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DNA extraction and qPCR protocol for sensitive detection of *Trypanosoma cruzi* in blood

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Brooke E. White[1], Rick Tarleton[1]

[1]University of Georgia

Brooke E. White
University of Georgia

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We use this protocol and it's working

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Abstract

This protocol summarizes all steps for "deep-sampling" DNA samples extracted from blood that is screened for presence of *Trypanosoma cruzi*. Through a combination of careful handling, large 384 well capacity qPCR instruments, and DNA fragmentation, this protocol allows highly sensitive detection of the agent that causes Chagas disease.

Guidelines

Contamination Precautions: All steps should be performed in dedicated Biosafety Cabinet. All equipment should be reserved for this use only and not shared between other projects or areas of the lab.
Spray work surface with 70% ethanol and apply UV light to cabinet at least 15 minutes before beginning work.
Use LookOut DNA Erase spray to supplement cleaning cabinet surface.
Using multichannel pipettes is an essential part of contamination control for our protocol. When 384 well plates are used, pipetting 96 wells at one time is preferred (we like the Integra mini 96).
All components (primers, probes, itaq mix) for qPCR master mix should be aliquoted into single use tubes when possible to avoid repeated opening of the same tube. This also prevents multiple freeze/thaw cycles for qPCR components.
When working with large amounts of *T. cruzi* DNA - such as when preparing amplification standards- prepare and process these separately from normal samples. Processing samples with very high amounts of *T. cruzi* DNA (higher than would normally be encountered in host blood) poses a high risk for contamination of other samples, even when taking all precautions outlined here. Do not extract DNA from high parasite standard samples at the same time as blood samples being tested for parasite DNA.

Materials

Kits:
Omega Biotek E.Z.N.A. Blood DNA Maxi Kit (item # D2492-03)

Chemicals and reagents:
PBS (Gibco 10010023)
100 % ethanol
LookOut DNA Erase spray
BioRad iTaq Universal Probes Supermix
Nuclease free water (Ambion AM9937)
Primers
Taqman probes
IAC (g-block from IDT)

Consumables and other material:
50 mL conical tubes
1.5 mL snap cap tubes
0.5 mL snap cap tubes
96-Well Polypropylene PCR Plates, Low Profile, Thin Wall, Skirted, White/Clear
or Hard-Shell 384-Well PCR Plates, thin wall, skirted, clear/white #HSP3805
Pipette basins
Microseal 'B' PCR Plate Sealing Film, adhesive, optical #MSB1001

Equipment:
Nanodrop
vortex mixer
pipettes and appropriate filter tips
Biosafety cabinet (UV sterilization)
swing bucket centrifuge with capacity for 15ml and 50ml conical tubes
qPCR software Bio-Rad CFX Manager Software (version 3.1)
Shake-N-Bake instrument set to 70°C (other incubator equipment can also work here: it just needs to be able to reach 70°C and have a moveable platform to mix samples during incubation)
C1000 Touch Bio-Rad CFX96 real-time PCR detection system or
CFX Opus 384 Real-Time PCR System (#12011452)
Bio-rad mini chiller with variable speed pump (170-3654)
Branson SFX250 Sonifier with support stand and converter clamp
3 inch Cuphorn attachment
Integra Mini 96 pipettor

Before start

Setting up a dedicated work space inside a Type II Biosafety Cabinet away from common pathogen DNA producing parts of the lab is key. Using this dedicated clean space and following the contamination prevention guidelines described in this protocol have enabled us to extract hundreds of DNA samples and run thousands of pcr reactions while avoiding cross contamination.
Note: See "Guidelines" section of this protocol for more details on contamination prevention.

1. Pre-heat Shake-N-Bake (or similar incubator) to 70°C.
2. Place 50 mL conical tube of elution buffer in incubator.
3. Turn on cuphorn chiller to pre-chill at 4°C.
4. Prepare all reagents for DNA extraction. Add isopropanol and ethanol to respective buffers as directed in E.Z.N.A. maxi kit.

DNA extraction

2h 3m

1 Take 3-10 mL blood freshly collected in K_2EDTA (purple top) blood collection tubes preserved on ice and centrifuge at 2000 g for 15 minutes to separate plasma from blood cells. 15m
Note: If blood is previously separated and frozen, thaw completely before starting extraction.

2 Transfer plasma layer to storage tube(s) with pre-printed labels detailing sample information and date and store at -80 for use as needed. 15m

3 **DNA Extraction Part 1 (Lysis)**

3.1 Transfer the cell pellet from blood into a nuclease-free 50 mL conical tube and bring the volume up to 10mL with PBS
Note: You can use plasma, whole blood, or the cell pellet here but we have found the cell pellet to be the most sensitive.

3.2 Add 250 µL Proteinase K solution (included with kit)

3.3 Vortex to mix well.
Note: Tubes should be tightly closed before vortexing.

3.4 Add 10.2 mL BL Buffer (included with kit).

3.5 Add 20 µL RNase A (Included with kit).

3.6 Vortex to mix thoroughly.

3.7 Incubate at 70 °C in Shake-N-Bake incubator for 30 minutes, vortexing the tube once at the halfway point. 30m
Note: Shaker platform should be on medium rocking speed during incubation.

4 DNA Extraction Part 2 (DNA isolation)

4.1 Add 10.3 mL 100% ethanol to the lysed blood mixture and vortex to mix.

4.2 Insert a column into a 50mL collection tube, both from the kit.

4.3 Transfer the lysed sample to the column, including any precipitates.

4.4 Centrifuge column and tube at 4,000 rcf for 5 minutes. 5m

4.5 Repeat Steps 4.3 + 4.4 until entire sample is transferred. Each transfer will be approximately 15 mL. 5m

4.6 Discard the filtrate in the tube, retaining the collection tube.

4.7 Add 5 mL HBC Buffer (included with kit) to the column.
Note: HBC Buffer must be diluted w/ isopropanol before use.

4.8 Centrifuge at 4,000 rcf for 3 minutes. 3m

4.9 Discard the filtrate and retain the collection tube.

4.10 Add 15 mL DNA Wash Buffer (included with kit) to the column.
Note: Wash Buffer must be diluted w/ethanol before use.

4.11 Centrifuge at 4,000 rcf for 5 minutes. 5m

4.12 Discard filtrate and reuse the collection tube.

4.13 Add 15 mL DNA Wash Buffer for a second wash step.

4.14 Centrifuge at 4,000 rcf for 5 minutes. 5m

4.15 Discard filtrate and reuse the collection tube.

4.16 Centrifuge the empty column at 4,000 rcf for 10 minutes to dry the column. 10m
Note: This is critical to remove any trace ethanol that could interfere with downstream applications.

4.17 Transfer the column into a new nuclease-free 50 mL tube
Note: Conical tube not provided with kit

4.18 Dry the column further at 70 °C for 10 minutes inside the Shake-N-Bake incubator. 10m

4.19 Wipe down the inside of the centrifuge with bleach followed by water to prevent corrosion.
Buckets and bucket inserts should also be washed thoroughly during this time.
Note: This step is important for contamination prevention.

4.20 Add 500 µL HOT (70°C) Elution Buffer (included with kit) directly to the filter inside the column.

4.21 Let sit at room temperature for 3-5 minutes. 5m

4.22 Centrifuge at 4,000 rcf for 5 minutes. 5m

4.23 Transfer eluted volume back onto the filter inside the column and add 150 µL fresh Elution Buffer.

4.24 Let sit at room temperature for 3-5 minutes. 5m

4.25 Centrifuge at 4,000 rcf for 5 minutes. 5m

4.26 Transfer eluted material into nuclease-free 1.5 mL microcentrifuge tube.

4.27 Measure DNA quantity.

4.28 Store eluted DNA at -20°C (or 4°C if planning PCR soon).

DNA Fragmentation with Cuphorn Sonication 30m

5 Wrap 1.5 mL microcentrifuge tubes containing extracted DNA in parafilm

5.1 Place samples in floating foam rack

5.2 Place in chilled cuphorn

5.3 Set sonifier to continuous mode with a time interval of 50 seconds and 60% amplitude

5.4 Start sonifier and repeat sonication 5 times for a total of 5 cycles of 50 seconds with 60% amplitude

5.5 Place samples back on ice and continue preparations for qPCR

qPCR SETUP 3h

6 After fragmenting, dilute DNA to desired concentration (200 ng/µl) in ultrapure water. Preferably in 96 well plates to make transfer to qpcr plate easier. 30m

7 Recipe for master mix for each pcr plate 20m

	A	B	C
	Component	Volume per 20µl reaction	Volume per 96 well plate (106 reactions)
	premix itaq univeral probe mix	10	1060
	Cruzi 1 primer (10 µM)	1.5	159
	Cruzi 2 primer (10 µM)	1.5	159
	IAC primer FW (10 µM)	0.2	21.2
	IAC primer Rev (10 µM)	0.2	21.2
	Cruzi 3 probe	0.1	10.6
	IAC probe	0.1	10.6
	Water	0.4	42.4

8 Add 14 µl mastermix + 1 µl IAC (25 pg/ µl) + 5 µl diluted sample DNA to each well 10m

8.1 Include no template controls (NTC) and a sample with known *T.cruzi* DNA concentration in each plate.
Note: For our typical assay, we run samples in 384 well plates and include 16 NTC reactions in each plate.

8.2 Spin down briefly before beginning cycler program.

9 Follow cycling conditions listed below. Be sure to select "all channels" when running protocol 2h

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	A	B
	PCR temperature	time
	95	3 min
	95	15 sec
	58	1 min
	repeat steps 2 and 3 ×50	

conditions for Bio Rad Opus 384

11 Export program file at the end of protocol and analyze using CFX Manager software

Supplementary Information

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	A	B
	Component	sequence
	Cruzi 1	ASTCGGCTGATCGTTTTCGA
	Cruzi 2	AATTCCTCCAAGCAGCGGATA
	IAC F	ACCGTCATGGAACAGCACGTA
	IAC R	CTCCCGCAACAAACCCTATAAAT
	Cruzi3 probe	5'-Fam-CACACACTGGACACCAA-NFQ-MGB-3'
	IAC probe	5′ -VIC-AGCATCTGTTCTTGAAGGT-NFQ-MGB-3′

Primer and probe details

12.1

	A	B
	IAC (Internal Amplification control) (g-block from IDT)	TGTTCTTAAAGTAACCGTCATGGAACAGCACGTACCGATTTA TAAGATTGCTGGAGAAATGACTGGATTTGGAGCATCTGTTCT TGAAGGTGTTTTAGCTTTCGTCTTGGTTTATACTGTGTTCAC GGCTAGCGATCCCAGACGTGGGCTACCTTTAGCAGTGGGAC CTATATTTATAGGGTTTGTTGCGGGAGCCAATGTTCTAGCCG C

IAC details

Protocol references

* Cruzi 1/2/3 primers/probe are from Duffy et al 2009, 2013 PLoS NTD, as originally from Piron et al 2007 Acta Trop; IAC primers/probe are from Duffy et al 2009, 2013 PLoS NTD