Standard operating procedures for the Loopamp *Trypanosoma brucei* Detection kit

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Background

Loop-mediated isothermal amplification (LAMP) of DNA was first described by Notomi *et al.* (2000) and has to-date been pursued for diagnosis of a variety of diseases. The test has been used for the detection of human African trypanosomiasis (HAT) in a number of studies (Kuboki et al., 2003). In 2007, Thekisoe *et al.* described LAMP targeting the 5.8S rRNA-internal transcribed spacer 2 (ITS2) gene for detection of *T.b. gambiense*, reporting analytical sensitivity of up to 0.01 trypanosome in the tested sample. The following year, Njiru *et al.* (2008a) published LAMP that amplifies the random insertion mobile element (RIME) that is diagnostic of the sub-genus *Trypanozoon*. The sensitivity observed was 0.001 trypanosomes/ml, attributed to the high copy number of RIME (500 copies/haploid genome; Bhattacharya *et al.* 2002). Subsequent work was targeted to the serum resistance associated (SRA) gene that is specific for *T.b. rhodesiense*; the resultant LAMP test could detect 10 trypanosomes/ml of blood (Njiru *et al*, 2008b).

As a result of its promising performance, the RIME sequences were used as the target in developing the Loopamp *Trypanosoma brucei* Detection kit by FIND and Eiken Chemical Company in Japan, to be used to detect HAT cases. Since the launch of the kit in 2011 during the conference of the International Scientific Council for Trypanosomiasis Research and Control (ISCTRC) in Bamako, Mali (http://www.finddiagnostics.org/media/press/110915.html, accessed 2/11/2013), the kit has been used in a number of clinical and laboratory studies with very promising results (Matovu et al., 2012; Mitachi et al., 2013; Kitibwa et al., 2013; Matovu et al., 2013a,b).

This document describes the Standard Operating Procedures (SOPs) that have been developed based on our experiences with the Loopamp *Trypanosoma brucei* Detection kit. Several methods for preparing the DNA template are described, and are regularly updated as more experience is gained using the kit. The sampling method used depends on the form of disease and the circumstances in the field, i.e., whether samples have to be collected and sent to a reference laboratory where LAMP is installed, or whether the sampling and the test itself are performed in the same place. In the latter case, fresh blood would be the sample to use, as it requires minimal processing. When the kit is used on samples from *T.b. rhodesiense* patients, the best performance has

been observed when samples are diluted 100 times, while for *T.b. gambiense,* the buffy coat diluted 10 times is the preferred sample type.

Most of the laboratory studies have relied on experimental *T. brucei* infections for which it is easy to manipulate parasite numbers in the tested samples. In natural infections, parasitaemia varies depending on the form of disease, and from individual to individual. We have therefore described a procedure for concentrating parasites from the blood in order to increase chances of detecting cases with low parasitaemia. This is important for *T.b. gambiense* HAT, which is generally associated with lower parasitaemia than *T.b. rhodesiense* HAT.

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NB: For all manipulations involving LAMP and human samples, always wear gloves and change them often. Use different gloves for the different stages of the test, i.e., taking sample from patient, preparing sample for the reaction (heat treatment, centrifugation), preparing the kit itself, and adding samples into the LAMP reaction tube. If several technicians are available, consider assigning each of the tasks to a different person. They could regularly interchange roles as deemed necessary.

SOP 1.0: Standard Operating Procedures for Preparation of Template for use with the Loopamp *Trypanosoma brucei* Detection kit

Date of issue : 23 March 2012 (updated on 8 November 2013)

Purpose: This SOP provides guidance for preparation of templates for loop-mediated isothermal amplification (LAMP) of DNA. The test must be performed following the manufacturer's instructions.

Applicability: The designated Quality Assurance/Quality Control (QA/QC) Officer, Laboratory Personnel, Laboratory Supervisors and Project Coordinator/Principal Investigator (PI).

A) Materials and Supplies

Pipettes

Either of the pipettes below is required

1. 10-100 µl **OR**
2. 20-200 µl

Pipette tips

All tips should preferably be those with filters

1. Sterile pipette tips (0-200 µl, "medium tips")
2. Sterile pipette tips (100-1000 µl)

Other supplies

1. Heparinized tubes or capillaries
2. Eppendorf tubes
3. Whatman filter paper (we have only tested Whatman Qualitative circles, cat#1001)
4. 15ml Falcon tubes

B) Equipment

1. Eppendorf centrifuge
2. Bench centrifuge
3. Water bath or heating block
4. Freezer (if long-term storage is required)

C) Procedures

i. Template from whole blood

1. Take 10 µl of heparinized blood.
2. Add to an Eppendorf tube containing 90 µl sterile/distilled/PCR grade water.
3. Incubate in a water bath or heating block set at 90^{o}C for 10 minutes.
4. Spin the tube for 3 minutes at maximum speed in an Eppendorf centrifuge (If not possible let the tube stand upright for 10 min).
5. Pipette the required template (5 µl) from near the surface (to avoid transferring debris into the reaction).
6. For future use, pipette off supernatant into a clean sterile Eppendorf tube and store frozen.

ii. Whole Blood Treated with SDS

1. Put 475 µl blood into a sterile Eppendorf tube.
2. Add 25 µl of 10% SDS solution (final SDS concentration=0.5%).
3. Close the tube.
4. Mix well by gently inverting 10 times.
5. Allow to stand for 10 minutes at room temperature.
6. Mix again as in 4 above.
7. Apply 100 µl on a labelled filter paper, OR.
8. Apply 20 µl on a microscopy slide and prepare a smear as in v below.

9. For template using fresh blood treated with SDS, take 10 µl and process as in C) i above.

iii. Template from red blood cell lysed and concentrated blood

1. Pipette 9 ml of Qiagen RBC lysis solution (cat. # 158902) into a 15 ml Falcon tube.
2. Add 3 ml of heparinized whole blood to the Falcon tube containing the lysis solution.
3. After gentle but thorough mixing, allow to stand for 10 minutes at room temperature. The red blood cells in the sample will be lysed, leaving white blood cells and any parasites intact.
4. Centrifuge the tubes at 1,000 x *g* for 15 minutes to form a pellet.

   Note: The centrifugation speed in revolutions per minute (RPM) that should be used to obtain an acceleration of 1,000 x g can be calculated using the following formula:

   Speed (RPM) $= \sqrt{10^8 / (1.118 \times R)}$

   where R is the radius of the centrifuge rotor in cm (from center of rotor to sample).
5. Take 200 µl of supernatant from the centrifuged tube and keep it in a separate tube.
6. Decant all the remaining supernatant from the centrifuged tube.
7. Re-suspend the pellet in the 200 µl supernatant from step 5 above.
8. Take 10 µl and process as in C) i above, for use in the LAMP reaction.

iv. Preparing Whatman filter papers with whole or lysed blood

A large Whatman filter paper can be folded twice and cut with scissors to give 4 pieces.

1. Label a clean Whatman filter paper with the sample code.
2. Carefully spread 100 µl of whole blood/or lysate obtained in C) ii 7 above onto the paper using a pipette. Start from the centre of the paper, slowly spreading outwards in a circular manner until the whole sample is absorbed.
3. Air dry for 30 minutes at room temperature (you can rest it on clean disposable tissue).
4. Enclose the paper in a sealable plastic or paper bag containing silica. Each sample must be stored separately to avoid cross-contamination.
5. Store in a dry place.

v. Preparing microscopy slides using whole blood

1. Take 20 µl blood.
2. Spread it onto a labelled slide to form a well spread out smear.
3. Allow to air dry for 30 minutes.
4. Store in slide box until ready to use.

vi. Preparing buffy coat from blood

1. Take 3-5 ml of heparinized blood into a centrifuge tube (preferably a 5 ml Falcon or Nunc cryotube to give best separation).
2. Spin at 800 x *g* for 10 minutes.
3. Use a Pasteur pipette to carefully remove the plasma, leaving the buffy coat undisturbed.
4. Then carefully pipette off the buffy coat into a clean tube (it will go with some residual plasma; aim to collect more than 100 µl of buffy coat/plasma mixture).
5. Mix the buffy coat with the residual plasma to get a homogeneous mixture. This tube is the stock of buffy coat.
6. Transfer 114 µl of stock buffy coat to a tube containing 6 µl of SDS 10% (final SDS concentration=0.5%)
7. Close the tube.
8. Mix well by gently inverting 10 times.
9. Allow to stand for 10 minutes at room temperature.
10. Mix again as in 8 above.
11.

Options for using the SDS-lysed buffy coat obtained under 10.:

a) Take 10 µl of lysed buffy coat and process it for the LAMP reaction as in C) i above, **OR**

b) Spread 100 µl of lysed buffy coat onto a Whatman filter paper as in C) iv above, **OR**

c) Store at -20°C, -80°C or in liquid nitrogen for future use

vii. Preparing template from a Whatman filter paper spotted with whole/lysed blood or buffy coat

1. Use a disc punch to cut one 7mm disc from the filter paper, and let it drop into a labeled Eppendorf tube.
2. Add 50 µl double-distilled water.

3. Incubate at 90°C for 10 min.
4. Spin for 3 minutes at max speed to pellet the filter disc and debris (optional).
5. Pipette 5 µl of template from near the surface and put it into the LAMP reaction tube.
6. Pipette the remaining supernatant from step 5 into a labeled sterile Eppendorf tube and store in a freezer (-20°C, or -80°C) for future use.

viii. Preparing template from microscopy slides

1. Take 100 µl PCR grade water.
2. Add to cover the entire blood smear.
3. Allow to stand for 2 minutes.
4. Pipette up and down 5 times to extract all the contents of the soaked smear.
5. Put the suspension into a labeled Eppendorf tube.
6. Heat at 90°C for 10 minutes.
7. Centrifuge at maximum speed for 3 minutes.
8. Pipette 5 µl of template from near the surface and put into the LAMP reaction tube.

SOP 2.0: Standard Operating Procedures for the Loopamp *Trypanosoma brucei* Detection kit

Date of issue : 23 March 2012 (updated on 8 November 2013)

Purpose: This SOP provides guidance for performing the loop-mediated isothermal amplification (LAMP) of DNA for trypanosome detection using Loopamp *Trypanosoma brucei* Detection kit developed by Eiken Co.

Applicability: The designated Quality Assurance/Quality Control (QA/QC) Officer, Laboratory Personnel, Laboratory Supervisors and Project Coordinator/Principal Investigator (PI).

Materials and Equipment

Pipettes

The following pipettes are required

1. 0-10 µl (1 pipette for LAMP area)
2. 10-100 µl or 20-200 µl (1 for LAMP reagent preparation area and 1 for template preparation area)

Pipette tips

All tips should preferably be those with filters

1. Sterile pipette tips (0-10 µl, “small tips”)
2. Sterile pipette tips (0-200 µl, “medium tips”)

Equipment

1. LAMP incubator
2. Centrifuge

Designated experimental areas:

1. Sample preparation area

2. Reagent preparation area
3. LAMP area

A) Preparing the template

Refer to SOP 1.0 for preparation of various templates; 5 µl should be used in each reaction.

B) Preparing the reaction tubes

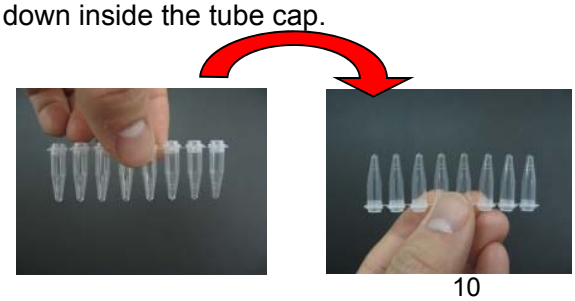
The Loopamp *Trypanosoma brucei* Detection kit is supplied as strings of 8 tubes each with the reagents dried down on the insides of the tube caps. One sealed pouch contains six (6) such strings. The LAMP incubator takes a maximum of 16 tubes in a single run. For every run there should be a tube for a negative and a positive control. This means a maximum of 14 patient samples can be tested in a single run.

1. Prepare a list of serial numbers of the reaction tubes and the corresponding samples you will add into each tube.
2. Carefully remove one string of 8 vials from the sealed pouch, then seal the pouch again.
3. Label each vial with the serial numbers.
4. Place the vials into the reaction tube stand.
5. Add 25µl of the 'negative control' buffer into the tube labeled "-" and immediately close it.
6. Add 20µl of 'negative control' control buffer into each test reaction tube.
7. Add 5µl of the sample (crude template) into each corresponding reaction tube.
8. Close the tubes immediately.
9. Add 25µl of 'positive control' buffer to its designated tube, close immediately and label "+".

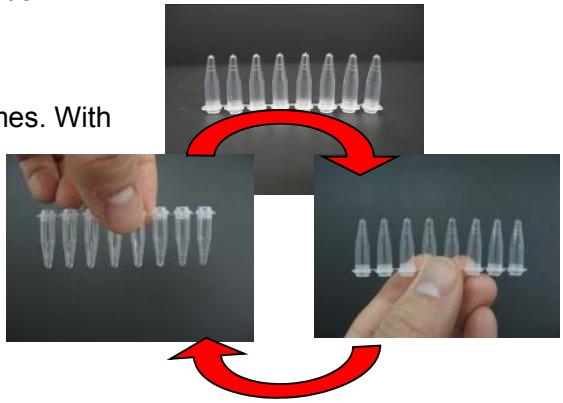
C) Amplification reaction

Once all reaction tubes have been filled and all lids have been closed, the sample has to be mixed with the reaction master mix that is dried down inside the tube cap.

1. Pick the string of reaction tubes from the reaction tube stand. Turn the tubes upside down, and shake firmly so that the solution moves into the inside of the tube caps.



2. Place the reaction tubes cap-side down on the lab bench for 2 minutes. This allows the solution to reconstitute the dried LAMP reagents inside the tube caps. Make sure no fluid is stuck at the end of the reaction tube.



3. Invert (turn upside down) the reaction tubes 5 times. With each inversion, shake the tubes so that the solution transfers from the caps to the bottoms of the tubes, or from the bottoms to the caps. This ensures proper mixing and dissolving of the LAMP reagent.

4. Finally, shake the reaction tubes downward to collect the solution at the bottom of the tubes.

5. Immediately place the reaction tubes in the LAMP incubator and close the bonnet.

6. Press the "start" switch on the LAMP incubator to start the LAMP reaction. Set the timer for 40 minutes.

7. When the beep signals completion of the reaction, press the "stop" switch on the LAMP incubator.

8. Remove the tubes from the LAMP incubator and insert them in the fluorescence unit.

E) Reading results

- The sample is positive if fluorescence is present (sample glows); this means the sample contains parasite DNA.
- The sample is negative if there is no fluorescence (sample does not glow); this means the sample does not contain trypanosome DNA.
- Be sure to check the positive and negative controls for the expected results. If the controls do not show the expected results, the test results are invalid (false).
- Record your results into the lab note book.

F) Interpretation of results

1. Positive reactions exhibit fluorescence under blue light.
2. Negative reactions remain clear (do not fluoresce under blue light).

G) Quality Control Measures

NB: Results of the LAMP assay are read by fluorescence and do not require any manipulation of the amplification product. The reaction tube should therefore not be opened after the reaction, to avoid contamination by amplicon. However, contamination cannot be totally avoided and could occur either during manipulation of samples, through gloves, door handles, lab coats, water taps or sharing of pipettes.

1. Laboratory coats and disposable latex gloves must be worn at all times while working.
2. Set up the reactions in special areas on the bench or dedicated room.
3. Wipe the working bench with a dilute solution of sodium hypochlorite before setting up. NB: sodium hypochlorite is corrosive and should not come in contact with your samples or pipettes.
4. Any spills must be cleaned up immediately with copious amounts of liquid disinfectants.
5. Change disposable latex gloves often as you work.
6. All waste (tubes, pipette tips, gloves, paper towels) must be disposed of in a biohazard bag, and autoclaved prior to disposal.