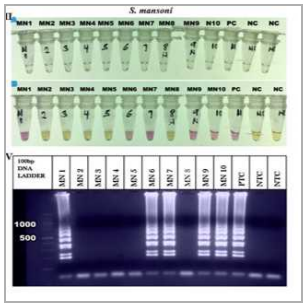

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Schistosome DNA Isolation and Colorimetric LAMP protocol for the detection of Schistosomiasis

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ABSTRACT

Schistosomiasis keeps devastating the world despite many measures put in place by control programs. Molecular diagnostics has played a significant role in the detection and monitoring of the treatment of the disease. Loop-medicated isothermal amplification has been developed with increased sensitivity and specificity to help the diagnosis of schistosomiasis. This protocol aids the successful isolation of schistosome DNA from urine and stool using Quick-DNA Miniprep kits (ZymoResearch, USA) with slight modification. Progressively, specific-specific Schisto-LAMP assay has been developed from the cytochrome c oxidase subunit 1 (COX1) gene of the schistosome mitochondrial genome. In addition, to effectively detect successful amplification, a laboratory-based colorimetric buffer which is pH dependent, was used. The neutral red dye changes from pale yellow to pink when positive and to a deep yellow color if negative. This protocol spells out the performance of two LAMP assays to detect *S. haematobium* and *S. mansoni* in urine and stool, respectively.

GUIDELINES

1. Schistosoma-DNA Extraction from Stool (Zymo Kits) Protocol
2. Schistosoma-DNA Extraction from Urine (Zymo Kit Plus) Protocol
3. Preparation of 2X colorimetric solution mix protocol
4. Preparation of 50 mM Neutral Red Dye Stock protocol.
5. Preparation of 25X Dye Stocks of Neutral red protocol.
6. Preparation of 10X SCH-LAMP Primer mix protocol
7. Preparation of Colourimetric LAMP reaction protocol
8. Agarose Gel Electrophoresis Protocol

MATERIALS TEXT

DNA isolation

- Quick-DNA Miniprep kits (ZymoResearch, USA)
- Sterile 1.5ml microcentrifuge tube
- Sterile 2.0ml microcentrifuge tube
- Phosphate buffered saline tablet (PBS) – 500g
- polyvinylpolypyrrolidone (PVPP) - 500g
- Dithiothreitol (DTT)
- Proteinase K
- Cryobox
- Nuclease-free water
- Glass beads: 1mm and 0.2-0.4mm

Polymerase Chain Reaction

- PCR tube (8 strip)
- OneTaq® 2X Master Mix with Standard Buffer (New England Biolabs)
- 1 M Tris-HCl, pH 7.5 (500 mL)
- dNTP mix

Colorimetric Buffer

- 2 M KCl
- 100 mM MgSO4
- Tween-20
- Betaine
- KOH
- 50 mL Falcon tubes
- Autoclavable Lab Bottles (500mL)
- Neutral red powder

LAMP Reaction

- LAMP Primer (SH and SM)
- Bst. 2.0 WarmStart DNA polymerase (8000 units)

Consumables

- Filtered tips 100-1000µL
- Filtered tips 2-20µL
- Filtered tips 20-200µL
- Yellow tips (1000/bag)
- Tips clear 0.1-10µL (1000/bag)

Agarose Gel

- NaOH
- Disodium ethylenediamine tetraacetate.$2H_2O$ (EDTA)
- Tris-Base
- Acetic acid
- Ethidium Bromide (EtBr)
- Bromophenol Blue
- Xylene Cyanol FF
- Glycerol
- DNA Ladder
- Agarose powder
- Glass beaker or flask

Equipment

- Vortex mixer
- Incubator
- Centrifuge
- MagNa Lyser
- Thermocycler (Gradient)
- Heating block
- Micropipette (10, 20, 200, 1000µL)
- Microwave
- Horizontal Gel electrophoresis apparatus
- UV transilluminator

SAFETY WARNINGS

SAFETY WARNINGS

Please handle ethidium bromide with care.

BEFORE START INSTRUCTIONS

Make sure urine and stool specimens are well stored since storage can affect the integrity of the DNA.
Please make to sterilize all consumables to be used for this experiment.
Please work in a sterile environment to prevent carryover contamination.
pH adjustment of the colorimetric buffer must be made with a well-cleaned pH meter to prevent contamination.

Schistosoma-DNA Extraction from Stool (Zymo Kits) Protocol

1 0.12-gram stool is suspended in 300µL of PBS (1X phosphate-buffered saline) with 2% PVPP (polyvinylpolypyrrolidone) in a 2ml microcentrifuge tube containing glass beads and vortex.

2 Freeze the sample for 1 hour at -50 ºC or overnight at -20 ºC.

3 Defrost and bead beat in MagNA lyser for 5 minutes at 3000 rpm.

4 Add 400µL Genomic Lysis Buffer and 10µL Proteinase K to the mixture and mix completely by vortexing for 15-30 seconds, then incubate at 56ºC overnight (14-16 hours).

5 Mix by vortexing at 3000rpm for 15-30 seconds and centrifuge at 10,000 x g for one minute.

6 Transfer the supernatant to a Zymo-Spin™ IIC Column in a Collection Tube. Centrifuge at 10,000 x g for one minute. Discard the Collection Tube with the flow-through.

7 Transfer the Zymo-Spin™ IIC Column to a new Collection Tube. Add 200µl of DNA Pre-Wash Buffer to the spin column. Centrifuge at 10,000 x g for one minute.

8 Add 500µl of g-DNA Wash Buffer to the spin column. Centrifuge at 10,000 x g for one minute. Repeat the centrifugation to dry the spin column.

9 Transfer the spin column to a clean microcentrifuge tube. Add 70µl DNA Elution Buffer to the spin column. Incubate for 15-30

minutes at room temperature and then centrifuge at top speed for 30 seconds to elute the DNA.

10 Repeat the elution step into a different microcentrifuge tube as the second elution. The eluted DNA can be used immediately for molecular-based applications or stored ≤-20ºC for future use.

Schistosoma-DNA Extraction from Urine (Zymo Kit Plus) Protocol

11 Mix urine sample by pipetting up and down, and aliquot 300µL in a sterile 1.5ml microcentrifuge.

12 Add 200µL of PBS (1X phosphate-buffered saline) with 2% PVPP (polyvinylpolypyrrolidone) to the sample and vortex.

13 Add 300µL BioFluid & Cell Buffer and 10µL Proteinase K to the mixture and mix completely by vortexing for 15-30 seconds, then incubate at 56ºC overnight (14-16 hours).

14 Mix by vortexing at 3000rpm for 15-30 seconds and centrifuge at 10,000 x g for one minute.

15 Add 300µL Genomic Binding Buffer to the digested sample. Mix thoroughly or vortex for 10-15 seconds.

16 Transfer the supernatant to a Zymo-Spin™ IIC Column in a Collection Tube. Centrifuge at 10,000 x g for one minute. Discard the Collection Tube with the flow-through.

17 Transfer the Zymo-Spin™ IIC Column to a new Collection Tube. Add 200µl of DNA Pre-Wash Buffer to the spin column. Centrifuge at 10,000 x g for one minute.

18 Add 500µl of g-DNA Wash Buffer to the spin column. Centrifuge at 10,000 x g for one minute. Repeat the centrifugation to dry the spin column.

19 Transfer the spin column to a clean microcentrifuge tube. Add 70µl DNA Elution Buffer to the spin column. Incubate for 15-30 minutes at room temperature and then centrifuge at top speed for 30 seconds to elute the DNA.

20 Repeat the elution step again in a different microcentrifuge tube as the second elution. The eluted DNA can be used immediately for molecular-based applications or stored ≤-20ºC for future use.

Preparation of 2X colorimetric solution mix protocol

21

A	B	C	D
Component	Volume (µL)	2X Concentration	1X Concentration
1 M Tris-HCl, pH 7.5	10	2 mM	1 mM
10 mM dNTP mix	1400	2.8 mM	1.4 mM
2 M KCl	250	100 mM	50 mM
100 mM MgSO4	800	16 mM	8 mM
Tween-20	10	0.2% v/v	0.1% v/v
Betaine	0.938g	1.6 M	0.8 M
ddH2O	1500		
Total Volume	5000		

Note: Adjust pH to 8.6 – 8.8 with 1 M KOH, and top up with ddH_2O to the 5000 µL mark.

Preparation of 50 mM Neutral Red Dye Stock protocol.

22 Weigh 0.7222g of Neutral red powder in a beaker and dissolve in 40 mL ddH_2O.

23 Mix thoroughly with a magnetic stirrer, and adjust the pH to 8.6 – 8.8 with 1 M KOH.

24 Top up the solution to the 50 mL mark with ddH_2O.

Preparation of 25X Dye Stocks of Neutral red protocol.

25

A	B	C

A	B	C
Component	Volume (µL)	25X Concentration
50 mM Stock Dye solution	50	2.5 mM
ddH2O	950	
Total Volume	1000	

Preparation of 10X SCH-LAMP Primer mix protocol

26

A	B	C
Species	Primer Name	Sequence, 5' - 3'
S. haematobium	Sh-COX1-Loop-F	ATCCATAATACCCAAACGATG
	Sh-COX1-Loop-B	GGTTGGTTTAGATTATTTGACTGC
	Sh-COX1-F3	CTGGATTTGGAATAGTTAGTCAT
	Sh-COX1-B3	TTTATACCTGTAGGAATCCCTAT
	Sh-COX1-FIP	TCGAAGCCATAGCACAAATCAAGAGGATAAGTAATAATGATTCATCG
	Sh-COX1-BIP	GCTTAGGAAGTGTAGTTTGAGCCCACTGAACTAAAAAATATAGCAGTC
S. mansoni	Sm-COX1-Loop-F	TCAAAACATAAACCTCTGGAT
	Sm-COX1-Loop-B	TAGTATGCTTAGGTAGAGTAG
	Sm-COX1-F3	CGATCCTATTTTGTTTCAGCATT
	Sm-COX1-B3	TCCAGTTAACGAATCAAAGC
	Sm-COX1-FIP	CCTAACTATACCAAAACCCGGAAGATTTTGGTTTTTTGGTCATCCA
	Sm-COX1-BIP	GGATTGATTTGCGCTATGGCTCATAAACATATGATGACCCCAT

A	B	C
Component	Volume (µL)	1X Concentration
100 µM FIP	32	1.6 µM
100 µM BIP	32	1.6 µM
100 µM F3	4	0.2 µM
100 µM B3	4	0.2 µM
100 µM Loop-F	8	0.4 µM
100 µM Loop-B	8	0.4 µ
ddH2O	112	
Total Volume	200	

Note: LAMP primer mix should be prepared independently for the two schistosome species

Preparation of Colourimetric LAMP reaction protocol

27

A	B	C
Component	Volume (µL)/rxn	Master mix volume (µL)/100 rxn
2X Colorimetric solution	10	1000
10X LAMP primer mix	2	200
2.5 mM Neutral red dye	0.8	80
ddH2O	2.4	240
Bst. 2.0 WarmStart DNA polymerase (8000 units)	0.8	80
DNA Template	4	*
Total Volume	20	

Note 1.: Do not centrifuge the master mix; only pulse vortex to mix thoroughly. This is because the centrifugation will precipitate the neutral red dye reducing its concentration in the solution, thus, making it non-uniform. This is done before the addition of the DNA template. Again, do not centrifuge the mixture after adding the sample DNA. Instead, mix briefly with the pipette tip.

Note 2.: A standard LAMP incubation protocol is set at 63°C(*S. haematobium* detection) and 65°C (*S. mansoni* detection) for 90 minutes, followed by 80°C for 20 minutes to inactivate the enzyme. A positive LAMP amplification shows a pink color change from pale or light yellow (before incubation). A negative LAMP assay shows a deep yellow color.

Agarose Gel Electrophoresis Protocol

28 The successful LAMP amplification can be confirmed with agarose gel electrophoresis and visualized under UV light. The steps for the gel preparation is as follows;

Step 28 includes a Step case.

Reagent Preparation

Gel Pre-Staining

Gel Preparation Procedure