Instructions for Use
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Human Diagnostics Worldwide
*380263-C

English

REF 975000

Loopamp™ MALARIA Pv Detection Kit

INTENDED USE

The Loopamp™ MALARIA Pv Detection Kit is a qualitative *in vitro* diagnostic test to detect the *Plasmodium vivax* DNA extracted from human blood samples in patients suspected Malaria infection. The kit aids in the diagnosis of *Plasmodium vivax* infection and is intended to be used in professional laboratories and hospitals by adequately trained personnel. The result can be interpreted either by automated turbidimeter or visually under UV irradiation.

TEST PRINCIPLES

This product is based on the nucleic acid amplification method, LAMP (Loop-mediated Isothermal Amplification), developed by Eiken Chemical Co., Ltd.

The characteristics of the LAMP method are as follows: (1) only one enzyme is needed, and the amplification reaction proceeds under isothermal conditions;[1),2)] (2) it has extremely high specificity because of the use of four primers recognizing six distinct regions on the target; (3) it has a high amplification efficiency and can produce a high concentration of amplified product in a short time, which makes visual or automated detection possible.[3),4)]

The *P. vivax* (Pv)-specific primers are designed to detect the mitochondrial DNA of Pv. The alignment analysis has confirmed the targeted DNA sequences to be specific for Pv-sequence.

The test DNA solution extracted from blood samples is dispensed into a reaction tube. Then, the strand displacement DNA polymerase, deoxynucleotide triphosphates (dATP, dCTP, dGTP, and dTTP), calcein, reaction buffers, and Malaria Pv-specific primers are stored in the dried form in the cap of the reaction tube. This dried LAMP reagent (Malaria Pv detection reagent (dMAL Pv)) is dissolved when the DNA solution is added. Then, the reaction tube is incubated at 65.0 °C, and the DNA is amplified by the strand displacement DNA polymerase per the LAMP reaction.

The detection of amplified products is based on the turbidity of magnesium pyrophosphate (a white precipitate produced as a by-product of DNA amplification).[3)] Alternatively, visual detection under UV light can be used. Before the DNA amplification, calcein in the reagent is in the quenched state, as it is bound to manganese ions. At the start of the DNA amplification, pyrophosphate ions generated out-compete the manganese ions for binding sites, thereby making calcein fluorescent.[4)]

KIT CONTENTS

Reagents are stable until the date on the label, assuming the container remains unopened at a storage temperature of 2–30 °C. The reagents are also confirmed stable after opening the container when adhered to these procedural directions.

Malaria Pv detection reagent (dMAL Pv) ····· 2 x 48 tubes
The following reagents in the dried form are contained in each reaction tube:
Bst DNA polymerase*a:
Deoxynucleotide triphosphates
Magnesium sulfate
Calcein
Manganese chloride
Primers*b:
Positive control Mal Pv (PC PV)*c: ····· 1 x 1.0 mL
Negative control Mal (NC Mal) ····· 3 x 0.5 mL
30 µL dropper ····· 5 x 12 droppers

*a: *Bst* DNA polymerase derived from *Bacillus stearothermophilus* is a strand displacement DNA polymerase that lacks 5′→3′ exonuclease activity.

*b: Primers designed for the mitochondrial DNA of Pv, purified from synthesized oligonucleotides by HPLC.

*c: The PC PV contains a product resulting from the *in vitro* amplification of an artificial gene designed from the mitochondrial DNA of Pv (GenBank No. AF055587).

Abbreviations of the following reagents, their Lot No., and the manufacturer (EKN), are printed on the containers as follows:

Reagents	Labelling on the tube	Code on the cap
Positive control Mal Pv	PC PV Lot No., EKN	PC PV
Negative control Mal	NC Mal Lot No., EKN	NC Mal

*Metrological Traceability Information

Positive control is prepared from Plasmodium vivax Salvador I (GenBank No. AF055587), as there is no international standard for Plasmodium DNA. The plasmid DNA including the targeted region of Plasmodium vivax Salvador I genomic DNA used as a template to amplify PC PV DNA fragment. The fragment is quantitated with the photospectrometric analysis, and DNA concentration of PC PV is adjusted at 2,000 copies/µL.

WARNINGS AND PRECAUTIONS

(1) For *in vitro* diagnostic use only.
(2) This product is only designed to detect the DNA of Pv parasites in human blood samples. Do not use it for other purposes.
(3) When using this product, always follow these Instructions for Use.
(4) Do not freeze the reagents.
(5) Do not use any expired reagent.
(6) Do not mix reagents from different Lots.
(7) Do not replenish any reagent.
(8) The performance of the Loopamp™ MALARIA Pv Detection Kit depends on the operator proficiency and adherence to procedural directions. Testing should be done by adequately trained personnel strictly according to the instructions provided.
(9) Exposure to heat, humidity, and light might deteriorate the dMAL Pv. Thus, remove only the required number of reaction tubes (sum of samples and controls) and re-seal the aluminium pouch immediately.
(10) Do not remove the desiccant from the aluminium pouch. High humidity can deteriorate the dried LAMP reagent in the reaction tubes.
(11) Read the instruction manual and ensure that the required equipment (turbidimeter or incubator) is available before commencing the procedure.
(12) Blood samples pose a potential risk for infection. Thus, use universal precautions to minimize biohazard.[5)]
(13) The PC PV and the NC Mal contain a small amount of sodium azide as a preservative. As sodium azide is classified as toxic, avoid any contact with eyes, mouth, or skin.
(14) In case of accidental contact of any reagent with eyes, mouth, or skin, immediately rinse the affected site with running water and seek medical advice.
(15) Do not dilute or add the PC PV to the samples. Use the PC PV only as described in this package insert to avoid DNA contamination.
(16) Store the PC PV and any positive blood samples separately from the other kit reagents.
(17) The cap of each reaction tube contains the dMAL Pv in the dried form. Do not touch the inside of the cap.
(18) Before using the reaction tubes, check carefully if they have any cracks or scratches. Damaged tubes might give false results and lead to DNA contamination of the incubator and work area.
(19) 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.
(20) When UV light is used for visual fluorescence judgment, do not stare directly at it. As UV light is harmful to the eyes, even watching for a short period can irritate eyes and cause symptoms similar to conjunctivitis. Instead, use a glass screen or wear protective goggles/glasses or a UV-blocking eye mask whenever looking directly at UV light.
(21) Refer to the manual of the incubator. When the HumaLoop M or the Real-Time Turbidimeter HumaTurb C+A is used, remove the reaction tubes from the incubator carefully to avoid burns.

(22) Do not use the PC PV as a positive control of the Loopamp™ MALARIA Pan Detection Kit and the Loopamp™ MALARIA Pf Detection Kit. Do not use PC of other kits as a positive control for this kit.

WASTE DISPOSAL

(1) Do not open the tubes after DNA amplification. Leave the cap closed and dispose of the used tubes as medical waste by incineration or after double bagging with sealable plastic bags.
(2) Never autoclave or reuse the reaction tubes, as amplified products will disperse and cause contamination.
(3) The main material for the reaction tubes and reagent tubes is PP; for the reaction tube tray, PET; for the aluminium pouch, aluminium; and for the kit case, paper.
(4) Dispose of any other reagent, container, or labware per local regulations.

SPECIMEN COLLECTION

(1) Blood samples should be used immediately after collection.
(2) Collect blood in a separate room from the LAMP amplification room. Aerosols containing the Pv DNA can be generated during blood collection and could cause contamination.
(3) DO NOT USE EDTA and citrate as anticoagulants for blood collection if the result is to be read by fluorescence. Instead, the use of heparin as an anticoagulant is recommended.

MATERIALS REQUIRED BUT NOT PROVIDED

- Loopamp™ PURE DNA Extraction Kit (optional)
- HumaHeat (optional)

For visual fluorescence detection

(For HumaLoop M)

- HumaLoop M

(For other incubator using UV light)

- Incubator (temperature accuracy: ±0.5 °C; with hot bonnet)
- Heating block
- UV light or Blue LED light (wavelength: 240–260 nm and 350–370 nm)
- Goggles/glasses or a UV-blocking eye mask (optional)

For real-time turbidity detection

- HumaTurb C+A

For reagent and sample mixing

- Micropipettes (10–100 µL or 20–200 µL) and pipette tips with a filter
- Centrifuge for microtubes (optional)
- Centrifuge for eight connected tubes (optional)
- HuMax ITA, Micro Centrifuge (optional)

PREPARATION OF SAMPLE DNA SOLUTION

To extract the DNA from blood samples, the PURE method and the Boil & Spin method are recommended. For more information, refer to the latest version of "Manual of Standard Operating Procedures for malaria LAMP" (SOP).

Pay attention to the following critical points for the PURE method.

- **Samples : Non-anticoagulated/heparinized whole blood or dried blood spot**
- **Sample volume : 30 µL (whole blood) or 6-mm blood spot punch (dried blood spot)**
- **Additive : Add 30 µL of 334-mM NaCl solution (not included in the Loopamp™ PURE DNA Extraction Kit) to the Heating Tube before heating**
- **Heating : For 5 minutes at 75 °C**

PREPARATION OF REAGENTS

(1) Malaria Pv detection reagent

Remove the required number of tubes from the aluminium pouch and place them on the rack (sum of samples and controls).

Note: After removing the required tubes, re-seal the aluminium pouch with any unused tubes immediately.

(2) Negative control Mal（NC Mal）

Flick (or spin) down the tube to collect the content to the bottom of the tube. Pipette 30 µL of the NC Mal into the Heating Tube provided in the Loopamp™ PURE DNA Extraction Kit. Follow the SOP to process the NC Mal (hereinafter extracted NC Mal is called "negative control solution").

Note: A negative control solution should be used with every run.

(3) Positive control Mal Pv (PC PV)

Flick (or spin) down the tube to collect the content to the bottom of the tube.

Note: The PC PV should be used with every run.

MEASUREMENT PROCEDURE

Reagent and sample mixing

(1) Turn on the HumaLoop M or the Real-Time Turbidimeter HumaTurb C+A.
(2) Dispense 30 µL of the extracted DNA solution into a reaction tube using the Loopamp™ PURE DNA Extraction Kit and close the cap.
(3) Dispense 30 µL of the negative control solution into a reaction tube using the Loopamp™ PURE DNA Extraction Kit and close the cap.
(4) Dispense 30 µL of the PC PV into a reaction tube using a pipette or provided dropper and close the cap.
(5) Flick (or spin) down all tubes to collect the solution to the bottom of the tubes.

Note: Ensure that the liquid level is in the middle of the two lines on a reaction tube to ensure 30 µL of pipetting.

(6) Reconstitute the dried reagents in the cap by inverting the reaction tubes and collecting the DNA solution in the cap. Leave the tubes standing upside down for 2 minutes to reconstitute the dried reagents.
(7) Invert the reaction tubes five times to mix the contents. Ensure that that the dried reagents in the cap are fully dissolved.
(8) Flick (or spin) down all tubes to collect the solution to the bottom of the tubes.

Amplification

For visual fluorescence detection

(For HumaLoop M)

(1) Check that the temperature of the HumaLoop M is 65.0 °C.
(2) Place the reaction tubes in the HumaLoop M and press the green button to start the LAMP reaction (40 minutes at 65.0 °C). Refer to the HumaLoop M instruction manual for details on how to operate the incubator.
(3) Confirm the completion of polymerase inactivation (automatically completed by the HumaLoop M). Take all reaction tubes out of the HumaLoop M.

(For other incubator using UV light)

(1) Set the incubator temperature to 65.0 °C (with hot bonnet temperature set to 10 °C above the reaction temperature or as near to this figure as possible – temperature accuracy: ±0.5 °C). Wait until the displayed temperature reaches the set value.
(2) Place the reaction tubes and then start amplification reaction (for 40 minutes at 65.0 °C).
(3) After 40 minutes, inactivate the polymerase using the heating block (for 5 minutes at 80 °C or 2 minutes at 95 °C) to terminate the reaction.

For real-time turbidity detection with HumaTurb C+A (see the flowchart of the procedure)

(1) Configure the Real-Time Turbidimeter HumaTurb C+A for detection with this product.
(2) Check whether the displayed temperature reaches 65.0 °C (allow the turbidimeter to warm up for 20 minutes before use).
(3) Place the reaction tubes and start measurement.
(4) Watch the turbidimeter display to check the positive and negative controls for any increase in the turbidity. If the turbidity increases in the positive control but not in the negative control, the amplification reaction is proceeding correctly (Fig. 1). If this is not the case, the amplification reaction might be proceeding in the wrong way. In such a case, retest the affected samples.
(5) Confirm the completion of polymerase inactivation (automatically completed by the turbidimeter). Take all reaction tubes out of the Real-Time Turbidimeter HumaTurb C+A and discard them without opening.

Amplification plots by Malaria Pv detection reagent

(Analyzer : Real-Time Turbidimeter HumaTurb C+A)

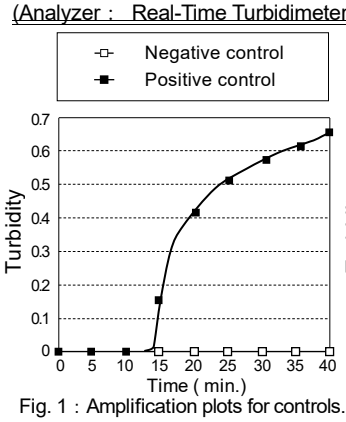

Fig. 1 : Amplification plots for controls.

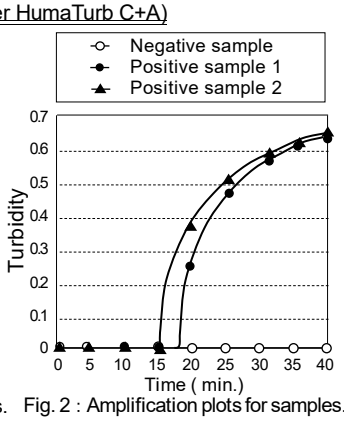

Fig. 2 : Amplification plots for samples.

PROCEDURAL NOTES

(1) The LAMP reaction is very sensitive, and any contamination with even small amounts of the amplified product could lead to false-positive results.
(2) Separate the sample preparation and the amplification areas.
(3) Clean benches with over 0.5% sodium hypochlorite before and after performing the test.
(4) Take all necessary measures to avoid contamination, in particular, change gloves after transferring the blood or if the gloves come in contact with the DNA solution.
(5) When handling this product, avoid microbial contamination and nuclease contamination. Even a small amount of contamination of the reaction tube from sweat or saliva might decompose the DNA and cause a false result.
(6) Furthermore, read the SOP when performing DNA extraction.
(7) The DNA solution should ideally be used immediately after preparation; if it is impossible, the DNA solution can be stored at room temperature and used within 72 hours.
(8) **(For HumaLoop M or other incubator using UV light)**
If bubbles are present, flick (or spin) the tubes to release them.
(For Real-Time Turbidimeter HumaTurb C+A)
As bubbles in the reaction solution may interfere with the turbidity measurement and cause a false result, avoid forming any bubble when mixing reagent and sample solution. If bubbles occur, spin or flick the tube to release them.
(9) The dMAL Pv should be fully dissolved. Any undissolved portion could influence the performance, such as decreasing the sensitivity. In particular, keep the tubes standing upside down for 2 minutes.
(10) The PC PV contains a high copy number of the control DNA. Avoid any contamination of other samples with the PC PV. Dispense the samples and the negative control solution and close all reaction tubes before dispensing the PC PV.
(11) Flick (or spin) down the PC PV tube before opening it to collect the content to the bottom of the tube. Close the tube immediately after dispensing the PC PV.
(12) 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.
(13) When the HumaLoop M or the Real-Time Turbidimeter HumaTurb C+A is used, polymerase inactivation is automatically performed.
(14) For other incubators, when visual fluorescence judgment is chosen, inactivate the polymerase (for 5 minutes at 80 °C or 2 minutes at 95 °C) before reading; else, false results will be caused.
(15) Do not reuse any amplified product in the tubes for electrophoresis or other applications.

INTERPRETATION OF RESULTS

For visual fluorescence detection

(For HumaLoop M)

Place each reaction tube in the Fluorescence Detection Unit, irradiate and observe the tube from the side.

(For other incubator using UV light)

Irradiate the bottom of each reaction tube and observe from the side through goggles/glasses or a UV-blocking eye mask.

For a valid run, the following results must be obtained when read at the specified time:

- Positive control: green fluorescent light is emitted.
- Negative control: no fluorescent 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 fluorescent light is emitted.
- Negative sample: no fluorescent light is emitted.

For real-time turbidity detection with HumaTurb C+A

After confirming that the turbidity increases in the positive control but not in the negative control, evaluate samples per the following criteria (Figs. 1 and 2).

- Positive: some increase is observed in turbidity.
- Negative: no increase is observed in turbidity.

Notes:

(1) The minimum detection sensitivity of the Loopamp™ MALARIA Pv Detection Kit is 7.5 copies per test. In the case of a negative test result, patients with any persisting or worsening symptoms should be considered for retesting, and other possible causes of symptoms should also be considered and investigated. Furthermore, the LAMP assay is highly sensitive and may detect low-level parasitaemia that is not the direct cause of the presenting symptoms. Hence, the patient's clinical condition should always be considered when making a final diagnosis and determining management.
(2) Although the primers have been designed to target a region containing a relatively small number of variations, *Plasmodium vivax* may further acquire variations in this region and become less sensitive to this product. Thus, a negative test does not always rule out infection by Pv.
(3) This is a kit for qualitative detection; it is not designed for quantitative measurement. Therefore, the intensity of the fluorescent light observed or the rise time of turbidity measured by the Real-Time Turbidimeter HumaTurb C+A does not correlate with the template DNA concentration.

INTERFERING SUBSTANCES

Our in-house studies revealed that the turbidimetry measurement was not affected by the presence of Heparin-Na (2,600 units/dL), Heparin-Li (2,600 units/dL), EDTA-2Na (300 mg/dL), EDTA-2K (380 mg/dL), EDTA-3K (340 mg/dL), sodium citrate (7.6%), free bilirubin (66.3 mg/dL), conjugated bilirubin (67.0 mg/dL), chyle (formazine turbidity: 5,433), and haemolytic haemoglobin (1,567 mg/dL). EDTA may cause false-positive results when the result is read by fluorescence.

Regarding drugs, our in-house studies revealed that the measurement was not affected by the presence of proguanil (0.6 µg/mL), chloroquine (1.1 µg/mL), quinine (26.7 µg/mL), doxycycline hydrochloride (10.0 µg/mL), mefloquine (4.7 µg/mL), primaquine (0.5 µg/mL), artemisinin (2.6 µg/mL), loxoprofen sodium (17.7 µg/mL), acetaminophen (9.0 µg/mL), isoniazid (23.3 µg/mL), ethambutol (5.7 µg/mL), rifampicin (26.6 µg/mL), pyrazinamide (116.7 µg/mL), clarithromycin (12.4 µg/mL), streptomycin (133.3 µg/mL), cefotaxime sodium (333.3 µg/mL), and levofloxacin (7.5 µg/mL).

PERFORMANCE CHARACTERISTICS

(1) Accuracy

In testing the following samples:

- Negative sample (concentration: 0 copy/test)
- Positive sample 1 (100 copies/test)
- Positive sample 2 (1,000 copies/test)

The negative sample should test negative, while the positive samples 1 and 2 should test positive.

(2) Within-run reproducibility

In testing five negative and five positive samples simultaneously, the negative samples should test negative throughout, while the positive samples should test positive throughout.

(3) Limit of detection

7.5 copies/test

(4) Cross-reactivity

The measurement system tested positive for *Plasmodium vivax* and negative for other pathogens, as detailed in the table below:

Plasmodium genus	
Plasmodium vivax	Positive
Plasmodium falciparum	Negative
Plasmodium ovale	Negative
Plasmodium malariae	Negative
Plasmodium knowlesi	Negative
Other pathogens	
Trypanosoma brucei	Negative
Trypanosoma cruzi	Negative
Leishmania donovani	Negative
Leishmania chagasi	Negative
Toxoplasma gondii	Negative
Entamoeba histolytica	Negative
Giardia intestinalis	Negative
Theileria parva	Negative
Mycobacterium tuberculosis	Negative
Other	
Human genomic DNA	Negative

(5) Information about a calibrator

The performance test for this product used the plasmid DNA

containing the mitochondrial DNA of Pv as a calibrator.

(6) Clinical performance

Malaria is caused by certain parasites of the *Plasmodium* genus, transmitted via the bites of infected mosquitoes. After developing in the liver for some time, blood-stage parasites are released, which enter red blood cells, lysing them during subsequent reproduction, and cause symptoms, including fever. The LAMP assay detects the DNA from blood-stage parasites.

From 560 blood samples collected in a study in Peru,[6] the DNA was extracted using the Boil & Spin method and tested by the Malaria LAMP using Pv-specific primers. The sensitivity and specificity of the Malaria Pv-specific primers against nested PCR were 84.6% and 92.0%, respectively.

Malaria LAMP vs. nested PCR	
Diagnostic sensitivity	84.6% (78.4-89.6)
Diagnostic specificity	92.0% (88.8-94.5)
Positive predictive value	82.7%
Negative predictive value	92.9%
Likelihood ratio + (sensitivity/(1-specificity))	10.575
Likelihood ratio - ((1-sensitivity)/specificity)	0.1674

ORDERING INFORMATION

Product Code	Product Name	Contents
975000	***Loopamp™ MALARIA Pv Detection Kit***	***96 tests***
962000	HumaLoop M	1 Main Unit 1 Fluorescence Detection Unit
970000	Loopamp™ PURE DNA Extraction Kit	90 tests
963200	HumaTurb C+A	1 Control Unit 1 Amplification Unit
980000	HuMax ITA	Micro Centrifuge
964000	HumaHeat Incubator	Heating Block

NOTICE

In case of occurrence of any serious incident that has occurred in relation to the device shall be reported to the authorised representative, the manufacturer and the competent authority of the Member State in which the user and/or the patient is established.

REFERENCES

1) Notomi T., et al.: Nucleic Acids Research 28, No. 12, e63 (2000)
2) Nagamine K., et al.: Clin. Chem. 47, No. 9, 1742–1743 (2001)
3) Mori Y., et al.: Biochem. Biophys. Res. Commun. 289, No. 1, 150–154 (2001)
4) Tomita N., et al.: Nat. Protoc. 3, No. 5, 877–882 (2008)
5) The guideline for the bio-safety and bio-hazard (by the Japanese Society for Bacteriology): Japanese Journal of Bacteriology 54, No. 3, 667–715 (1999)
6) The study was conducted in collaboration between FIND and the University Peruana Cayetano Heredia (UPCH).

Flow chart

Operation procedure for real-time turbidity detection

Preparation of the sample solution

Prepare sample DNA solutions by extracting the DNA from collected blood samples.

Reagent preparation

Take the required number of reaction tubes of the dMAL Pv.

(For samples, negative and positive controls)

Mixing of the reagent and sample solution

Transfer 30 µL of sample or control solution into each reaction tube.

Use the negative control solution as negative control.
Use the PC PV as positive control.
(The positive control should be prepared at last.)

Invert the reaction tubes to collect the solution in the cap. Leave the tubes standing upside down for 2 minutes.

Invert the reaction tubes five times to mix the contents and then spin them down.

(Avoid making any bubbles.)

Amplification

Place the reaction tubes in the reaction block of the turbidimeter.

As directed in the instructions for use of the turbidimeter, start reaction and measure and evaluate the turbidity (for 40 minutes at 65.0 °C).

Confirm the completion of polymerase inactivation (for 5 minutes at 80 °C or 2 minutes at 95 °C). Take all reaction tubes from the turbidimeter and discard them without opening. Be careful not to damage the tubes.

TABLE OF SYMBOLS

REF Catalog number	Consult instructions for use	Expiration date
IVD *In vitro* diagnostic medical device	Manufacturer	Temperature limitation
LOT Batch code	Contains sufficient for <n> tests	EC REP Authorized Representative in the European Community

IVD CE 0123

Importer

EC REP **HUMAN Gesellschaft für Biochemica und Diagnostica mbH**
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