SOP-1

1. PURPOSE/SCOPE

This procedure describes DNA extraction from triatomines

2. MATERIALS

2.1 Reagents and Supplies:

Qiagen DNeasy Blood & Tissue Kit
100% ethanol, molecular biology grade
Bleach
Cavicide
Disposable scalpel blades
Transfer pipets
Petri dishes or weighing dishes
50 ml conical tubes
2.0 ml safe-lock tubes
5 mm stainless steel bead
1.5 ml tubes
2.0 ml collection tubes
10 µl – 1 ml aerosol barrier tips
Paper towels
Bleach pan

2.2 Equipment:

Biological Safety Cabinet (BSC)
TissueLyser II
Vortex
Heated water bath or heated dry block
Microcentrifuge capable of 20,000 g
Micropipettes
(Optional) Spectrophotometer

3. SAFETY POLICY

All dissecting and grinding procedures are done inside a BSC.
For all volumes greater than 1 ml, use plastic serological pipets and pipet aids.
For volumes of 1 ml or less, use aerosol barrier filter tips and micropipettes.
All procedures must also conform to safety outlined in the Biosafety Manual and the CDC BMBL.

4. PROCEDURE

4.1. Rinse the insect in 30 ml of 0.1% bleach in 50 ml conical tube for 1 min, wash in 30-40 ml of water, transfer the insect to a disposable Petri dish or stacked weighing dishes.

4.2. Use disposable scalpel blade and transfer pipet to cut posterior third of the insect abdomen.

4.3. Transfer the tissue to a 2.0 ml safe-lock tubes containing 5 mm stainless steel bead. Disinfect all disposables in 10% bleach for 30 min before discarding.

4.4. Add 200 µl of AL buffer, load the tubes in TissueLyser II, and triturate for 3 min at 25 Hz. Surface disinfect the tubes by wiping with cavicide-soaked paper towel.

4.5. Spin down briefly, add 180 µl of ATL buffer and 20 µl of proteinase K, vortex for a few seconds.

4.6. Incubate at 56°C for 15 min (or more), vortexing occasionally.

4.7. Spin in microcentrifuge at maximum speed for 1 min.

4.8. Collect 300 µl of supernatant avoiding insect debris, transfer to a 1.5 ml tube, add 150 µl of 100% ethanol, vortex, spin down briefly.

4.9. Load the sample onto the column, spin at ≥6000 g for 1 min.

4.10. Transfer the column to new collection tube, aspirate sample leftovers from the rim on the filter. Add 500 µl of AW1 buffer, spin at ≥6000 g for 1 min.

4.11. Transfer the column to new collection tube, aspirate sample leftovers from the rim on the filter. Add 500 µl of AW2 buffer, let stand open for 5 min. Spin at 20,000 g (or maximum speed) for 3 min.

4.12. Very carefully (so that flow-through won't touch the tip of the column; alternatively, spin AW2 for 1 min, then in new collection tube for 2 more min) transfer the column to 1.5 ml tube, aspirate sample leftovers from the rim on the filter.

4.13. Add 200 µl of AE buffer, let stand for 1 min, spin at ≥6000 g for 1 min. Vortex eluted DNA solution, spin down briefly.

4.14. (Optional) Measure DNA concentration on a spectrophotometer.

4.15. Sample can be directly used in PCR, or stored frozen.

SOP-2

1. PURPOSE/SCOPE

This procedure describes *T. cruzi* detection PCR and agarose gel analysis

2. MATERIALS

2.1 Reagents and Supplies:

DNA polymerase kit (we use Phusion Hot Start II)
TCZ1 primer 5' – cgagctcttgcccacacgggtgct – 3'
TCZ2 primer 5' – cctccaagcagcggatagttcagg – 3'
(directly from JC Pizarro, DE Lucero, and L Stevens, 2007)
Td16S F primer 5' – tgttcgcctgtttaacaaaaacat – 3'
Td16S R primer 5' – gaaaaaattacgctgttatccctaa – 3'
(adapted from DF Lyman, FA Monteiro, AA Escalante, C Cordon-Rosales, DM Wesson, JP Dujardin, and CB Beard, 1999)
6xEZ-Vision One Dye-as-Loading Buffer (or regular loading dye and ethidium bromide)
DNA marker ladder
Agarose
TAE buffer
Ice
1.5 ml tubes
PCR tubes or strips
10 µl – 1 ml aerosol barrier tips

2.2 Equipment:

PCR hood or separate PCR room
Thermocycler
Vortex
Microcentrifuge
Horizontal gel electrophoresis system
Power supply
UV transilluminator/gel documentation system
UV-protective eyewear
Micropipettes

3. SAFETY POLICY

Always use UV-protective eyewear when working with UV transilluminator.
For volumes of 1 ml or less, use aerosol barrier filter tips and micropipettes.
All procedures must also conform to safety outlined in the Biosafety Manual and the CDC BMBL.

4. PROCEDURE

4.1. Phusion Hot Start II reaction composition:

17.75 µl H_2O
5 µl 5xHF buffer

0.5 µl 10 mM dNTP
0.25 µl 100 µM forward primer
0.25 µl 100 µM reverse primer
1 µl DNA sample
0.25 µl Phusion Hot Start II DNA polymerase
For other DNA polymerases use manufacturer's recommendations.

4.2. Thaw PCR components and keep them on ice.

4.3. In PCR hood or PCR room prepare PCR master mix for (2xN+1) 25 µl reactions. If the DNA polymerase is not a "hot-start" type, it should be done on ice. Master mix lacks primers and DNA sample.

4.4. Split master mix in two, add primers TCZ1 – TCZ2 in one tube and primers Td16S F – Td16S R in the other.

4.5. Aliquot 24 µl in PCR tubes or strips, add 1 µl of DNA sample. Use 1 µl of *T. cruzi* DNA sample (0.01 – 10 pg/µl) as positive control and 1 µl of *T. cruzi*-negative sample or H_2O as negative control.

4.6. Run PCR cycles. Phusion Hot Start II conditions:

98° 30 s
  98° 10 s |
  64° 20 s | 35 cycles
  72° 20 s |
72° 7 min
For other DNA polymerases use manufacturer's recommendations (including annealing temperature).

4.7. Cast 1% agarose gel prepared in TAE buffer.

4.8. Add 5 µl of EZ-Vision One buffer, load samples onto the gel flanking with DNA marker ladder, run at 5-7 V/cm in TAE buffer.

4.9. Visualize on UV transilluminator/gel documentation system. TCZ1 – TCZ2 primers will produce 188 bp band indicating *T. cruzi*-positive sample; Td16S F – Td16S R primers will produce 430 bp band indicating valid DNA extraction/absence of PCR inhibition.

4.10. Interpretation of the results:

TCZ reaction	+	--	+	--
Td16S reaction	+	+	--	--
***T. cruzi* DNA status**	**positive**	**negative**	**positive**	**indeterminate**

SOP-3

1. PURPOSE/SCOPE

This procedure describes analysis of triatomoine blood meal.

2. MATERIALS

2.1 Reagents and Supplies:

High fidelity DNA polymerase kit (we use Phusion Hot Start II)
Taq DNA polymerase kit (we use HotStarTaq)
12S F primer   5' – cccaaactgggattagataccc – 3'
12S R primer   5' – gtttgctgaagatggcggta – 3'
(T Kitano, et al. 2007)
6xEZ-Vision One Dye-as-Loading Buffer (or regular loading dye and ethidium bromide)
DNA marker ladder
Agarose
TAE buffer
Qiagen MinElute Gel Extraction Kit
Ice
1.5 ml tubes
PCR tubes or strips
10 µl – 1 ml aerosol barrier tips

2.2 Equipment:

PCR hood or separate PCR room
Thermocycler
Vortex
Microcentrifuge
Horizontal gel electrophoresis system
Power supply
UV transilluminator/gel documentation system
UV-protective eyewear
Micropipettes
Computer with internet access

3. SAFETY POLICY

Always use UV-protective eyewear when working with UV transilluminator.
For volumes of 1 ml or less, use aerosol barrier filter tips and micropipettes.
All procedures must also conform to safety outlined in the Biosafety Manual and the CDC BMBL.

4. PROCEDURE

4.1. 12S PCR Amplification

4.1.1. Fill bucket with ice (ice machine located on 4th floor).

4.1.2. Retrieve the following reagents from the -20°C freezer. Thaw, vortex, and spin down the tubes.

5x HF Buffer
10 mM dNTP
100 µM 12S F primer
100 µM 12S R primer
DEPC-treated water aliquot

4.1.3. Thaw, vortex, and spin down the tubes in microcentrifuge.

4.1.4. Set up and label PCR tubes.

4.1.5. Create master mix in 1.5ml sterile tube using the following formula (multiply µl by the number or rxn tubes you need to set up, be sure to multiply by 0.5 extra to allow for loss due to pipetting):

Note: do not take the Phusion HSII out of the -20°C freezer until you are prepared to add it to master mix. Once the appropriate volume is aspirated from the stock container immediately place back in the -20°C.

	µl/rxn	Multiplier:___
Nuclease-free water	16.6	
5x HF	5	
10 mM dNTP	0.5	
100 µM 12S F	0.1	
100 µM 12s R	0.1	
Phusion HSII	0.2	
Master mix volume/rxn	22.5	
DNA sample volume/rxn	2.5	

4.1.6. Add 22.5 µl of mastermix to each reaction tube.

4.1.7. Add 2.5 µl of DEPC-treated water to the negative control tube.

4.1.8. Add 2.5 µl of you positive control to the appropriate tube.

4.1.9. Add 2.5 µl of you sample to the appropriate tubes.

4.1.10. Seal lid tightly on PCR tubes, vortex, and spin down.

4.1.11. Place PCR tubes in thermo cycler. Select 'saved programs' and then select Phusion from the list.

4.1.12. Run PCR cycles. Phusion Hot Start II conditions:

98° 30 s
  98° 10 s |
  60° 20 s | 35 cycles
  72° 20 s |
72° 7 min

For other DNA polymerases use manufacturer's recommendations (including annealing temperature).

4.1.13. Once the thermo cycler has completed its program remove the PCR tubes from the machine.

4.1.14. The second round of PCR will be performed with the amplified 12S samples (steps 1-13).

4.1.15. Retrieve the following reagents from the -20°C freezer. Thaw, vortex, and spin down the tubes.

5x HF Buffer
10 mM dNTP
100 µM 12S F primer
100 µM 12S R primer
DEPC-treated water aliquot

4.1.16. Thaw, vortex, and spin down the tubes in microcentrifuge.

4.1.17. Set up and label PCR tubes.

4.1.18. Create master mix in 1.5ml sterile tube using the following formula (multiply µl by the number or rxn tubes you need to set up, be sure to multiply by 0.5 extra to allow for loss due to pipetting):

Note: do not take the Phusion HSII out of the -20°C freezer until you are prepared to add it to master mix. Once the appropriate volume is aspirated from the stock container immediately place back in the -20°C.

	µl/rxn	Multiplier:___
Nuclease-free water	18.6	
5x HF	5	
10 mM dNTP	0.5	
100 µM 12S F	0.1	
100 µM 12s R	0.1	
Phusion HSII	0.2	
Master mix volume/rxn	24.5	
DNA sample volume/rxn	0.5	

4.1.19. Add 24.5 µl of mastermix to each reaction tube.

4.1.20. Add 0.5µl of DNA from previous amplification to each respective tube.

4.1.21. Seal lid tightly on PCR tubes, vortex, and spin down.

4.1.22. Place PCR tubes in thermo cycler. Select ‘saved programs’ and then select Phusion from the list.

4.1.23. Run PCR cycles. The second round Phusion Hot Start II conditions:

98° 30 s
98° 10 s |
65° 20 s | 30 cycles
72° 20 s |
72° 7 min

For other DNA polymerases use manufacturer’s recommendations (including annealing temperature).

4.1.24. Once PCR is complete take both amplification samples to the 5th floor lab.

4.1.25. Add 4µl of EZ-Vision One buffer to each of the samples. Vortex the samples and then spin them down in the microcentrifuge.

4.1.26. Run the samples out on a 1% agarose gel (TAE buffer) along with 2-log ladder, at constant voltage 180 for 50 minutes.

4.1.27. Visualize on UV transilluminator/gel documentation system. Excise positive bands (about 215 bp) using sterile scalpel and place into 1.5ml tube. Once all bands have been excised move on to gel extraction.

Note: If positive band is bright enough in first round amplification to excise, use it; if not use second round amplification band.

4.2. Gel Extraction

4.2.1. Excise the DNA fragment from the agarose gel with a clean scalpel and place in sterile 1.5 ml tube.

4.2.2. Gel slices should be around 100 mg. If it looks much larger weigh the gel slice to determine size. Add 3 volumes of Buffer QG to 1 volume of gel. Typically, use 400 µl to cover up to 133 mg gel slices.

4.2.3. Incubate at 50°C in heat block for 10 minutes or until gel has fully dissolved. Tap tube every 2-3min to mix.

4.2.4. Add 1/3 of Buffer QG volume (typically, 133µl) of isopropanol to the sample and mix by inverting.

4.2.5. Place a MinElute spin column in a 2 ml collection tube. Apply sample to the MinElute column and centrifuge for 1min at full speed. Discard flow-through (in liquid hazardous waste).

4.2.6. Add 500 µl of Buffer QG to the MinElute column and centrifuge for 1min. Discard flow through.

4.2.7. Add 500µl of Buffer PE to MinElute column, let column stand open for at least 5 min before centrifuging for 1 min. Discard flow through.

4.2.8. Add 500µl of Buffer PE to MinElute column and spin for 1min. Discard flow through.

4.2.9. Centrifuge the column in an empty 2 ml collection tube for 1 min to remove residual ethanol from Buffer PE.

4.2.10. Place MinElute column into a clean 1.5 ml tube. To elute DNA, add 10 µl of Buffer EB to the center of the MinElute membrane. Let column stand for 1 min and then centrifuge the column for 1 min.

4.2.11. DNA can be frozen or used for further downstream application.

4.2.12. Preform nanodrop analysis to measure DNA concentration. Expected yield: 10-30 ng/µl.

4.3. 3’A Extension

4.3.1. Retrieve the following reagents from the -20°C freezer. Thaw, vortex, and spin down the tubes.

10x PCR Buffer
10 mM dNTP

4.3.2. Thaw, vortex, and spin down the tubes in microcentrifuge.

4.3.3. Set up and label PCR tubes.

4.3.4. Create master mix in 1.5ml sterile tube using the following formula (multiply µl by the number or rxn tubes you need to set up, be sure to multiply by 0.5 extra to allow for loss due to pipetting):

Note: do not take the HotStarTaq out of the -20°C freezer until you are prepared to add it to master mix. Once the appropriate volume is aspirated from the stock container immediately place back in the -20°C.

	µl/rxn
Nuclease-free water	9.7
10x PCR Buffer	1.5
10 mM dNTP	0.3
HotStarTaq	0.5
Master mix volume/rxn	12
DNA sample volume/rxn	3

4.3.5. Add 12 µl of mastermix to each reaction tube.

4.3.6. Add 3 µl of DEPC-treated water to the negative control tube.

4.3.7. Add 3 µl of you 12S amplified and purified sample to the appropriate tubes.

4.3.8. Seal lid tightly on PCR tubes, vortex, and spin down

4.3.9. Place PCR tubes in thermo cycler. Select 'saved programs' and then select ADD from the list.

4.4. Ligation and Trasformation

4.4.1. Retrieve the following reagents from the -20°C freezer. Thaw, vortex, and spin down the tubes.

2x Rapid Ligation Buffer

pGEM-T Easy Vector

4.4.2. Thaw, vortex, and spin down the tubes in microcentrifuge.

4.4.3. Set up and label 1.5 ml tubes.

4.4.4. Create master mix in 1.5ml sterile tube using the following formula (multiply µl by the number or rxn tubes you need to set up, be sure to multiply by 0.5 extra to allow for loss due to pipetting):

Note: do not take the T4 DNA Ligase out of the -20°C freezer until you are prepared to add it to master mix. Once the appropriate volume is aspirated from the stock container immediately place back in the -20°C.

	µl/rxn
Nuclease-free water	0.5
2x Rapid Ligation Buffer	2.5
pGEM-T Easy Vector	0.5
T4 DNA Ligase	0.5
Master mix volume/rxn	4
DNA sample volume/rxn	1

4.4.5. Use 1µl of the 3'A extended DNA straight into ligation.

4.4.6. Allow mixture to incubate overnight at 4°C (or can be incubated at room temperature if less than overnight).

4.4.7. Thaw JM109 competent cells on ice and aliquot by 20 µl in pre-chilled 1.5 ml tubes.

4.4.8. Mix 2 µl of the ligation reaction with JM109 cells. Leave on ice for 20 min. During that time place agar plates and a tube rack into 37°C incubator, to be used in steps 4.4.11 and 4.4.12. Also, turn on the heat block and fill up few wells with deionized water, to be used in step 4.4.9.

If competent cells remaining, flash freeze by mixing ethanol with dry ice and place tube in bath until frozen.

4.4.9. Incubate reaction at 42°C for 45 seconds.

4.4.10. Move reaction back to ice for 2 minutes.

4.4.11. Add 200 µl of SOC media and incubate at 37°C for 30 min.

4.4.12. Add 70µl of bacterial suspension to LB agar plate with ampicillin and spread using disposable spreader. Carbenicillin is a good substitution for ampicillin. IPTG/X-Gal plates are also good for further colony selection.

4.4.13. Allow plates to incubate overnight at 37°C.

4.5. M13 PCR Amplification

4.5.1. Retrieve the following reagents from the -20°C freezer. Thaw, vortex, and spin down the tubes.

10x PCR Buffer
Q-solution
10 mM dNTP
100 µM M13 F primer
100 µM M13 R primer

4.5.2. Thaw, vortex, and spin down the tubes in microcentrifuge.

4.5.3. Set up and label PCR tubes.

4.5.4. Create master mix in 1.5ml sterile tube using the following formula (multiply µl by the number or rxn tubes you need to set up, be sure to multiply by 0.5 extra to allow for loss due to pipetting):

Note: do not take the HotStarTaq out of the -20°C freezer until you are prepared to add it to master mix. Once the appropriate volume is aspirated from the stock container immediately place back in the -20°C.

	µl/rxn
Nuclease-free water	13
10x PCR Buffer	2
Q-solution	4
10 mM dNTP	0.4
100µM M13 R	0.2
100µM M13 F	0.2
HotStarTaq	0.2
Master mix volume/rxn	20

DNA sample volume/rxn	1 colony

4.5.5. Add 20µl of master mix to each tube

4.5.6. Identify and label 10 colonies from each plate that are single colonies.

4.5.7. Pick colonies with pipet tip and inoculate into master mix, mix by pipetting up and down.

4.5.8. Place tubes to PCR machine and run on saved protocol 'HotStarTaq':

95° 15 min
  94° 30 s |
  60° 30 s | 25 cycles
  72° 30 s |
72° 10 min

For other DNA polymerases use manufacturer's recommendations (including annealing temperature).

4.5.9. Load on 1% agrose gel and run out samples as above.

4.5.10. Excise all positive amplifications (about 480 bp).

4.5.11. Extract DNA from 5 gel slices per construct following MinElute gel extraction protocol used previously, elute in 15 µl of EB. Save the rest of gel slices at 4°C.

4.5.12. Submit 5µl of the extracted samples for sequencing with sample of 5 µM M13 R to Lone Star Labs.

4.5.13. Clean up the sequence between 12S primers, BLAST it to infer the blood meal source.