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LAMP-PCR targeting HSP70 for screening Chagas disease

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Sneider Gutierrez[1], Jessy Condori[2], Shirley Equilia[3], Edith Malaga[2], Jean Karla Velarde[3], Luciana Basma[4]

[1]Johns Hopkins University Bloomberg School of Public Health, Baltimore, MD, USA.;
[2]Universidad Peruana Cayetano Heredia, Lima, Perú.; [3]Asociación Benéfica Prisma (PRISMA). Lima, Perú;
[4]Universidad Católica Boliviana San Pablo. Santa Cruz, Bolivia

Sneider Gutierrez
Jons Hopkins University

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We use this protocol and it's working

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Abstract

The Loop-mediated isothermal amplification LAMP-PCR$_{TcHSP70}$ assay described here targets the *HSP70* gene of *T. cruzi* (Gene ID: TcCLB.511211.170). It has been designed to identify multiple *T. cruzi* lineages without cross-reacting with *T. rangeli* or *Leishmania* spp.

Guidelines

LAMP-PCR$_{TcHSP70}$ assay can be run using Sybr green or using the Cas12a system.

Materials

- 1.5 mL vials
- 8-vial 0.2 mL PCR strips
- 1.5 mL racks
- 0.2 mL racks
- 1000 µL tips
- 200 µL tips
- 20 µL tips
- 10 µL extra -ong tips
- Thermo block compatible with PCR tubes (eg. Benchmark BSH1002 Corning LSE digital dry bath heater with block 6 × 0.2 mL Strips)
- 1.5 mL and 0.2 mL tubes spinner (eg. Benchmark C1012 MyFuge12 Mini Centrifuge CN Z742583)
- Vortex
- Phone camera
- Blue LED lamp (only when using Cas12a system)

Troubleshooting

Safety warnings

- Do not open amplified products in the area where the master mix is prepared or DNA is seeded.

Before start

- Make sure all materials and reagents are available and prepared as described in the reagent preparation section

- Thaw the DNA samples in advance and keep them at 4°C.

Reagents list

1 **List of reagents required for the LAMP-PCR TcHSP70 assay.** Some reagents are essential, while others are exclusively needed when integrating the Cas12a system.

1.1 **Primers for the LAMP-PCR$_{TcHSP70}$ assay:**

	A	B	C	D	E	F	G	H
	Primer	**Label**	**Sequence 5-3′**	**Primer length**	**Purification method**	**DNA Oligo**	Essential	
	TcH_1	FIP (F1c-F2)	TCGCGCTGTTCCTTGTCCTGCGGCCTGAGCAAGGCGGACATTG	43	Standard Desalting	25 nmole	Yes	
	TcH_2	BIP (B1c-B2)	CGGTCTTGAGAACTACGCATTTTCGTGTCGGCCTCCTCAATCTTG	45	Standard Desalting	25 nmole	Yes	
	TcH_3	F3	AAGCGCAACCAGATTGTCATCACG	24	Standard Desalting	25 nmole	Yes	
	TcH_4	B3	TCCACGGCACTCGTAATCGTGT	22	Standard Desalting	25 nmole	Yes	
	TcH_5	FL	CTTGGCAGCCTCGGACACCA	20	Standard Desalting	25 nmole	Yes	
	TcH_6	BL	TAAACGAGCCGAACGTCGC	19	Standard Desalting	25 nmole	Yes	

1.2 General reagents for LAMP-PCR$_{TcHSP70}$ assay

	A	B	C	D	E
	Reagent	**Concentration**	**Privider**	**Catalog number**	Essential
	Ultrapure H2O	NA	Sigma Aldrich	W4502	Yes
	Deoxynucleotide (dNTP) Solution Mix	10mM	New England Biolab	N0447S	Yes
	Magnesium Sulfate (MgSO4) Solution	100mM	New England Biolab	B1003S	Yes
	Betaine	5M	Sigma Aldrich	B0300-5VL	Yes
	Isothermal Amplification buffer	20mM	New England Biolab	B0537S	Yes
	Bst 2.0 DNA Polymerase - 8000 units	10X	New England Biolab	M0537L	Yes
	SYBR™ Green I Nucleic Acid Gel Stain - 10,000X concentrate in DMSO. Catalog number: S7563	10,000X	Thermo Fisher Scientific	S7563	Yes

1.3 Reagents for LAMP-PCR$_{TcHSP70}$ assay coupled to Cas12a system

	A	B	C	D	E
	Reagent	**Concentration**	**Privider**	**Catalog number**	**Essential**
	NEBuffer™ r2.1	10 X	Nee England Biolabs	B6002S	Only for Cas12a system
	Alt-R™ L.b. Cas12a (Cpf1) Ultra	100 µg	IDT	na	Only for Cas12a system

	A	B	C	D	E
	ssDNA probe: /56-FAM/TTATTATT/3IABkFQ/	250 nmole DNA Oligo	IDT	customized	Only for Cas12a system
	Alt-R™ L.b. Cas12a crRNA, Standard Desalting IE HPLC Purification*	10 nmol	IDT	customized	Only for Cas12a system
	RNAase Inhibitor	20 U/ µl	Thermo Fisher Scientific	FIC-N8080119	Only for Cas12a system
	Mineral oil	na	Sigma Aldrich	M8662	Only fo Cas12a system

* Alt-R™ L.b. Cas12a crRNA sequence:
/AlTR1/rUrArArUrUrUrCrUrArCrUrArArGrUrGrUrArGrArUrCrGrUrCrArArUrGrCrGrCrUrCrGrCrGrCrUrGrU/AlTR2/

Reagents preparation

2 Preparing Reagent Stock Solutions: Reagents not described here do not require additional preparation.

2.1 Primer Preparation

- Spin down lyophilized primers (SG11-SG16) for 3 minutes at 1000xg.
- Resuspend primers at a concentration of 100 µM in ultrapure H_2O.
- Vortex for 5 seconds.
- Take 100 µL of the 100 µM stock solution and dilute it with 900 µL of ultrapure water to prepare a 10 µM stock solution.
- Vortex for 5 seconds.
- Spin down.
- Prepare 200 µL aliquots of each individual primer at 10 µM to avoid multiple freeze-thaw cycles.
- Store at -20°C until use.

2.2 Preparing additional reagents for LAMP-PCR$_{TcHSP70}$ assay

Diluting SYBR™ Green I Nucleic Acid Gel Stain - 10,000X

- Prepare a dilution of 1:10 in molecular water.
- Vortex for 5 seconds.
- Make aliquots of 200µL.
- Store aliquots protected from light at -20°C until use

2.3 **Preparing additional reagents for LAMP-PCR$_{TcHSP70}$ assay coupled to the Cas12a system**

***Reconstitution of* Alt-R™ L.b. Cas12a (Cpf1) Ultra**

- The Alt-R™ L.b. Cas12a is provided in solution at 10 µg/µL. 100 µg of LbCas12a Ultra nuclease = 670 pmol, equivalent to an initial concentration of 67 µM.
- For each 10 uL of 67 µM Cas12a add 660 of ultrapure H2O to prepare a 1 µM stock solution.
- Vortex for 5 seconds.
- Store at -20°C until use.

***Reconstitution of* ssDNA probe:** /56-FAM/TTATTATT/3IABkFQ/ 250 nmol

- Spin down the lyophilized sDNA probe for 3 minutes at 1000xg.
- Add 392 µL of ultrapure water to the lyophilized ssDNA probe to make a 100 µM stock solution. Mix well to ensure complete dissolution.
- To prepare a 10 µM working stock, dilute the 100 µM stock solution by adding 10 µL of the 100 µM solution to 90 µL of ultrapure water.
- Prepare 10 µL aliquots of the 10 µM working stock.
- Store aliquots protected from light at -20°C until use.

Reconstitution of Lyophilized crRNA: *Alt-R™ L.b. Cas12a crRNA, 10 nmol*

- Spin down lyophilized crRNA for 3 minutes at 1000xg.
- Add 1 mL of ultrapure water to the lyophilized crRNA to make a 10 µM stock solution. Vortex for 5 seconds.
- Take 100 µL of the 10 µM stock solution and dilute it with 900 µL of ultrapure water to prepare a 1 µM working solution.
- Aliquot 100 µL of the 1 µM solution into separate tubes.
- Store aliquots at -20°C until use, or at -80°C for long-term storage.

RNAase Inhibitor at 20 U/µL

- Prepare aliquots of 50 uL to avoid multiple thawing cycles.
- Store aliquots at -20°C until use.

Preparing the master mix

3

Thaw all reagents at room temperature except by the enzymes and gRNA.

Mix the reagents at room temperature as described in the following table:

	A	B	C	D
	Primer ID	**Work concentration**	**Stock concentration**	**µL for 1 reaction**
	Primer SG_11-FIP	40 pmol	10 µM	4
	Primer SG_12-BIP	40 pmol	10 µM	4
	Primer SG_13-F3	5 pmol	10 µM	0.5
	Primer SG_14-B3	5 pmol	10 µM	0.5
	Primer SG_15-LF	5 pmol	10 µM	0.5
	Primer SG_16-LB	5 pmol	10 µM	0.5
	Betaine	1M	5M	5
	Ultrapure H2O	NA	NA	3.38
	Isothermal amplification buffer	1X	10X	2.52
	Bst pol 2.0	8U	8,000 units/ml	1
	MgSO4	0.8mM	100mM	0.8
	dNTPs	2.5mM	10 mM	0.5

Vortex the mix for 3 seconds

Spin down

Aliquot 23 µL of the master mix per vial of 0.2 mL and use immediately or store it at -20°C for up to 8 months until use.

Note: It is recommended to use 8-vial 0.2 mL PCR strips, as they facilitate taking pictures for tube alignment. To make tube handling easier, it is also recommended to use scissors to cut them into sets of four vials. This facilitates opening and closing the tubes when loading SYBR Green and DNA.

When using the Cas12a system instead of SYBR Green

Prepare the Cas12a-gRNA complex by mixing the reagents described in the following table:

	A	B	C
	Reagent	**Stock concentration**	**µL for 1 reaction**
	NEB Buffer r2.1	10x	4
	gRNA	1 uM	2.5
	lbCas12a	1 uM	2.5
	ssDNA probes	10 uM	3
	RNAase Inhibitor	4U/uL	0
	Water	n/a	8

Incubate the mix at 37 °C for 40 minutes to generate the Cas12a-gRNA complex

DNA loading

4
- To 23 µL of the previously prepared master mix, add 2 µL of DNA and mix by pipetting ten times.

The DNA seeding can be performed at room temperature when using the Bst2 polymerase.

- Replace the DNA template with 2 µL of ultrapure water for the negative control.
- Replace the DNA template with 2 µL of positive control (DNA extracted from *T. cruzi*)

4.1 **When using SYBR green for visualization:**

- Add 2 µL of 1:10 diluted SYBR Green I fluorescent dye to the lid of the tube.
- Carefully close the tube.
- Proceed to the incubation section.

4.2 **When using the Cas12a system for visualization:**

- Add 35 µL of mineral oil.

- Add 20 µL of the Cas12a-gRNA complex to the lid of the tube.
- Carefully close the tube.
- Proceed to the incubation section.

Incubation

5 Incubation time changes depending on the visualization method

5.1 **When using SYBR green for visualization:**

- Incubate the reaction at 65 °C for 60 min.

5.2 **When using the Cas12a system for visualization:**

- Incubate the reaction at 65 °C for 40 min.
- Spin down the reaction mixture for 5-10 seconds to combine Cas12a-gRNA complex and the LAMP reaction.
- Incubate again at 37 degrees C for 20 minutes.

Visualization

6 Two options for visualization

6.1 **When using SYBR green for visualization:**

- Spin down the incubated reaction and register the change of color from “brown” to “green” for positive amplification:

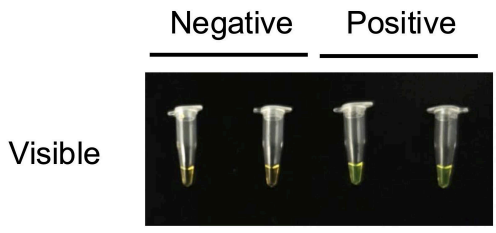

Figure 1. Visualization of LAMP-Amplified Products with the Naked Eye

6.2 **When using the Cas12a system for visualization:**

- Spin down the incubated reaction and visualize the tubes under LED blue light.

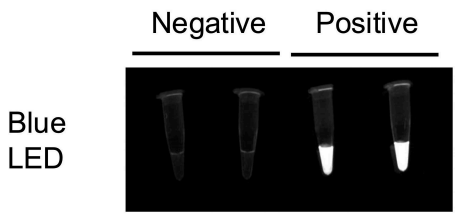

Figure 2. Visualization of LAMP-Amplified Products Using the Cas12a System and Blue LED Light

Agarose gel

7 - Before opening the tube inactivate the enzymes at 80°C for 10 minutes to stop the reaction.

Note: open the tubes in an area exclusively dedicated for electrophoresis.

- Mix 3 µL of the amplified products with 3 µL of 2X loading buffer.
- Load the mix on a 2% agarose gel, pre-stained with 0.2 µg/mL ethidium.
- Load 3 µL of 100 bp DNA Ladder for product size reference.
- Run the electrophoresis at 100V for 30-35 minutes

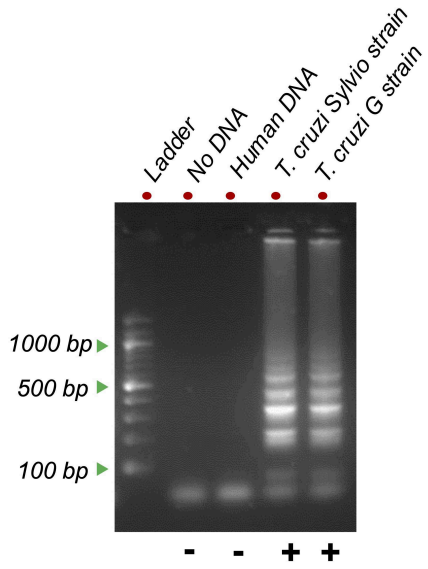

Figure 3. Amplification Products Visualized on Agarose Gel. No amplification (-). Positive amplification (+).

Protocol references

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