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Title of resource
GN_21: Protocol for analysis of FTA cards for *Leishmania* parasites
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DOI
Description
Protocol for analysis of FTA cards for *Leishmania* parasites. Describes the process of gDNA or RNA extraction from FTA cards (from lab or field work), and real time PCR for detection of parasites. Limits of detection for the PCR assay are also provided. Protocol from the Gnatwork project "The use of FTA cards to monitor *Leishmania* infection and infectiousness in sand flies and midges" created for processing of samples collected during laboratory work at LSHTM and field work in Ethiopia and Ghana (Oct 2018 – Sep 2019).
Intended use
Scientific research use and training purposes.
Restrictions on use
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Resource history
N/A

GN_21: Protocol for analysis of FTA cards for *Leishmania* parasites

When using this protocol, the following should be referenced:
Mojca Kristan, Tom Walker, Matt Rogers (London School of Hygiene and Tropical Medicine)

A. Introduction

This protocol outlines the steps required to extract nucleic acids (gDNA or RNA) from FTA cards and determine the presence of *Leishmania* parasites using real time PCR.

B. Materials

Equipment and Reagents

- Qiagen 96 collection tubules (cat no. 19560)
- Qiagen RNA 96 extraction kits (cat no. 74182)
- Qiagen DNeasy 96 Blood & Tissue Kits (cat no. 69582)
- AirPore Tape Sheet
- SYBR Green Master mix (Roche Diagnostics)
- qPCR machine
- Applied Biosystems High Capacity cDNA Reverse Transcription kit
- FastStart SYBR Green Master mix (Roche Diagnostics)

C. Method

Preparation of FTA cards prior to nucleic acid extraction

C.1 Individual FTA cards are cut into four quarters to allow the segments to be placed within Qiagen 96 collection tubules (cat no. 19560) that are compatible with both Qiagen RNA 96 extraction kits (cat no. 74182) and Qiagen DNeasy 96 Blood & Tissue Kits (cat no. 69582). To account for variation in the location of saliva expectorated on the FTA cards, all four segments should be included allowing the extraction of 24 FTA cards in a 96 well extraction format.

gDNA extraction

D.1 A working solution of 40 µl proteinase K + 360 µL Buffer ATL from the Qiagen DNeasy 96 Blood & Tissue Kits is added to Qiagen 96 collection tubules containing FTA card segments (fully submerging the cards).

D.2 Samples are incubated at 56°C for 24 hours. Place a weight on top of the caps during the incubation. Mix occasionally during incubation to disperse the sample, or place on a rocking platform.

D.3 Carefully remove the caps and add 400 µl Buffer AL-ethanol (50:50 mix) to each sample.

D.4 Place two DNeasy 96 plates on top of S-Blocks. Mark DNeasy 96 plates for later sample identification. Remove and discard the caps from the collection microtubes. Carefully transfer the lysis mixture of each sample to each well of the DNeasy 96 plates.

D.5 Seal each DNeasy 96 plate with an AirPore Tape Sheet. Centrifuge for 4 min at 6000 rpm.

D.6 Remove the tape. Carefully add 500 µl Buffer AW1 to each sample. Note: Ensure that ethanol has been added to Buffer AW1 prior to use.

D.7 Seal each DNeasy 96 plate with new AirPore Tape Sheet. Centrifuge for 2 min at 6000 rpm.

D.8 Remove tape. Carefully add 500 µl Buffer AW2 to each sample. Note: Ensure that ethanol has been added to Buffer AW2 prior to use.

D.9 Centrifuge for 15 min at 6000 rpm. Do not seal the plate with AirPore Tape.

D.10 Place each DNeasy 96 plate in correct orientation on a new rack of Elution Microtubes.

D.11 To elute the DNA, add 100 µl Buffer AE to each sample, and seal the DNeasy 96 plates with new AirPore Tape Sheets. Incubate for 1 min at room temperature (15–25°C). Centrifuge for 4 min at 6000 rpm.

D.12 Use new caps or sealing foil to seal the Elution Microtubes for storage at -20° or -80°C.

RNA extraction and generation of cDNA

E.1 Add 300 µl of Buffer RLT to Qiagen 96 collection tubules containing FTA card segments (submerging the cards).

E.2 Add 1 volume (300 µl) of 70% ethanol to each sample and mix by pipetting up and down 3 times.

E.3 Place two RNeasy 96 plates on top of S-Blocks. Mark RNeasy 96 plates for later sample identification.

E.4 Remove and discard the caps from the collection microtubes. Carefully transfer the lysis mixture of each sample to each well of the RNeasy 96 plates.

E.5 Seal each RNeasy 96 plate with AirPore Tape Sheet. Centrifuge for 4 min at 6000 rpm.

E.6 Remove the tape. Carefully add 500 µl Buffer RW1 to each sample.

E.7 Seal each RNeasy 96 plate with AirPore Tape Sheet. Centrifuge for 4 min at 6000 rpm.

E.8 Remove the tape. Carefully add 500 µl Buffer RPE to each sample.

E.9 Seal each RNeasy 96 plate with AirPore Tape Sheet. Centrifuge for 10 min at 6000 rpm.

E.10 Remove the tape. Carefully add 500 µl Buffer RPE to each sample.

E.11 Place each RNeasy 96 plate in correct orientation on a new rack of Elution Microtubes.

E.12 To elute the RNA, add 45 µl RNase-free water to each sample and seal the RNeasy 96 plates with new AirPore Tape Sheets. Incubate for 1 min at room temperature (15–25°C). Centrifuge for 4 min at 6000 rpm.

E.13 Use new caps or sealing foil to seal the Elution Microtubes for storage at -80°C.

E.14 To generate cDNA, RNA is reverse transcribed using an Applied Biosystems High Capacity cDNA Reverse Transcription kit. A final volume of 20 µl contains 10 µl RNA, 2 µl 10X RT buffer, 0.8 µl 25X dNTP (100 mM), 2 µl 10X random primers, 1µl reverse transcriptase and 4.2 µl nuclease-free water.

E.15 Reverse transcription is undertaken in a Thermal Cycler as follows: 25°C for 10 min, 37°C for 120 min and 85°C for 5 min and cDNA stored at –20°C.

Real time PCR analysis for detection of *Leishmania* parasites

F.1 qRT-PCR is undertaken targeting small hydrophilic endoplasmic reticulum-associated protein (*sherp*) and small subunit ribosomal RNA (*ssrRNA*) gene transcripts [1]*.

F.2 Reactions are prepared using 5 µl of FastStart SYBR Green Master mix (Roche Diagnostics), a final concentration of 1 µM of each primer, 1 µl of PCR grade water and 2 µl template DNA (or cDNA), to a final reaction volume of 10 µl.

F.3 Prepared reactions are run on a Roche LightCycler® 96 System for 15 minutes at 95°C, followed by 40 cycles of 95°C for 5 sec, 55°C for 5 secs and 72°C for 5 secs.

F.4 Amplification is followed by a dissociation curve (95°C for 10 seconds, 65°C for 60 seconds and 97°C for 1 second) to ensure the correct target sequence was being amplified.

F.5 PCR results are analysed using the LightCycler® 96 software (Roche Diagnostics).

G. References

1. *Giraud, E., *et al*. (2019). Quantifying Leishmania Metacyclic Promastigotes from Individual Sandfly Bites Reveals the Efficiency of Vector Transmission. *Commun Biol*, 2: 84.

Limit of detection

1. RNA was extracted from cultured *L. mexicana* parasites using Qiagen RNeasy mini kits

2. cDNA generated and ten-fold serial dilutions created (lowest dilution = 10^7 dilution of neat cDNA) and estimated limit of 2-3 parasites for sherp gene (Figure 1).

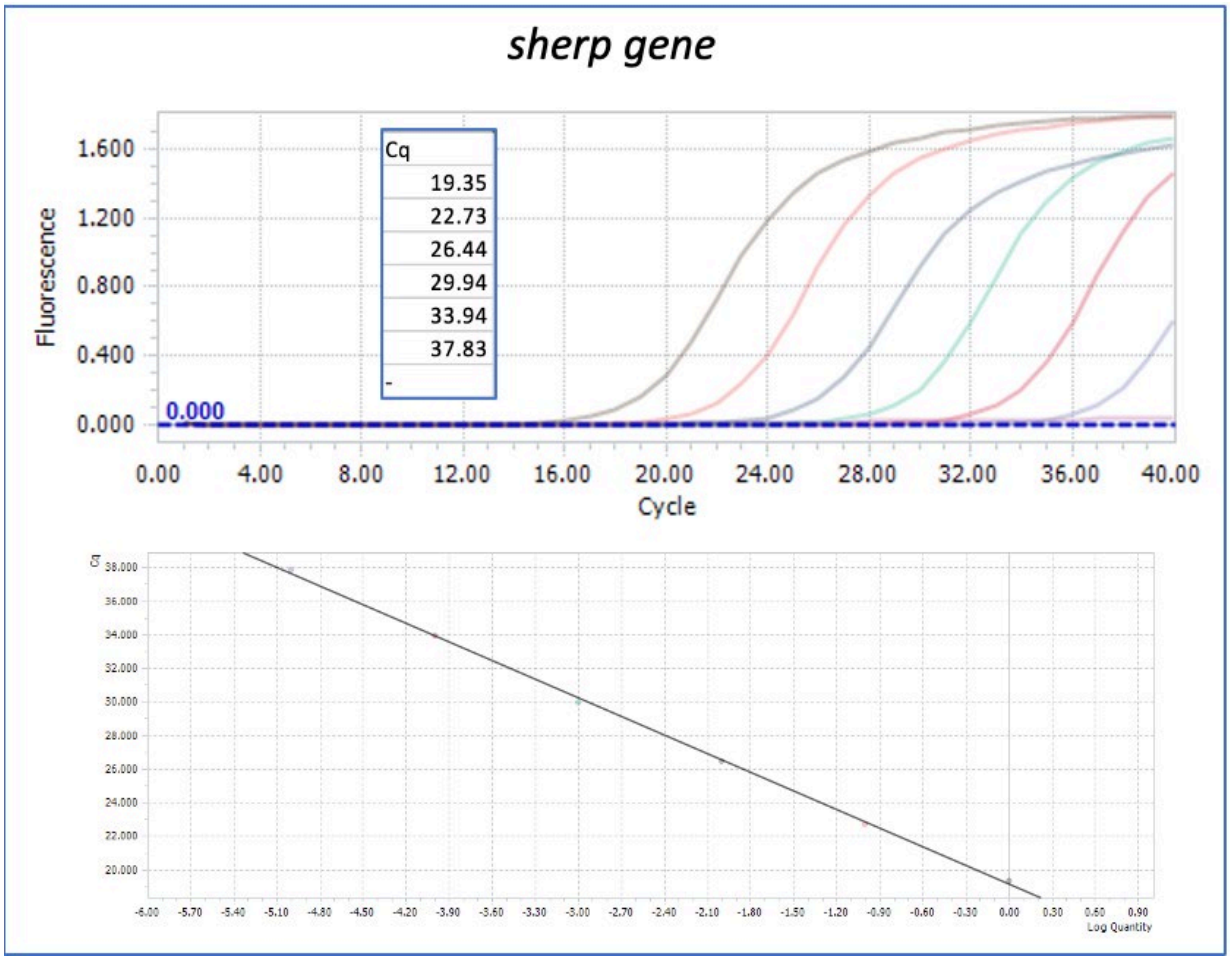

Figure 1. qRT-PCR targeting the sherp gene transcript on serial dilutions of cDNA from RNA extracted from cultured *L. mexicana* parasites.

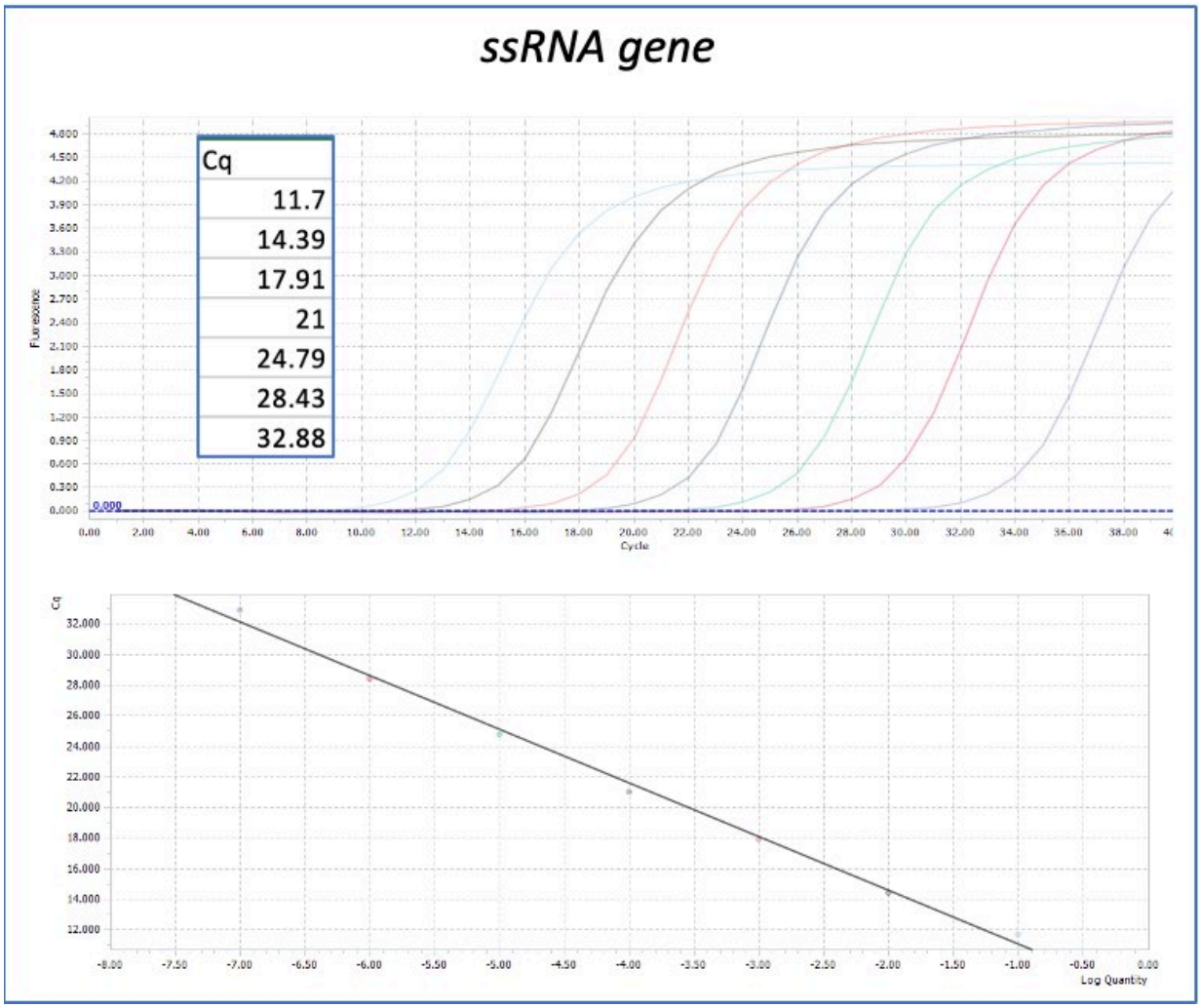

Figure 2. qRT-PCR targeting the ssRNA gene transcript on serial dilutions of cDNA from RNA extracted from cultured *L. mexicana* parasites.