurb C+A (REF: 963200 – Eiken Chemical Co./Human Desellschaft fur Biochemica und Diagnostica mbH)

Materials: Marking pen

Disposable gloves (powder free)

0.5% sodium hypochlorite

10–100-µL pipette

Sterile disposable tip for 10–100-µL pipette

Loopamp™ Malaria Pan Detection Kit (REF: 977000, Eiken Chemical Co.)

Kit contents:

Malaria Pan reaction tubes

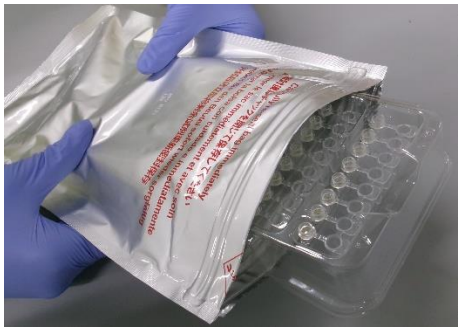
PC Mal

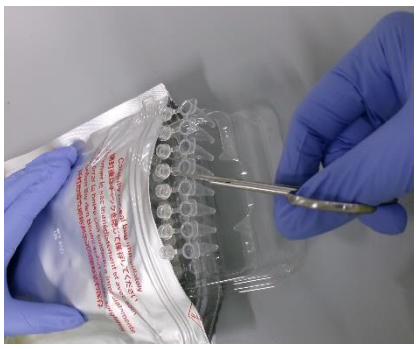
NC Mal

30-µL droppers

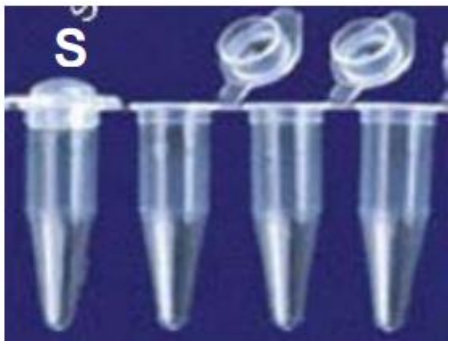
Procedure:

- Read the Instruction for Use for the Loopamp™ Malaria Pan Detection Kit carefully.
- Read the LF-160/HumaLoop M or LA-500/HumaTurb C+A instruction manuals carefully before using these devices.
- Turn on the incubator or heat block or the Loopamp™ LF-160/HumaLoop M or the LA-500/HumaTurb C+A and verify that the temperature has reached 65°C. Allow the LA-500/HumaTurb C+A to warm up for 20 min before use (set the “MALARIA” program in the respective amplification unit).
- Work in an amplification area separate from the sample preparation area.
- Clean the bench with 0.5% sodium hypochlorite.
- All the required materials should be placed after the bench has been cleaned.
- Discard the outer gloves when leaving the sample preparation area.
- Remove the required number of the reaction tubes from the aluminum pouch and put them in an appropriate rack (one reaction tube per sample, plus one for the positive control, and one for the negative control). Up to 14 samples can be amplified in one operation.

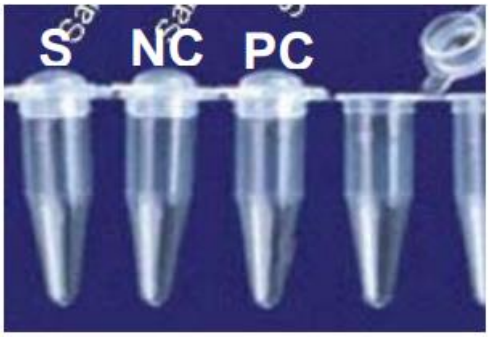




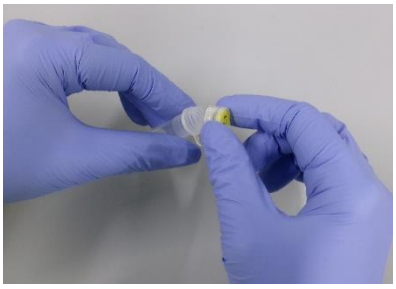
- After removing the required number of reaction tubes, reseal the aluminum bag immediately (close tightly) with any unused tubes.
- Flick or spin down the tubes with the PC Mal and NC Mal before using them to collect the content at the bottom of the tubes.
- Write the patient's name or code on the respective reaction tubes.
- Dispense 30 µL of S into the reaction tube and close the cap. Repeat this step for all samples to be amplified in the same run.



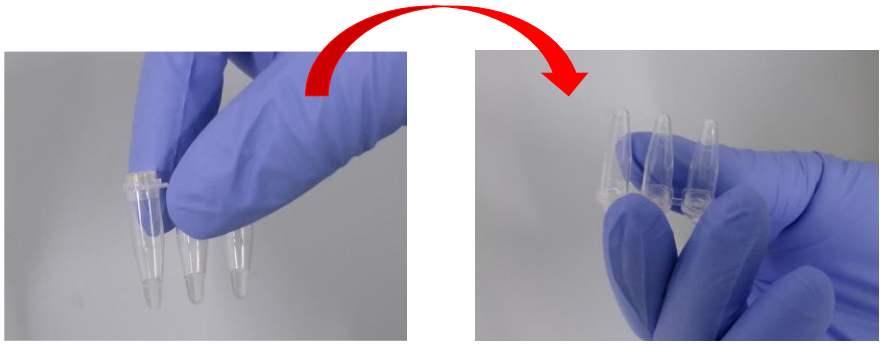
- Dispense 30 µL of the negative solution into the reaction tube and close the cap. Always dispense the PC Mal into the last tube as the final step. Dispensing earlier may cause contamination.



- Close the PC Mal tube immediately after dispensing the required volume into the reaction tube.

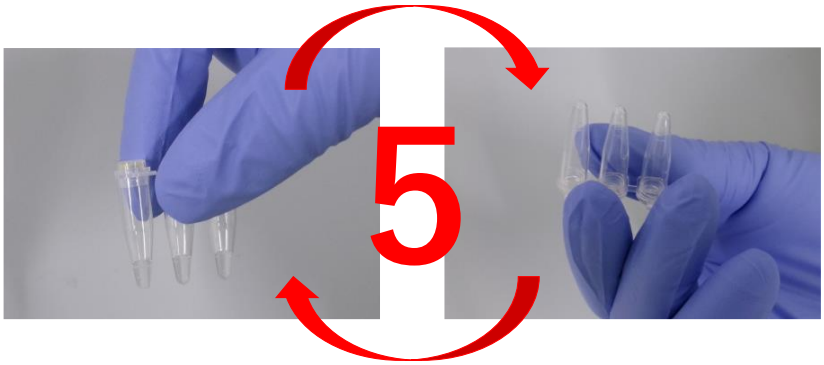


- Ensure that the liquid level is in the middle of the two lines on a reaction tube to ensure 30 µL of pipetting.
- Flick or spin down all tubes so that the solution collects at the bottom of the tubes.
- Pick up the reaction tubes from the rack and invert the tubes so that the solution collects in the cap.



- Leave the reaction tubes standing upside down on the rack for 2 min. This allows the DNA solution to dissolve the dried LAMP reagents inside the tube cap.

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



- Finally, flick or spin down the reaction tubes so that the solution collects at the bottom of the tubes.

- Immediately place the reaction tubes in the incubator or heat block or LA-500/HumaTurb C+A or LF-160/HumaLoop M (at 65°C) and close the bonnet.

OR

For incubator or heat block:

- Start the amplification reaction at 65°C and set timer for 40 min.
- At the end of amplification reaction, heat the tubes at 80°C for 5 min or 95°C for 2 min to inactivate the enzyme.

For LF-160/HumaLoop M

- Press the green button to start the LAMP reaction (turn on it for 20 min to warm up).
- Confirm the completion of the enzyme inactivation step (automatically completed).

For LA-500/HumaTurb C+A

- Check if the displayed temperature reached 65°C.
- Place the reaction tubes and start the measurement.
- Verify in the display the increase in turbidity for the PC.
- Confirm the completion of the enzyme inactivation step (automatically completed).

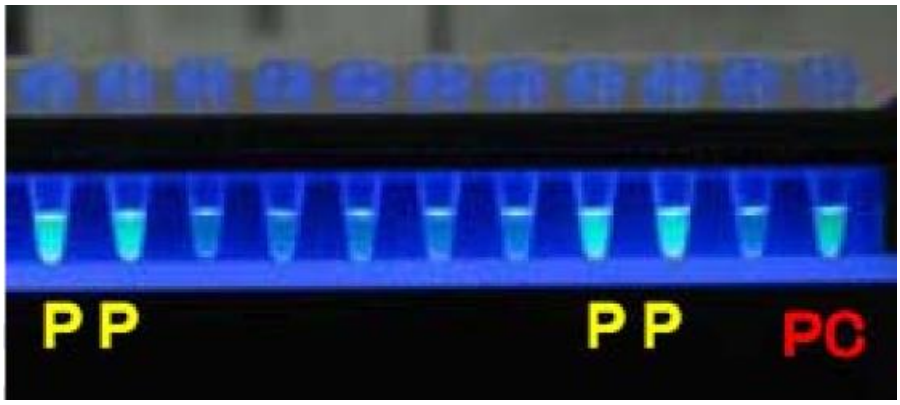
Detection and interpretation of results

For UV light

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

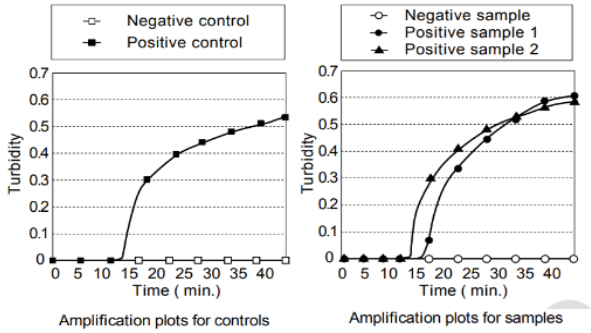
For LF-160/HumaLoop M

- Place the reaction tubes into the fluorescence visual unit, turn on the light and observe the tubes from the side.
- For valid run, the following results must be obtained:
    - Positive sample: Green light is emitted.
    - Negative sample: No light is emitted.
- If any control is invalid, all samples in that run should be reported as invalid, and the samples should be retested.



For LA-500/HumaTurb C+A

- Watch the display of the turbidimeter to check the positive and negative controls for any increase in turbidity.
- If the turbidity increases in the PC but does not in the NC, the amplification reaction is proceeding properly. If that is not the case, the amplification reaction might be proceeding the wrong way. In such a case, retest the affected samples from DNA extraction.
- After confirming that the controls are properly working, evaluate the samples in accordance with the following criteria:
    - Positive sample: Green light is emitted.
    - Negative sample: No light is emitted.



PURE METHOD

These SOPs present the use of the PURE method that has been evaluated with the whole blood samples of the patient. The PURE device is a specially designed series of interlocking plastic components that provide a closed system for sample processing and direct dispensing of DNA solution to reaction tubes.

Sample collection and storage

- These SOPs apply to fresh blood samples collected by finger prick, heparinized blood, and dried blood spot.
- The finger-pricked blood should be used immediately.
- Heparinized blood can be stored at 4°C for up to 2 weeks.
- For the PURE method, 30 µL of whole blood is required.

For dried blood spot, the following is recommended:

- Use Whatman 3-mm of printed filter mat A – Wallac (standard FTA™ card **do not** perform well in the LAMP assay and are not recommended).
- Dry the blood spot naturally for at least 3 h and then store them in sealable plastic bags with desiccant at room temperature in a dry place.
- Punch a 6-mm hole at the center of the dried blood spot and then put the punched spot into the tube with a tweezer. After punching the sample, punch out the wet paper with

0.5% sodium hypochlorite five times and the dry paper five times. The tweezer should be cleaned in the same way as the puncher. After cleaning, place them on the clean area.

DNA extraction by the PURE method

Equipment: Water bath / heat block at 75°C (acceptable temperature accuracy ± 0.5°C)
Timer
LF-160/HumaLoop M

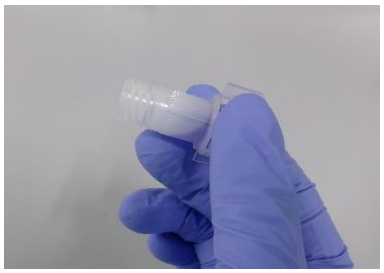
Material: Marking pen
Disposable gloves (powder free)
0.5% sodium hypochlorite
10–100-µL pipette
Sterile 10–100-µL pipette tips
334 mM NaCl solution
Loopamp™ PURE DNA Extraction Kit
Kit Contents:
Heating Tubes
Adsorbent Tubes
Injection cap
Loopamp™ Malaria Pan Detection Kit
Kit contents:
NC Mal

Procedure:

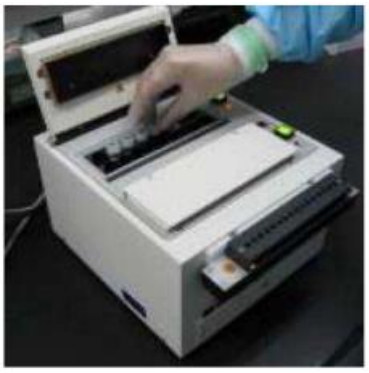
- Read the Instruction for Use for the Loopamp™ PURE DNA Extraction Kit carefully.
- Read the LF-160/HumaLoop M instruction manual carefully before using this device.
- Turn on the incubator or water bath or LF-160/HumaLoop M and confirm that the temperature has reached 75°C. Regarding LF-160/HumaLoop M, the temperature on the heat block should reached 75°C.
- Work with two pairs of disposable examination gloves (discard outer ones when completing sample processing and starting the LAMP reaction.)
- Work in a sample preparation area separate from the amplification area.
- Clean the bench with 0.5% sodium hypochlorite.
- Place all the required materials on the clean surface.
- Write the patient's name or code on the cap of the Heating Tubes.
- Transfer 30 µL of 334 mM NaCl solution to the Heating Tubes.
- Transfer 30 µL of whole blood or a 6-mm dried blood spot punch to the Heating Tubes with micropipette, transfer device for a finger prick, or a tweezer.
- Repeat this step for every sample. One Heating Tube is used for every sample.

- Transfer 30 µL of NC Mal into the Heating Tube.

- Invert the Heating Tubes three times.



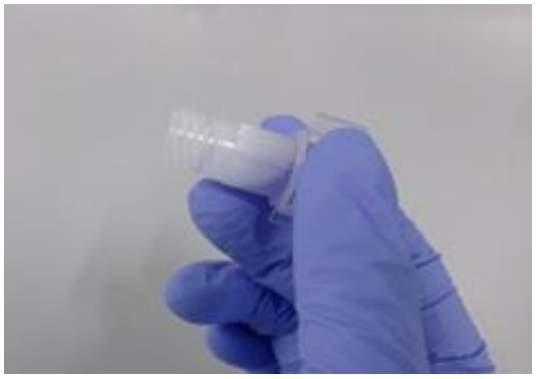
- Place the tubes in the heat block or water bath as 75°C and set the timer for 5 min, or in the heating block of LF-160/HumaLoop M so that the heating process is completed automatically.



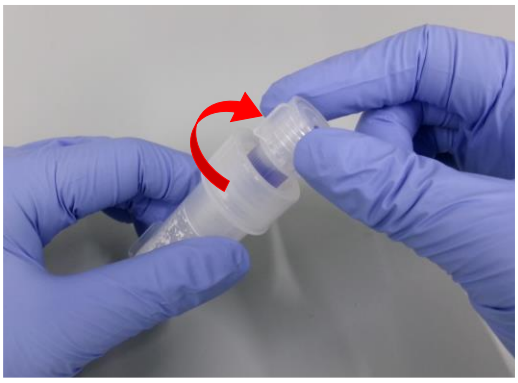
- Remove the tubes immediately in 5 min. Overheating might degrade the DNA.
- Let the Heating Tubes cool for 2 min.

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

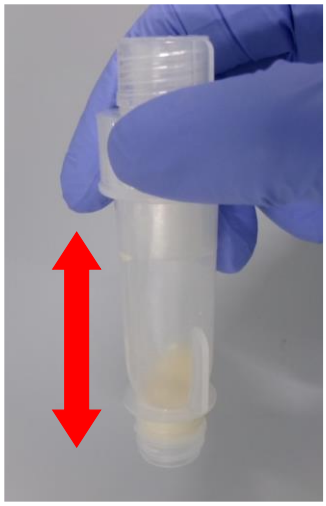
- Mix the Heating Tube with sample and NC Mal again by inverting the tubes three times.

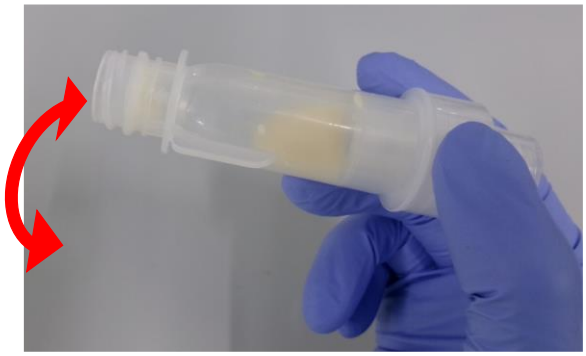


- Screw the Heating Tube firmly into area where the cap ring was removed from Adsorbent Tube. Ensure that the blue liquid is transferred from the Heating Tube to Adsorbent Tube.



- Shake the tube vertically 10 times and then swing it horizontally 10 times to mix the sample and NC Mal with the absorbent powder. Ensure complete mixing by repeated shaking motions until no white powder is clinging to the inside of the tube and the solution turns pale red-yellow).





- Place the tube horizontally over a clean surface while processing additional samples.
- Repeat these steps for every sample and NC Mal being sure of using a single Adsorbent Tube per sample. The DNA extracted from the PURE device should be transferred to the reaction tubes. The DNA solution and negative solution can be stored for 72 h.

LAMP amplification

Equipment: Incubator or heat block at 65°C with hot bonnet over 70°C and below 90°C (acceptable temperature accuracy ± 0.5°C)

- UV light or blue LED light (wavelength: 240–260 nm and 350–370 nm)
- Timer

or

LF-160/HumaLoop M (REF: 962000 – Eiken Chemical Co./Human Gesellschaft für Biochemica und Diagnostica mbH)

or

LA-500/HumaTurb C+A (REF: 963200 – Eiken Chemical Co./Human Gesellschaft für Biochemica und Diagnostica mbH)

Materials: Marking pen

Disposable gloves (powder free)

0.5% sodium hypochlorite

10–100-µL pipette

Sterile disposable tip for 10–100-µL pipette

Loopamp™ Malaria Pan Detection Kit (REF: 977000 - Eiken Chemical Co.)

Kit contents:

Malaria Pan reaction tubes

PC Mal

NC Mal

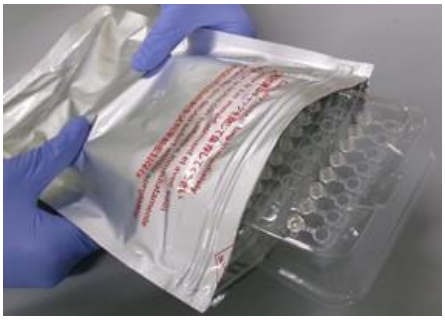
30-µL droppers

Sealable plastic bag

Protective goggles/glasses/face shield if using UV light or LF-160/HumaLoop M

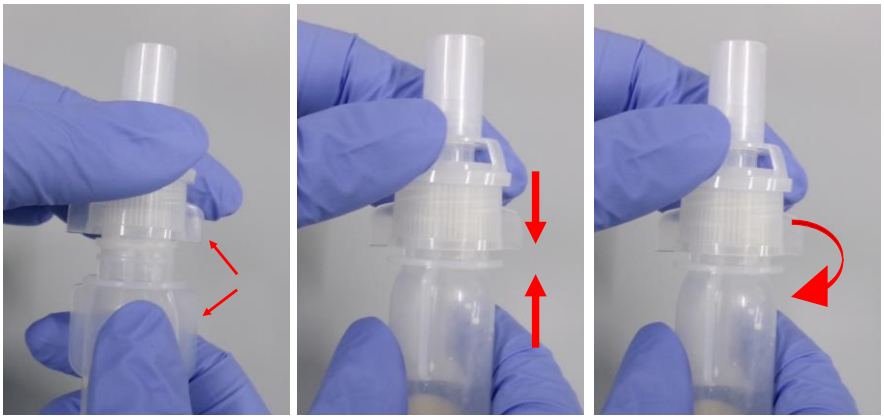
Procedure:

- Read the Instruction for Use for the Loopamp™ Malaria Pan Detection Kit carefully.
- Read the LF-160/HumaLoop M or LA-500/HumaTurb C+A instruction manuals carefully before using these devices.
- Turn on the incubator or heat block or the LF-160/HumaLoop M or the LA-500/HumaTurb C+A and verify that the temperature has reached 65°C. Allow the LA-500/HumaTurb C+A to warm up for 20 min before use (set the “MALARIA” program in the respective amplification unit).
- Work in an amplification area separate from the sample preparation area.
- Clean the bench with 0.5% sodium hypochlorite.
- Discard the outer gloves when leaving the sample preparation area.
- All the required materials should be placed after cleaned completely with 0.5% sodium hypochlorite.
- Remove the required number of the reaction tubes from the aluminum pouch and put them in an appropriate rack (one reaction tube per sample, plus one for the PC, and one for the NC). Up to 14 samples can be amplified in one operation.

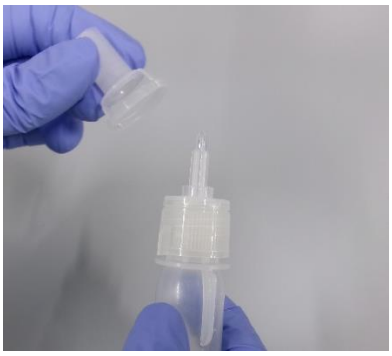


- After removing the required number of reaction tubes, reseal the aluminum pouch immediately (close tightly) with any unused tubes.
- Flick or spin down the tubes with the PC Mal before using them to collect the content at the bottom of the tube.
- Write the patient’s name or code on the respective reaction tubes.
- Attach the cap ring, which is removed from Adsorbent Tube previously to the nozzle of the injection cap.

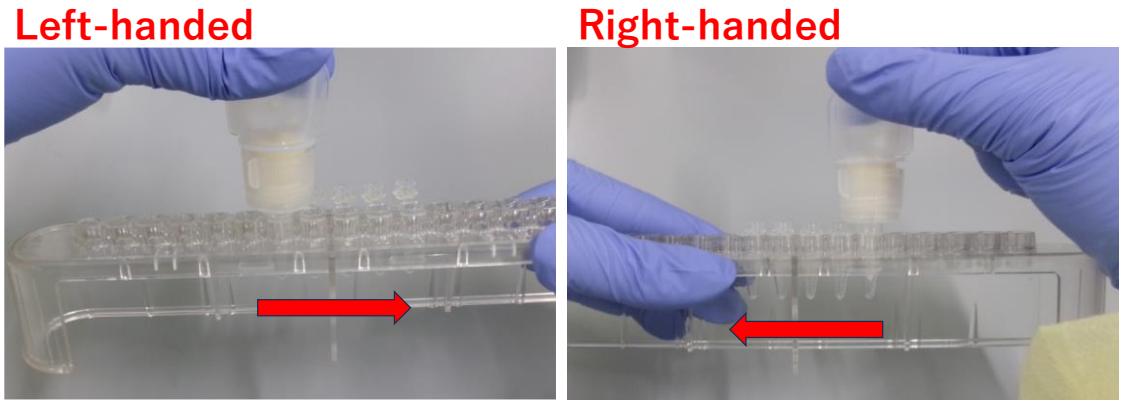
- Line up the wing of the injection cap with that of the inverted Adsorbent Tube with sample. Push the injection cap onto Adsorbent Tube until hearing a click and then screw the cap tightly.



- Shake and swing the PURE device to transfer the mixture to the injection cap.
- Remove the cap ring from the nozzle of the injection cap with the PURE device inverted.

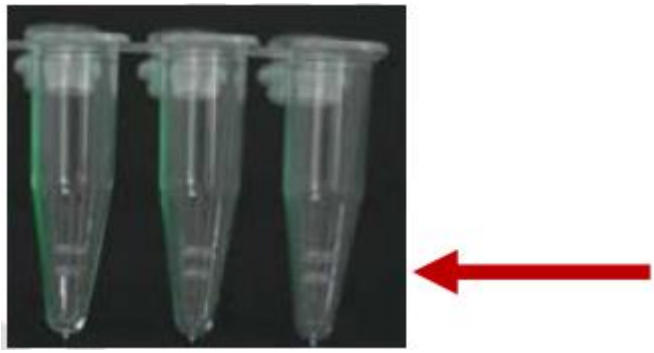


- Turn the PURE over and hold it with the injection cap facing down.
- Hold the rack with the reaction tubes up to your eye level and insert the nozzle of the PURE device completely into the reaction tube.

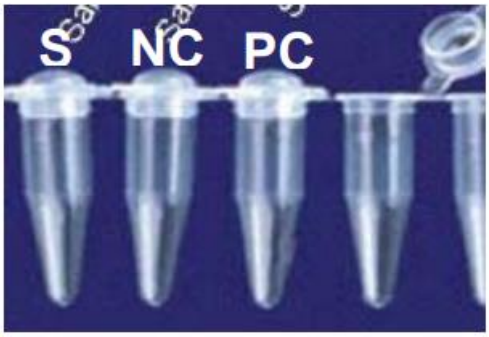


- Squeeze the PURE device for S and negative solution to drop into the reaction tube.
- Fill in the reaction tube up to the middle of the two lines on the reaction tubes to ensure 30 µL of volume and close the cap. For accurate test results, do not over-fill

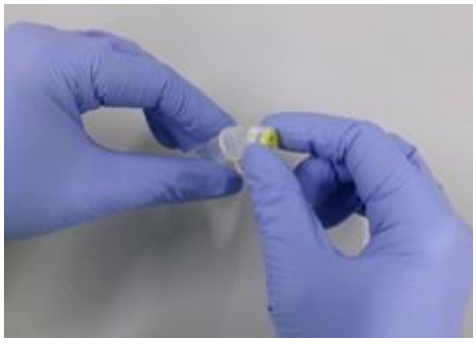
or under-fill the reaction tube. Repeat this step for all samples to be amplified in the same run.



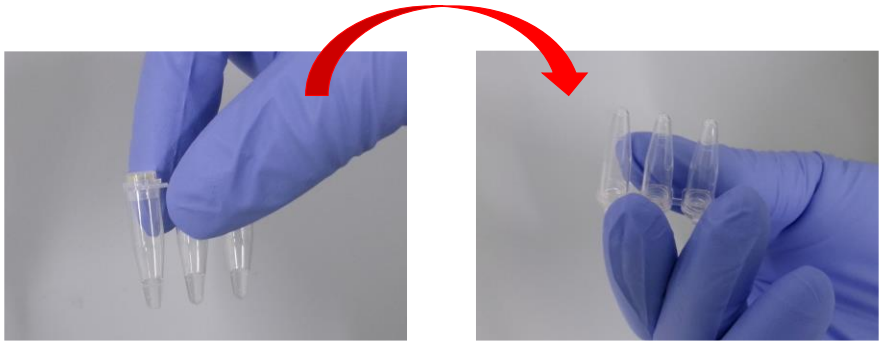
- Dispense 30 µL of the negative solution into the reaction tube and close the cap.
- Dispense 30 µL of PC Mal into the reaction tube and close the cap. Always dispense the PC Mal into the last tube as a final step. Dispensing earlier may cause contamination.



- Close the PC Mal tube immediately after dispensing the required volume into the reaction tube.

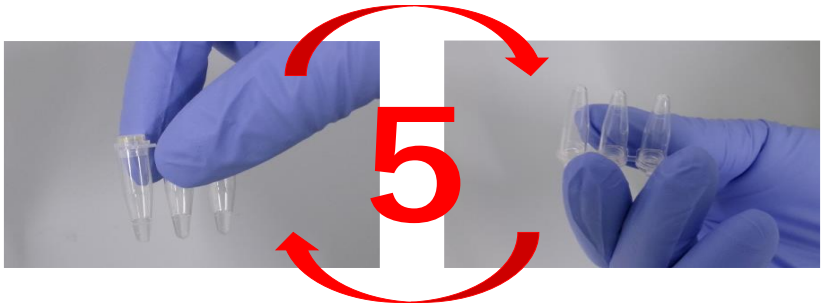


- Ensure that the liquid level is in the middle of the two lines on a reaction tube to ensure 30 µL of pipetting.
- Flick or spin down all tubes to make the solution collect at the bottom of the tubes.
- Pick up the reaction tubes from the rack and invert the tubes so that the solution collects in the cap.



- Leave the reaction tubes standing upside down on the rack for 2 min. This allows the DNA solution to dissolve the dried LAMP reagents inside the tube cap.

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



- Finally, flick or spin down the reaction tubes so that the solution collects at the bottom of the tubes.

- Immediately place the reaction tubes in the incubator or heat block or LA-500/HumaTurb C+A or LF-160/HumaLoop M (at 65°C) and close the bonnet.

OR

For incubator or heat block:

- Start the amplification reaction at 65°C and set the timer for 40 min.
- At the end of the amplification reaction, heat the tubes at 80°C for 5 min or 95°C for 2 min to inactivate the enzyme.

For LF-160/HumaLoop M

- Press the green button to start the LAMP reaction (turn on it for 20 min to warm up).
- Confirm the completion of enzyme inactivation step (automatically completed).

For LA-500/HumaTurb C+A

- Check if the displayed temperature reached 65°C.
- Place the reaction tubes and start the measurement.
- Verify in the display the increase in turbidity for the PC.
- Confirm the completion of the enzyme inactivation step (automatically completed).

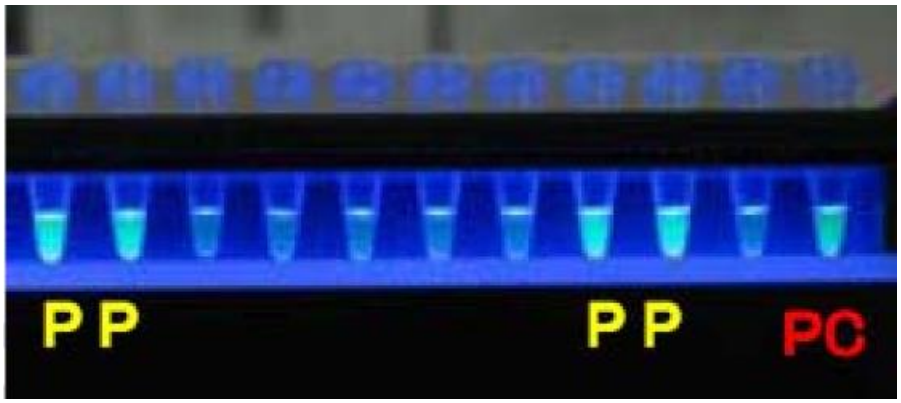
Detection and interpretation of results

For UV light

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

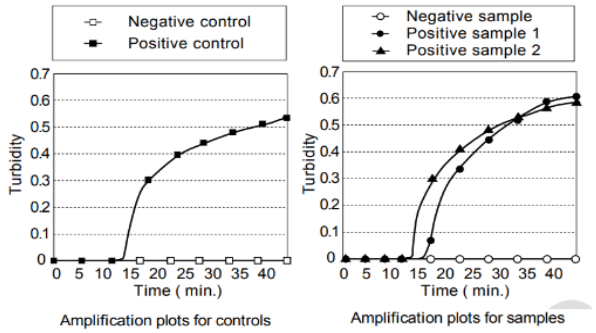
For LF-160/HumaLoop M

- Place the reaction tubes into the fluorescence visual unit, turn on the light, and observe the tubes from the sides.
- For valid run, the following results must be obtained:
  - Positive sample: Green light is emitted.
  - Negative sample: No light is emitted.
- If any control is invalid, all samples in that run should be reported as invalid, and the samples should be retested.



For LA-500/HumaTurb C+A

- Watch the display of the turbidimeter to check the positive and negative controls for any increase in turbidity.
- If the turbidity increases in the PC but does not in the NC, the amplification reaction is proceeding properly. If that is not the case, the amplification reaction might be proceeding the wrong way. In such a case, retest the affected samples from DNA extraction.
- After confirming that controls are properly working, evaluate the samples in accordance with the following criteria:
  - Positive sample: Green light is emitted.
  - Negative sample: No light is emitted.



WASTE DISPOSAL

- **Do not** open the tubes after DNA amplification. After DNA amplification, the reaction tubes contain very high concentrations of amplicon. Opening the tubes might cause DNA contamination in the working area, which poses a risk of subsequent false-positive LAMP results. Leave the cap closed and double bagged with sealable plastic bags before discarding them as medical waste for incineration.
- Dispose of any other reagents, containers, or labware in accordance with the local regulations.

ACKNOWLEDGMENTS

We would like to thank the Foundation for Innovative New Diagnostics (FIND) and Hospital for Tropical Disease (HTD) for the cooperation in developing the method for DNA extraction and LAMP amplification from blood.

CHANGE HISTORY

New version #/date	Old version #/date	No. of changes	Description of changes	Source of change request
1.0/AUG 12	-	-	-	-
2.0/DEC 5	1.0/AUG 12	NA	Replaced images. Enhanced clarity in explanations. Overall formatting improvements.	In-house review