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.

References

1. Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe, Amino N and Hase T (2000) Loop-mediated isothermal amplification of DNA. Nucleic. Acid. Res. 28, E63.
2. Kuboki, N., N. Inoue, T. Sakurai, F. Cello, D. J. Grab, H. Suzuki, C. Sugimoto and I. Igarashi (2003). Loop-Mediated Isothermal Amplification for Detection of African Trypanosomes. J. Clin. Microbiol. 41: 5517-5524.
3. Thekisoe OMM, Kuboki N, Nambota A, Fujisaki K, Sugimoto C, Igarashi I, Yasuda J, and Inoue I (2007) Species-specific loop-mediated isothermal amplification (LAMP) for diagnosis of trypanosomosis. Acta Trop. 102: 182-189.
4. Njiru ZK, Mikosza ASJ, Matovu E, Enyaru JCK, Ouma JO, Kibona SM, Thompson RCA, and Ndung'u JM (2008a). African trypanosomiasis: Sensitive and rapid detection of the sub-genus *Trypanozoon* by loop-mediated isothermal amplification (LAMP) of parasite DNA. Int. J. Parasitol. 38: 589-599.
5. Bhattacharya S, Barke A and Bhattacharya A (2002) Mobile genetic elements in protozoan parasites. J. Genetics 81, 73–86.
6. Njiru ZK, Mikosza ASJ, Armstrong T, Enyaru JCK, Ndung'u JM and Thompson RCA (2008b). Loop-Mediated Isothermal Amplification (LAMP) Method for Rapid Detection of *Trypanosoma brucei rhodesiense*. PLoS NTD 2, e147.
7. First field-based molecular diagnostic test for African sleeping sickness in sight. http://www.finddiagnostics.org/media/press/110915.html. Accessed 2/11/2013.
8. Matovu E., Kazibwe A.J., Mugasa C.M., Ndung'u J.M. and Njiru Z.K. (2012). Towards point-of-care diagnostic and staging tools for human African trypanosomiasis. J Trop Med: Article ID 340538.
9. Mitashi P, Hasker E, Mumba Ngoyi D, Pyana PP, Lejon V, Van der Veken W, Lutumba P, Buscher P, Boelaert M, Deborggraeve S. (2013) PLoS Negl Trop Dis 7(10): e2504. doi:10.1371/journal.pntd.0002504
10. Kitibwa A, Matovu E, Kazibwe A, Boobo A, Bieler S and Ndungu J (2013) Comparison of classical LAMP, LoopAmp *Trypanosoma brucei Detection* kit and PCR for detection of trypanosome DNA in experimental infections. *ISCTRC Conference, Khartoum, Sudan 8-12 September 2013.*
11. Matovu E, Mugasa C, Waiswa P, Kitibwa A, Boobo A, Kazibwe A and Ndungu J (2013a) Setting a model for elimination *Trypanosoma brucei rhodesiense* human African trypanosomiasis in Uganda. *ISCTRC Conference, Khartoum, Sudan 8-12 September 2013.*
12. Matovu E, Ojom J, Edielu A, Kitibwa A, Bieler S and Ndungu J (2013b) Evaluation of the LoopAmp kit for detection of *Trypanosoma brucei rhodesiense* in crude extracts of patient blood. *ISCTRC Conference, Khartoum, Sudan 8-12 September 2013.*

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 2.0: Standard Operating Procedures for Preparation of Template for use with the Loopamp™ *Trypanosoma brucei* detection kit

Version 2.1: Microscopy Center

	Name	**Signature**	**Date**
Prepared by	Enock Matovu	EM	04 July 2011
Reviewed by	Enock Matovu	EM	20 July 2015
Reviewed by	Enock Matovu	EM	25 May 2016
Reviewed by	Joseph Ndungu	JN	27 May 2016
Reviewed by	Sylvain Biéler	SB	3 June 2016
Approved by	Joseph Ndung'u	JN	10 June 2016

Purpose: This SOP provides guidance for preparation of templates for loop-mediated isothermal amplification (LAMP) of DNA using the Loopamp™ *Trypanosoma brucei* detection kit

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. 10-100µl **or** 20-200µl
2. 100-1000µl

Pipette tips

All tips must be those with filters

1. Sterile pipette tips (10-200µl, yellow tips)
2. Sterile pipette tips (100-1000µl, blue tips)

The tips for transferring buffy coat should preferably be wide mouthed, or cut with scissors about 5mm from the pointed end.

Other Supplies

1. Heparinised tubes
2. Eppendorf tubes
3. Whatman filter paper (Whatman Qualitative circles, cat#1001)
4. 15ml Falcon tubes

B) Equipment

1. Bench centrifuge

C) Procedures

Notes:

1. Prepare **one sample of whole blood** and **one of buffy coat** from each patient.
2. These will be spread onto filter papers after treatment with 5% SDS.
3. Samples dried on filter papers must be transferred to the LAMP centre as soon as possible after being collected.
4. All samples must be tested with LAMP within two weeks from the date of collection

i. Lysing Whole Blood with 5% SDS Solution

1. Take 450µl of heparinised blood into an eppendorf tube
2. Add 50µl of 5% SDS solution
3. Close the tube
4. Mix well by gently inverting 5 times
5. Allow to stand for 5 minutes at room temperature
6. Mix again as in 4 above
7. Use 100µl each to spread on 2 labelled filter papers as described in **iv** below

ii. Preparing Buffy Coat from Whole Blood

1. Take 5ml of heparinized blood into a centrifuge tube (a thin tube should be preferred for good cell separation)
2. Spin at 4000rpm (or 1000g) for 10 minutes
3. With a Pasteur pipette, carefully remove the plasma to leave the buffy coat undisturbed
4. Then carefully pipette off the buffy coat into a clean Eppendorf tube (it will go with some residual plasma; aim to have about 500µl of buffy coat/plasma suspension). If you use a pipette tip, ensure that it is wide mouthed.
5. Mix the buffy coat with the plasma to get a homogeneous mixture

iii. Lysing Buffy Coat with 5% SDS solution

1. With a pipette, determine the total volume of buffy coat collected
2. Add 1/9 volume of 5% SDS solution (Final SDS concentration=0.5%) e.g. 10µl SDS into 90µl buffy coat, 20µl SDS into 180µl buffy coat, etc.
3. Close the tube

4. Mix well by gently inverting 5 times
5. Allow to stand for 5 minutes at room temperature
6. Mix again as in 4 above
7. Use 100µl each to spread onto 2 labelled filter papers as described in **iv** below

iv. Preparing Whatman Filter papers with SDS-Lysed Blood or Buffy Coat

1. Label 2 clean Whatman filter papers each with the participant name, microscopy centre and date. At the end add **B** (for blood) or **BC** (for Buffy Coat)

2. Carefully spread 100µl of the lysate obtained in Ci and Ciii above onto each filter paper using a pipette. Start from the centre of the paper, and slowly spread 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 disposable tissue or hang with a peg, **taking care not to contaminate the sample!**)

4. Enclose each paper together with a silica gel sachet in individual sealable plastic bags/pouches

5. Store dry at room temperature in an air-tight container

6. Transfer the sealed filter papers to the LAMP centre as soon as possible

SOP 2.0: Standard Operating Procedures for Preparation of Template for use with the Loopamp™ *Trypanosoma brucei* detection kit			
Version 2.2: LAMP Centre			
	Name	**Signature**	**Date**
Prepared by	Enock Matovu	EM	04 July 2011
Reviewed by	Enock Matovu	EM	20 July 2015
Reviewed by	Enock Matovu	EM	25 May 2016
Reviewed by	Joseph Ndung'u	JN	26 May 2016
Reviewed by	Sylvain Biéler	SB	3 June 2016
Approved by	Joseph Ndung'u	JN	10 June 2016

Purpose: This SOP provides guidance for preparation of templates for loop-mediated isothermal amplification (LAMP) of DNA using the Loopamp™ *Trypanosoma brucei* detection kit

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. 1-10µl or 1-20µl
2. 10-100µl **or** 20-200µl
3. 100-1000µl

Pipette tips

All tips must be those with filters

1. Sterile pipette tips (1-20µl, white tips)
2. Sterile pipette tips (10-200µl, yellow tips)
3. Sterile pipette tips (100-1000µl, blue tips)

The tips for transferring buffy coat should preferably be wide mouthed, or cut with scissors about 5mm from the pointed end.

Other Supplies

1. Heparinised tubes
2. Eppendorf tubes
3. Whatman filter paper (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 will be required)

C) Procedures

Notes:

1. Prepare **one sample of whole blood** and **one of buffy coat** from each patient.
2. The LAMP centre will only test freshly lysed samples prepared at their centre, and filter papers from the other sites.
3. All samples on filter papers must be tested with LAMP within two weeks from the date of collection. Fresh samples prepared at the LAMP centre must be analysed on the day of sample collection, or kept frozen until analysis.
4. The working solution for lysis of samples is 5% SDS. If supplied with a 10% stock, start by diluting an aliquot of 1:1 with PCR water. In an eppendorf tube labelled with 5% SDS and date, pipette 500ul water and then add 500ul of the supplied 10% SDS solution.

i. Lysing Whole Blood with 5% SDS Solution

1. Take 450µl of heparinised blood into an eppendorf tube
2. Add 50µl of 5% SDS solution
3. Close the tube
4. Mix well by gently inverting 5 times
5. Allow to stand for 5 minutes at room temperature
6. Mix again as in 4 above
7. Process 20µl each for the LAMP reaction as described in **v** below

ii. Preparing Buffy Coat from Whole Blood

1. Take 5ml of heparinized blood into a centrifuge tube (a thin tube should be preferred for good cell separation)
2. Spin at 4000rpm (or 1000g) for 10 minutes
3. With a Pasteur pipette, carefully remove the plasma to leave the buffy coat undisturbed
4. Then carefully pipette off the buffy coat into a clean eppendorf tube (it will go with some residual plasma; aim to have about 500µl of buffy coat/plasma suspension). If you use a pipette tip, ensure that it is wide mouthed.
5. Mix the buffy coat with the plasma to get a homogeneous mixture.

iii. Lysing the Buffy Coat with 5% SDS solution

1. With a pipette, determine the total volume of buffy coat collected
2. Add 1/9 volume of 5% SDS solution (final SDS concentration=0.5%) e.g. 10µl SDS into 90µl buffy coat, 20µl SDS into 180µl buffy coat, etc.
3. Close the tube
4. Mix well by gently inverting 5 times
5. Allow to stand for 5 minutes at room temperature
6. Mix again as in 4 above
7. Process 20µl each for the LAMP reaction as described in **v** below

iv. Preparing Template from Whatman Filter Papers with SDS Lysed Blood or Buffy Coats

NB: This is only done for samples delivered from other centres. If a patient is at the LAMP centre, only freshly lysed blood and buffy coats are to be tested.

1. Cut one 7mm disc from the filter paper, using a disc punch, into a labeled eppendorf tube
2. Add 100µl double distilled water or PCR water
3. Heat at 90^{o}C for 10 min
4. Spin for 3 minutes at max speed to pellet the filter discs (optional)
5. Pipette template (5µl) off the surface and introduce into the LAMP reaction

v. Preparing Template from Freshly Lysed Blood or Buffy Coat

1. Label a clean eppendorf tube with participant codes, indicating with **B** or **BC**
2. Add 80µl of PCR water
3. To each tube add 20µl of the freshly lysed blood (in Ci above) or buffy coat (in Ciii above). **Observe patience as you pipette the viscous solution so that the required volume gets into the tip, preferably use a wide mouthed tip.**
4. Close the tube and heat at 90^{o}C for 10 min
5. Centrifuge for 3 minutes at max speed to pellet any cell debris
6. Pipette 5µl off the supernatant and introduce into the LAMP reaction

SOP 3.0: Standard Operating Procedures for LAMP			
	Name	**Signature**	**Date**
Prepared by	Enock Matovu	EM	02 March 2011
Reviewed by	Enock Matovu	EM	25 March 2013
Reviewed by	Sylvain Biéler	SB	3 June 2016
Approved by	Joseph Ndung'u	JN	10 June 2016

Purpose: This SOP provides guidance for performing the loop-mediated isothermal amplification (LAMP) of DNA using the Loopamp™ *Trypanosoma brucei* detection kit

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

Pipette tips
All tips must be those with filters
1. Sterile pipette tips (1-10 µl, "white tips")
2. Sterile pipette tips (10-200 µl, "yellow tips")

Equipment
1. LAMP incubator (with visual read-out or real-time reader)
2. Centrifuge

Designated experimental areas:
1. Sample (template) preparation area
2. Reagent preparation area
3. LAMP area

A) Template preparation
Refer to SOP 2.0 for preparation of the various templates. 5µl of test sample should be used in each reaction

B) Preparing the reaction tubes

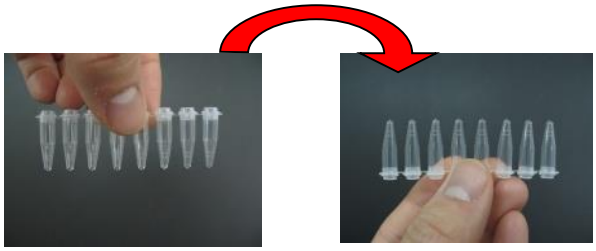
The LAMP kit is supplied as strings of 8 reaction tubes each. The Eiken LF-160 LAMP incubator can take a maximum of 16 tubes in a single run. For every run there should be a tube for negative and positive control. This means a maximum of 14 patient samples can be tested in a single run.

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 –ve 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

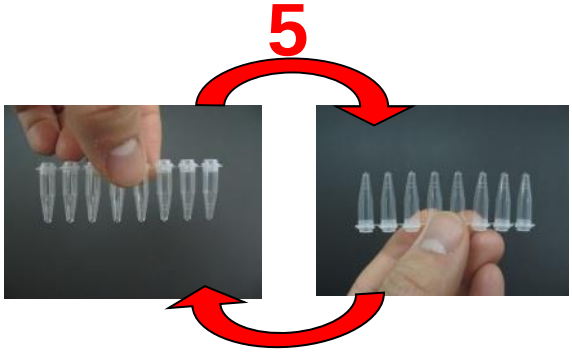
Once all reaction tubes have been filled and all lids have been closed, the sample needs to be mixed with the reaction master mix contained in the lid of the tube.

1) Pick up the row 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 reagent 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 bottom 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 into the bottom of the tubes.

5) Immediately place the reaction tubes into 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 the completion of the reaction, press the "stop" switch on the LAMP incubator.

8) Remove the tubes from the LAMP incubator and place them on the fluorescence unit.

D) Reading results

Turn on the fluorescent light by pressing the small button on the left of the fluorescence unit and observe the fluorescence of the samples.

- 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) and have to be repeated.
- Record your results into the lab note book

E) Quality control measures

NB: Contamination with LAMP amplicon or carryover template is mainly through gloves, door handles, lab coats, water taps and sharing of pipettes.

1. Laboratory coat and disposable latex gloves must be worn at all times while working.
2. Set up reactions in special areas on the bench or dedicated chamber.
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 the yellow biohazard bag, and autoclaved prior to disposal.