



Joint FAO/IAEA Programme
Nuclear Techniques in Food and Agriculture

STANDARD OPERATING PROCEDURES FOR DETECTION AND IDENTIFICATION OF TRYPANOSOME SPECIES IN TSETSE FLIES

Version 1.0



Tsetse fly



DNA extraction



PCR



Identification
Trypanosoma species

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The proper citation for this document is:

FAO/IAEA. 2019. Standard operating procedures for detection and identification of trypanosome species in tsetse flies, Van Den Abbeele J., Demirbas-Uzel G., Argilés Herrero R., Vermeiren L. and Abd-Alla A. (eds.), Food and Agriculture Organization of the United Nations/International Atomic Energy Agency. Vienna, Austria. 29 pp.

STANDARD OPERATING PROCEDURES
FOR
DETECTION AND IDENTIFICATION OF
TRYPANOSOME SPECIES IN TSETSE FLIES

Version 1.0

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Food and Agriculture Organization of the United Nations
International Atomic Energy Agency
Vienna, 2019

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1. INTRODUCTION

African trypanosomes are flagellated protozoa causing a wide range of diseases in both humans and animals, called African trypanosomiasis, a vector-borne disease restricted to sub-Saharan Africa. Human African trypanosomiasis (HAT; sleeping sickness) is a complex of protozoan infections, fatal if untreated, which can be caused by infection with either *Trypanosoma brucei gambiense* (gambiense HAT) or *T. brucei rhodesiense* (rhodesiense HAT). Both human infective sub-species are cyclically transmitted by tsetse flies (genus *Glossina*). Several other tsetse-transmitted trypanosome species like *T. congolense*, *T. vivax* and *T. brucei brucei* cause animal African trypanosomiasis, a devastating disease in livestock which is a serious constraint for agricultural development and a threat for the food security in many communities on the African continent. Besides transmission by tsetse, *T. vivax* can also be transmitted mechanically by other biting insects like tabanids.

The multitude of PCR-based methods that are currently available can identify trypanosomes at the subspecies level, are suitable for analysis of mixed infections and can be applied to samples where parasite numbers are low. Generic PCR-methods ('pan-species' methods) are less sensitive than species-specific PCR methods but allow for multiple trypanosome species to be identified with a single test (Gibson, 2009). Most generic methods, such as restriction fragment length polymorphism PCR (RFLP-PCR) and ribosomal length-based methods (targeting the ribosomal RNA gene locus), utilize primers that recognize a semi-conserved region of the trypanosome genome allowing the amplification of the target sequence in a broad range of trypanosomes. Here, trypanosome identification is made based on the length of the amplified regions as a direct result of the PCR (Adams *et al.*, 2006) or after an additional fragmentation step using a specific restriction enzyme (Geysen *et al.*, 2003). On the other hand, species-specific PCR-based methods are using primers that allow targeted amplification of only a subgenus or species-specific sequence. As for other organisms, the ribosomal RNA gene locus (including 18S and ITS1 sequences) has been a popular target for molecular detection methods. It is a highly expressed multicopy gene locus containing conserved and variable nucleotide sequences that offer targets for universal and/or specific primers.

Despite the existence of a multitude of different PCR-based methods, so far there is no simple test available that is suitable for the detection of all trypanosomes with sensitivity and specificity sufficient to differentiate between infections at the subspecies level, and usable for known, unknown and mixed infections (Hutchinson & Stevens, 2018).

In the procedure below, we provide an easy-to-use work flow that combines several different PCR-based methods that can be used for trypanosome detection and identification in field-caught tsetse flies and/or host blood samples collected from livestock and wildlife animals.

2. SCHEME FOR A STEP BY STEP IDENTIFICATION OF *GLOSSINA* SPECIES

The procedure for the identification of trypanosome species will depend on the starting material:

- (i) Tsetse flies freshly captured or stored in ethanol
- (ii) host blood collected on filter paper or stored in a different way

Tsetse flies can be identified as previously described in http://www-naweb.iaea.org/nafa/ipc/public/SOP-for-tsetse-species-identification-Final_8.pdf.

For the trypanosome identification of extracted DNA, we should follow the following steps (**Figure 1**):

Step 1: DNA will be extracted from the samples (either tsetse fly or vertebrate host blood as detailed in **section 3**). then proceed to step 2.

Step 2: Assess the DNA quality and quantity as detailed in **section 4** to ensure that an equal amount of the DNA is used during further processing steps.

Step 3: Perform a PCR with Trypanosomatidae 18S specific primers which can detect a wide range of trypanosome species in a DNA sample as detailed in **section 5**.

Step 4: Perform a Pan-species PCR with ITS1 specific primers which generate a PCR product with variable size that can be linked to a specific trypanosome species as detailed in **section 6.1**.

Step 5: Perform a PCR with species specific primers to validate results obtained in **step 4** as detailed in **section 6.2**.

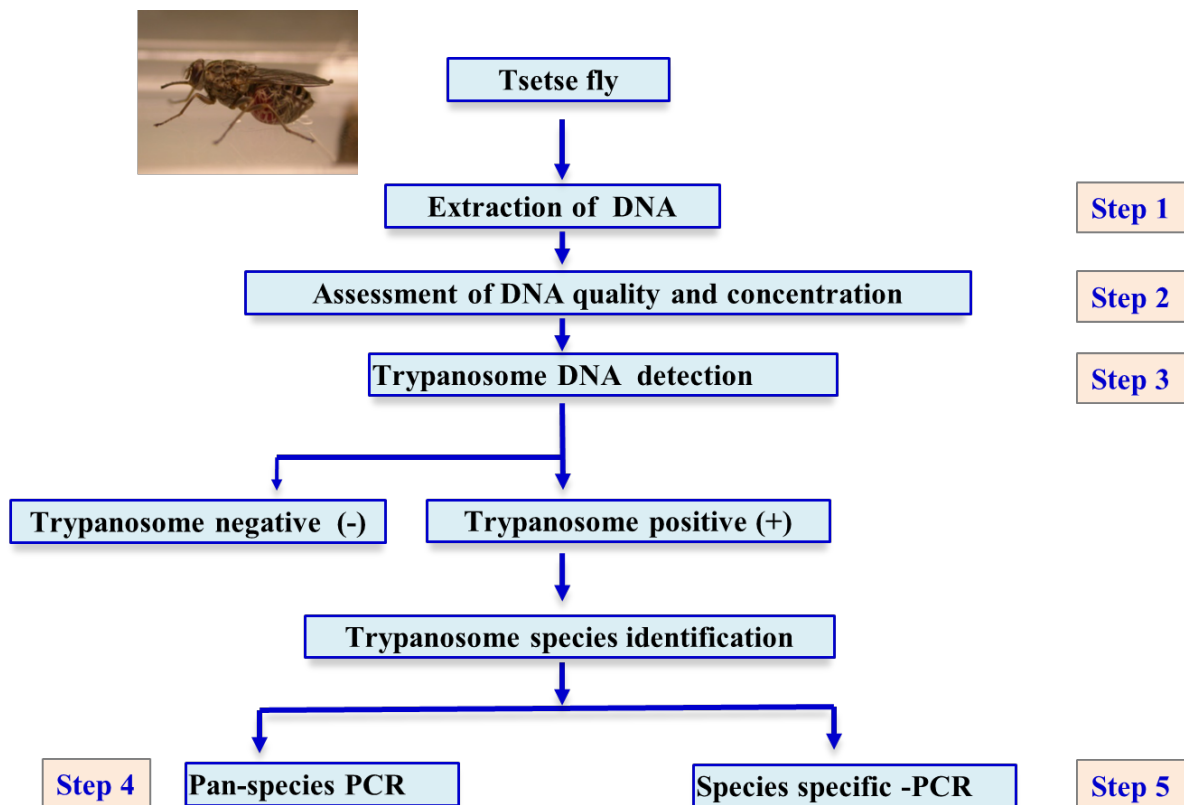


Figure 1. Schematic summary of step by step identification of African trypanosome species. The starting material can be the DNA samples of either tsetse fly or vertebrate host blood. Detailed information of the procedures and the expected results are explained below.

3. DNA EXTRACTION FROM TSETSE FLIES

- **Purpose**

To isolate total DNA from tsetse flies for trypanosome species identification after identifying the collected tsetse species following the SOP: http://www-naweb.iaea.org/nafa/ipc/public/SOP-for-tsetse-species-identification-Final_8.pdf. The DNA can be also extracted from vertebrate host blood using the same method.

- **Materials and equipment requirements**

This procedure requires the following materials and equipment:

- Fresh or conserved tsetse flies of unknown species preserved in alcohol
- Microtubes
- Microtube holders
- Sterile micropestles
- Liquid nitrogen (N₂)
- Autoclave
- Clean room
- Pipette (e.g. Pipetman set)
- Pipette tips (e.g. Pipetman tips)
- Water bath
- Microcentrifuge
- Refrigerator
- Deep freezer
- DNA extraction kit
- Spectrophotometer
- Sterile distilled water
- Gloves.

- **Required skills requirements**

Research or laboratory technician with the required skills for molecular biology work, including DNA extraction.

- **Procedure**

1. For live tsetse flies from colonies kept in the laboratory (use as negative control in downstream PCR reactions), select the flies randomly (preferably 10 days old) and proceed to step 3.
2. For field collected tsetse flies conserved in ethanol (or other solutions for conserving specimens, e.g. propylene glycol), wash off the alcohol and replace it with distilled water, then wash off the water and dry the fly on tissue paper.
3. Put each individual fly in a separate microtube, label each tube with a serial number.
4. Insert one sterile micropestle in each microtube.
5. Add liquid nitrogen to the microtube to freeze the fly and the micropestle, and immediately grind the fly using the micropestle to obtain homogenized fly tissue as a fine powder.
6. Proceed with the DNA extraction using DNA extraction kit following the supplier instructions: (e.g. DNAeasy kit:

http://mvz.berkeley.edu/egl/inserts/DNeasy_Blood_&_Tissue_Handbook.pdf

7. After eluting the DNA, quantify the DNA concentration using a spectrophotometer to ensure extraction of an equal DNA quantity from each fly (e.g. Nano Drop: for more information see <http://www.nanodrop.com/nucleicacid.aspx>).
8. The DNA samples can be stored in a refrigerator (between + 4-8 ° C) until required for the next step.

4. CHECKING DNA EXTRACT

Before proceeding the PCR for trypanosome detection and identification, it is important to verify the quantity and quality of the extracted DNA.

- **Purpose**

Different methods are used to verify quantity and quality of the extracted DNA

- **Scope**

This procedure is designed to correctly verify quantity and quality of the extracted DNA from tsetse fly and vertebrate host blood spot using spectrophotometer and specific PCR reaction.

- **Materials and equipment requirements**

This procedure requires the following materials and equipment:

- DNA samples extracted in Section 3.
- Clean room
- Air conditioning
- Spectrophotometer
- PCR work station
- PCR thermocycler
- Pipette (e.g. Pipetman set)
- Pipette tips (e.g. Pipetman tips)
- Refrigerator
- PCR master mix reagent
- Specific primers
- Agarose
- Gel electrophoresis apparatus
- DNA dye
- DNA ladder
- Sterile distilled water
- Gloves

- **Required skills**

Research or laboratory technician with the required skills for molecular biology work, including PCR technology and controlling PCR results on agarose gel.

- **Procedure**

A. Verification of quantity using the spectrophotometer

Note: This method is valid for DNA extracted samples from both tsetse and vertebrate blood samples

1. After eluting the DNA, quantify the DNA concentration using a spectrophotometer to ensure extraction of an equal DNA quantity from each fly (e.g. Nano Drop: for more information see <http://www.nanodrop.com/nucleicacid.aspx>) (**Figure 2**).

B. Verification of quality of tsetse DNA samples with specific PCR

Warning: The PCR detection method is very sensitive, and precautions should be taken to avoid cross contamination between samples (from instruments, equipment etc.).

1. Use 1.5 uL of the DNA extracted from each sample in a 25 uL PCR reaction.
2. Conduct PCR using *GpCAG 133* specific primers (GpCAG133F: ATTTTTCGTC AACGTGA and GpCAG133R: ATGAGGATGTTGTCCAGTTT) with negative (no DNA template) and positive DNA samples (positive DNA sample of known tsetse species). Use the following PCR programme for GPCAG 133 amplification: 5 min 94°C, 30 cycles of 94°C for 1 min, 58°C for 45 sec and 72°C for 90 sec, then 72°C for 10 min as described by Baker and Krafur, 2001. **Note:** that in case of using hot start DNA polymerase enzyme in the PCR reaction, the start denaturation should be extended to 15 min.
3. Separate the PCR products by electrophoresis on 2% agarose gel (**Figure 3**). DNA dye (Ethidium bromide, Safe green or etc.) can be used to stain the PCR product, although precautions should be taken to avoid contact with this chemical as it is a powerful mutagenic.
4. Visualize the results of electrophoresis using a gel documentation system (**Figure 4**).
5. Analyze the PCR results of each sample with the positive control and record the amplification of 180-220 bp band size/length of each sample using the DNA ladder (**Figure 5**). The absence of this band indicates non-adequate DNA quality or the presence of PCR-inhibiting components making the DNA sample not suitable for further analysis.

C. Verification of quality of host blood DNA samples with vertebrate cytochrome B PCR

Warning: The PCR detection method is very sensitive, and precautions should be taken to avoid cross contamination between samples (from instruments, equipment etc.).

1. Use 1.5 uL of the DNA extracted from each sample in a 25 uL PCR reaction.
2. Conduct PCR using vertebrate *cytochrome B* PCR specific primers (L14841 FW 5'-CCATCCAACATCTCAGCATGATGAAA-3' and H15149 Rev 5'-GCCCTCAGAATGATATTTGTCCTCA-3') including a negative (no DNA template) and a positive vertebrate DNA sample. Use the following PCR programme: 5 min 94°C, 40 cycles of 94°C for 30 sec, 52°C for 30 sec and 72°C for 30 sec, then 72°C for 10 min. **Note:** that in case of using Hot Start DNA polymerase enzyme in the PCR reaction, the start denaturation should be extended to 15 min.

3. Separate the PCR products by electrophoresis on 1.5% agarose gel (**Figure 3**). DNA dye (Ethidium bromide, Safe green or etc.) can be used to stain the PCR product, although precautions should be taken to avoid contact with this chemical as it is a powerful mutagenic.
4. Visualize the results of electrophoresis using a gel documentation system (**Figure 4**).
5. Analyze the PCR results of each sample with the positive control and record the amplification of 350 bp band size/length of each sample using the DNA ladder (**Figure 6**). This band should be present and indicates that the DNA extraction OK and the sample can be used for further analysis. The absence of this band indicates non-adequate DNA quality or the presence of PCR-inhibiting components making the DNA sample not suitable for further analysis.

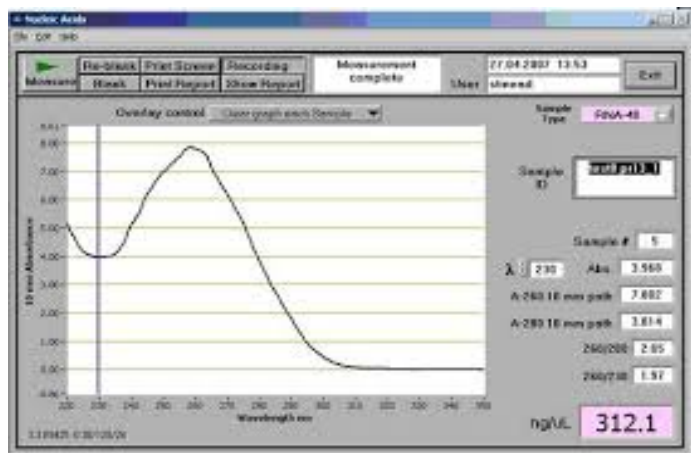


Figure 2. Nanodrop spectrophotometry.

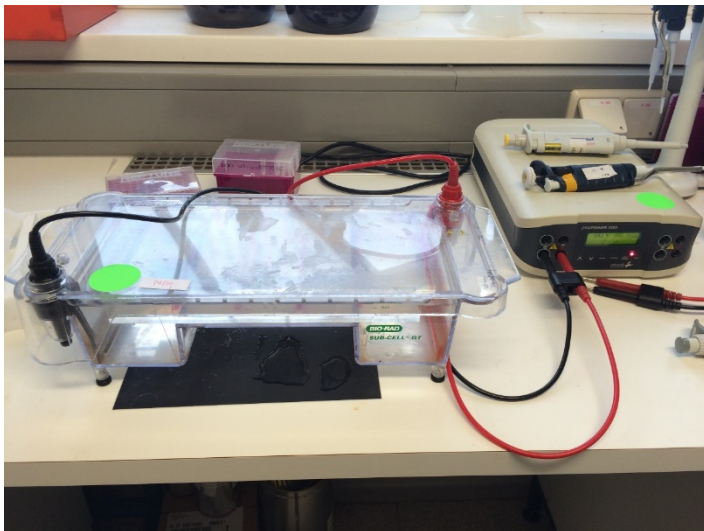


Figure 3. Gel electrophoresis system.

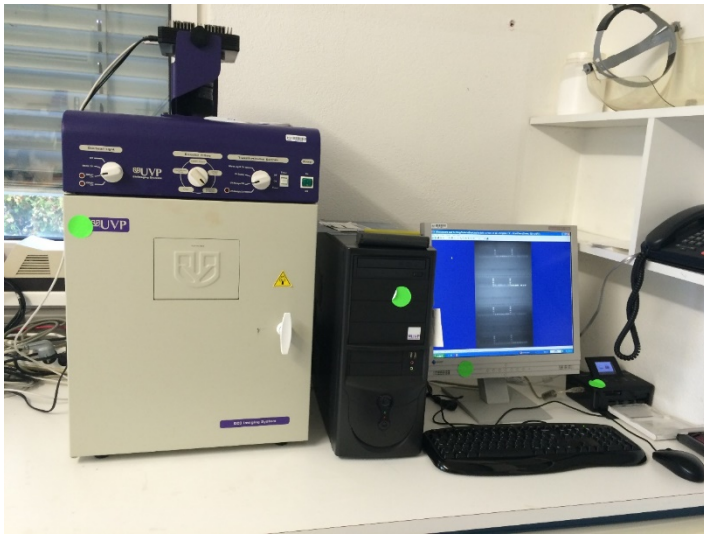
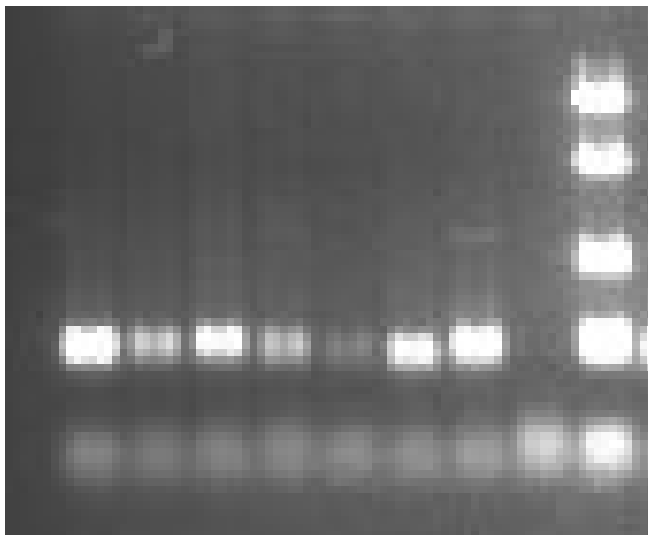
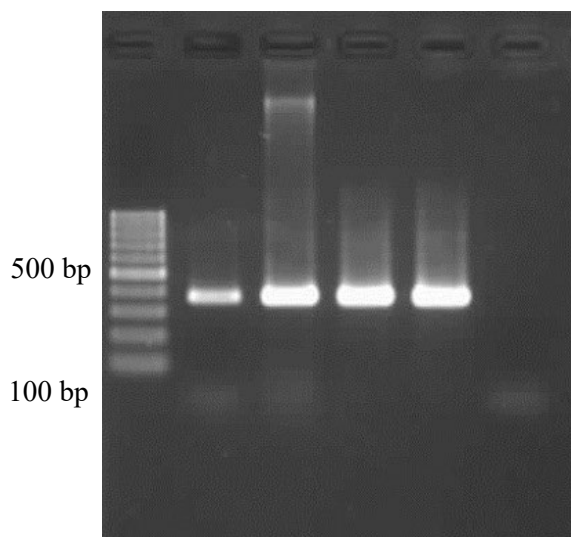


Figure 4. Gel documentation system.



← 200 bp GpCAG 133

Figure 5. 2 % agarose gel image showing the amplification of tsetse genomic DNA using GPCAG133 primers.



← Cytochrome B amplicon

Figure 6. 1.5% agarose gel image showing the PCR-amplification product with vertebrate cytochrome B specific primers. The presence of 350 bp PCR product indicate adequate DNA quality in the host blood extract for further analysis.

5. PCR-BASED DETECTION OF TRYPANOSOME DNA IN THE EXTRACT

Specific PCR will be used to detect trypanosome DNA in the extracted DNA samples from tsetse or host blood.

- **Purpose**

This method is used to detect trypanosome DNA in the extracted samples

- **Scope**

This method will allow to determine the status of trypanosome infection in the tested samples.

- **Materials and equipment requirements**

This procedure requires the following materials and equipment:

- DNA samples extracted in Section 3
- Clean room
- Air conditioning
- PCR work station
- PCR thermocycler
- Pipette (e.g. Pipetman set)
- Pipette tips (e.g. Pipetman tips)
- Refrigerator
- PCR master mix reagent
- *Trypanosomatidae* 18S specific primers
- Agarose
- Gel electrophoresis apparatus
- DNA dye (Ethidium bromide, Safe green or etc.)
- DNA ladder
- Sterile distilled water
- Gloves

- **Required skills**

Research or laboratory technician with the required skills for molecular biology work, including PCR technology and controlling PCR results on agarose gel.

- **Procedure**

Warning: The PCR detection method is very sensitive, and precautions should be taken to avoid cross contamination between samples (from instruments, equipment etc.).

1. Use 1.5 uL of the DNA extracted from each sample in a 25 uL PCR reaction.
2. Conduct PCR using *Trypanosomatidae* 18S specific primers (M18S-F-T: 5'-CGCCAAGCTAATACATGAACCAA-3' and M18S-R-T: 5'-TAATTCATTCATTCGCTGGACG-3') with negative (no DNA template) and positive known DNA sample. Use the following PCR programme for *Trypanosomatidae* 18S specific primers amplification: 5 min 94°C, 40 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec, then 72°C for 10 min as described by Deborggraeve et al., 2006. **Note:** in case of using Hot Start DNA polymerase enzyme in the PCR reaction, the start denaturation should be extended to 15 min.

3. Separate the PCR products by electrophoresis on 2% agarose gel (**Figure 3**). DNA dye (Ethidium bromide, Safe green or etc.) can be used to stain the PCR product, although precautions should be taken to avoid contact with this chemical as it is a powerful mutagenic.
4. Visualize the results of electrophoresis using a gel documentation system (**Figure 4**).
5. Analyze the PCR results of each sample with the positive control and record the presence or absence of a specific band (size/length:106 bp) using the DNA ladder (**Figure 7**). Presence of band indicates that the sample contained trypanosome DNA. These samples can be analyzed for further identification of the trypanosome species.

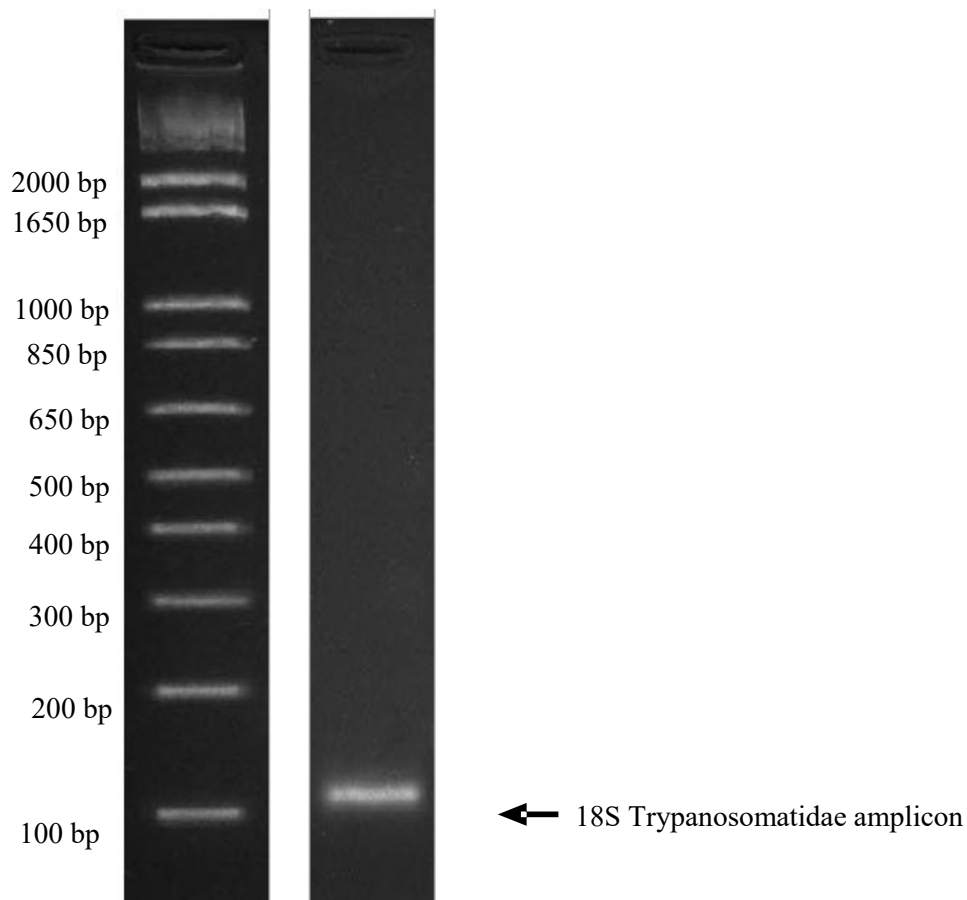


Figure 7. 2 % agarose gel image showing the PCR-amplification product with *Trypanosomatidae* 18S specific primers. The presence of a 106 bp PCR product indicates the presence of trypanosome DNA in the sample extract.

6. PCR BASED IDENTIFICATION OF TRYPANOSOME SPECIES

A combination of different molecular markers can be applied to distinguish trypanosome species as described below.

6.1. Identification of trypanosome species using a Pan-species PCR (ITS1-sequence based)

- **Purpose**

This method is used to identify and distinguish several trypanosome species/subspecies in a DNA sample based upon one PCR reaction.

- **Scope**

This procedure is designed to correctly detect and identify trypanosome species/subspecies in the extracted DNA samples from tsetse or host blood.

- **Materials and equipment requirements**

The following materials and equipment are needed:

- DNA samples extracted in Section 3
- Clean room
- Air conditioning
- PCR work station
- PCR thermocycler
- Pipette (e.g. Pipetman set)
- Pipette tips (e.g. Pipetman tips)
- Refrigerator
- PCR master mix reagent
- ITS1 specific primers (Adams et al, 2006)
- Agarose
- Gel electrophoresis apparatus
- DNA dye (Ethidium bromide, Safe green or etc.)
- DNA ladder
- Sterile distilled water
- Gloves

- **Required skills**

Research or laboratory technician with the required skills for molecular biology work, including PCR technology and controlling PCR results on agarose gel.

- **Procedure**

Warning: The PCR detection method is very sensitive, and precautions should be taken to avoid cross contamination between samples (from instruments, equipment etc.).

1. Use 1.5 uL of the DNA extracted from each sample in a 25 uL PCR reaction.

2. For this nested PCR two consecutive PCR reactions are carried out; a set of outer primers TRYP 3 and 4 are used in the first-round reaction, followed by inner primers TRYP 1 and 2 in the second round.
3. Conduct PCR 1 using ITS1 specific outer primers (TRYP3: 5'-TGCAATTATTGGTCGCGC-3' and TRYP4: 5'-CTTTGCTGCGTTCTT-3') with negative (no DNA template) and positive DNA samples of known trypanosome species). Use the following PCR programme for ITS1 first amplification: 5 min 94°C, 40 cycles of 94°C for 30 sec, 54°C for 30 sec and 72°C for 30 sec, then 72°C for 10 min as described by Adams *et al.*, 2006. **Note:** in case of using Hot Start DNA polymerase enzyme in the PCR reaction, the start denaturation should be extended to 15 min.
4. Use 0.5 uL of the PCR product from PCR1 in a 25 uL PCR reaction.
5. Conduct PCR2 using ITS1 specific inner primers (TRYP1:5'-AGGCCAAGTCATCCATCG-3' and TRYP2: 5'-TAGAGGAAGCAAAAG-3'). Use the following PCR programme for ITS1 nested amplification: 5 min 94°C, 40 cycles of 94°C for 30 sec, 54°C for 30 sec and 72°C for 30 sec, then 72°C for 10 min as described by Adams *et al.*, 2006. **Note:** in case of using Hot Start DNA polymerase enzyme in the PCR reaction, the start denaturation should be extended to 15 min.
6. Separate the PCR products by electrophoresis on 2% agarose gel (**Figure 3**). Ethidium bromide can be used to stain the PCR product, although precautions should be taken to avoid contact with this chemical as it is a powerful mutagenic.
7. Visualize the results of electrophoresis using a gel documentation system (**Figure 4**).
8. Analyze the PCR results of each sample with the positive control and record the band size/length of each sample using the DNA ladder (**Figure 8**).
9. Identify trypanosome species in the analyzed DNA sample based on ITS1 band size of reference trypanosome species (see **Figure 8**).

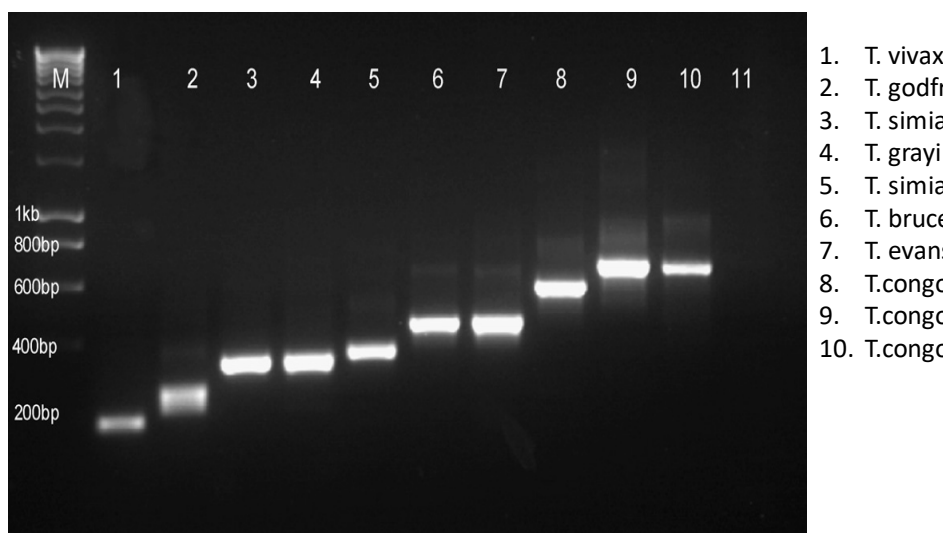


Figure 8. 2% agarose gel images showing the amplification of trypanosome genomic DNA using ITS1 specific primers (Adams *et al.*, 2006). The size of the PCR product varies and is linked to a specific trypanosome species.

Note: The absence of a *T. vivax* specific PCR product using these ITS1 primers does not indicate reliably the absence of this trypanosome species in the sample. For this, the *T. vivax* specific PCR described in 7.2 should be used.

6.2. Identification of trypanosome species using specific PCR

6.2.1. Identification of *Trypanosoma vivax*

- **Purpose**

This method is used to identify *Trypanosoma vivax* in extracted DNA samples.

- **Scope**

This procedure is designed to identify *Trypanosoma vivax* in the extracted DNA samples from tsetse or host blood.

- **Materials and equipment requirements**

The following materials and equipment are needed:

- DNA samples extracted in Section 3
- Clean room
- Air conditioning
- PCR work station
- PCR thermocycler
- Pipette (e.g. Pipetman set)
- Pipette tips (e.g. Pipetman tips)
- Refrigerator
- PCR master mix reagent
- *T. vivax* specific primers
- Agarose
- Gel electrophoresis apparatus
- DNA dye (Ethidium bromide, Safe green or etc.)
- DNA ladder
- Sterile distilled water
- Gloves

- **Required skills**

Research or laboratory technician with the required skills for molecular biology work, including PCR technology and controlling PCR results on agarose gel.

- **Procedure**

Warning: PCR detection method is very sensitive, and precautions should be taken to avoid cross contamination between samples (from instruments, equipment etc.).

1. Use 1.5 µL of the DNA extracted from each sample in a 25 µL PCR reaction.
2. Conduct PCR using specific primers based on the *T. vivax* proline racemase (*TvPRAC*) gene. (*TvPRAC*-F: 5'-CGCAAGTGGACCGTTTCGCCT-3' and *TvPRAC*-R: 5'-ACGCGGGGCGAACAGAAGTG-3') with negative (no DNA template) and positive DNA sample. Use the following PCR program for primers 5 min 94°C, 40 cycles of 94°C for 30 sec, 63°C for 30 sec and 72°C for 30 sec, then 72°C for 10 min as described by Fikru *et al.*, 2014.

Note: that in case of using Hot Start DNA polymerase enzyme in the PCR reaction, the start denaturation should be extended to 15 min.

3. Separate PCR products by electrophoresis on 1.5% agarose gel (**Figure 3**). DNA dye (Ethidium bromide, Safe green or etc.) can be used to stain the PCR product, although precautions should be taken to avoid any contact with this product as it is a powerful mutagenic.
4. Visualize the results of electrophoresis using gel documentation system (**Figure 4**).
5. Analyze the PCR results of each sample with the positive control and record the band size/length of each sample using the DNA ladder (**Figure 9**). The presence of a PCR product of 239 bp indicates *T. vivax* infection.

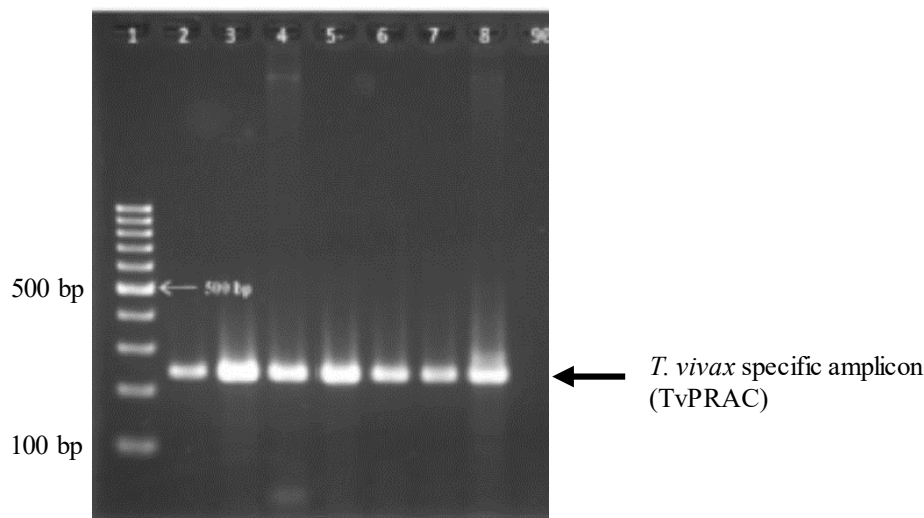


Figure 9. 2 % agarose gel image showing the PCR-amplification product with *T. vivax* specific primers (TvPRAC). The presence of a 239 bp PCR product indicates *T. vivax* infection (Fikru et al., 2014).

6.2.2. Identification of *Trypanozoon* spp (*T. brucei brucei*, *T. brucei rhodesiense*, *T. brucei gambiense*, *T. evansi*)

- **Purpose**

This method is used to identify one of the *Trypanozoon* spp in DNA samples.

- **Scope**

This procedure is designed to identify *Trypanozoon* spp (*T. brucei brucei*, *T. brucei rhodesiense*, *T. brucei gambiense*, *T. evansi*) in the extracted DNA samples from tsetse or host blood.

- **Materials and equipment requirements**

The following materials and equipment are needed:

- DNA samples extracted in Section 3
- Clean room
- Air conditioning
- PCR work station
- PCR thermocycler

- Pipette (e.g. Pipetman set)
- Pipette tips (e.g. Pipetman tips)
- Refrigerator
- PCR master mix reagent
- *Trypanozoon* spp specific primers
- Agarose
- Gel electrophoresis apparatus
- DNA dye (Ethidium bromide, Safe green or etc.)
- DNA ladder
- Sterile distilled water
- Gloves

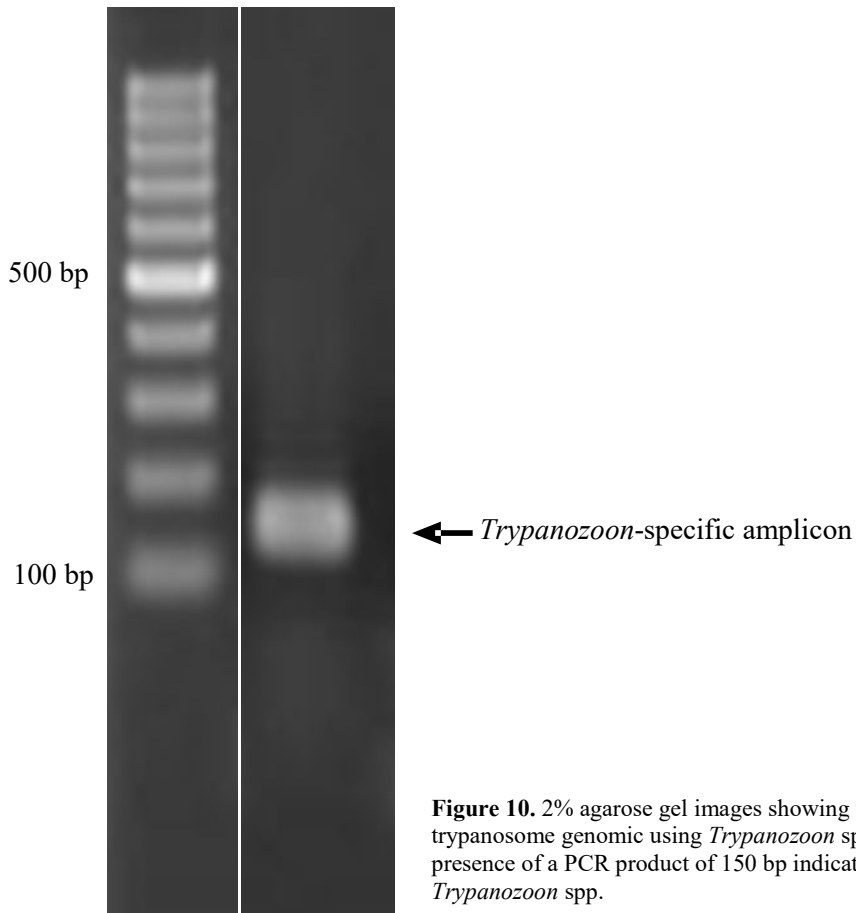
- **Required skills**

Research or laboratory technician with the required skills for molecular biology work, including PCR technology and controlling PCR results on agarose gel.

- **Procedure**

Warning: PCR detection method is very sensitive, and precautions should be taken to avoid cross contamination between samples (from instruments, equipment etc.).

1. Use 1.5 µL of the DNA extracted from each sample in a 25 µL PCR reaction.
2. Conduct PCR using *Trypanozoon* spp specific primers (M18S-II-F-Tb 5'-CGTAGTTGAACTGTGGGCCACGT-3' and M18S-II-R-Tb 5'-ATGCATGACATGCGTGAAAGTGAG-3') with negative (no DNA template) and positive DNA samples (positive DNA sample of known tsetse species). Use the following PCR program for primers 5 min 95°C, 40 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec, then 72°C for 10 min. **Note:** that in case of using Hot Start DNA polymerase enzyme in the PCR reaction, the start denaturation should be extended to 15 min.
3. Separate PCR products by electrophoresis on 1.5% agarose gel (**Figure 3**). DNA dye (Ethidium bromide, Safe green or etc.) can be used to stain the PCR product, although precautions should be taken to avoid any contact with this product as it is a powerful mutagenic.
4. Visualize the results of electrophoresis using gel documentation system (**Figure 4**).
5. Analyze the PCR results of each sample with the positive control and record the band size/length of each sample using the DNA ladder (**Figure 10**). The presence of band of 150 bp indicate the infection with one of the *Trypanozoon* spp (*T. brucei brucei*, *T. brucei rhodesiense*, *T. brucei gambiense*, *T. evansi*)



6.2.3. Identification of *T. brucei gambiense*

- **Purpose**

This method is used to identify one of the *T. brucei gambiense* in DNA samples.

- **Scope**

This procedure is designed to identify *T. brucei gambiense* in the extracted DNA samples from tsetse or host blood.

- **Materials and equipment requirements**

The following materials and equipment are needed:

- DNA samples extracted in Section 3
- Clean room
- Air conditioning
- PCR work station
- PCR thermocycler
- Pipette (e.g. Pipetman set)
- Pipette tips (e.g. Pipetman tips)
- Refrigerator
- PCR master mix reagent
- *T. brucei gambiense* specific primers
- Agarose
- Gel electrophoresis apparatus

- DNA dye (Ethidium bromide, Safe green or etc.)
- DNA ladder
- Sterile distilled water
- Gloves

- **Required skills**

Research or laboratory technician with the required skills for molecular biology work, including PCR technology and controlling PCR results on agarose gel.

- **Procedure**

Warning: PCR detection method is very sensitive, and precautions should be taken to avoid cross contamination between samples (from instruments, equipment etc.).

1. Use 1.5 µL of the DNA extracted from each sample in a 25 µL PCR reaction.
2. Conduct PCR using *T. brucei gambiense* specific primers (TgsGP-F: 5'-GCTGCTGTGTTCTGGAGAGC-3' and TgsGP-R: 5'-GCCATCGTGCTTGCCGCTC-3') with negative (no DNA template) and positive DNA sample. Use the following PCR program for primers 5 min 94°C, 45 cycles of 94°C for 30 sec, 63°C for 60 sec and 72°C for 60 sec, then 72°C for 10 min as described by Radwanska et al., 2002a. **Note:** that in case of using Hot Start DNA polymerase enzyme in the PCR reaction, the start denaturation should be extended to 15 min.
3. Separate PCR products by electrophoresis on 1.5% agarose gel (**Figure 3**). DNA dye (Ethidium bromide, Safe green or etc.) can be used to stain the PCR product, although precautions should be taken to avoid any contact with this product as it is a powerful mutagenic.
4. Visualize the results of electrophoresis using gel documentation system (**Figure 4**).
5. Analyze the PCR results of each sample with the positive control and record the band size/length of each sample using the DNA ladder (**Figure 11**). The presence of band of 308 bp indicate the infection with *T. brucei gambiense*.

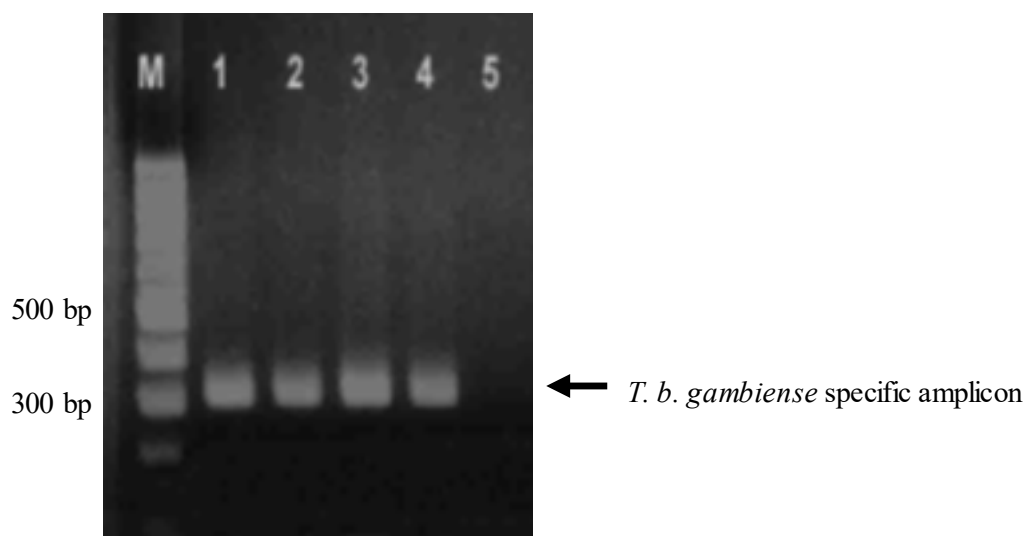


Figure 11. 2% agarose gel images showing the amplification of trypanosome genomic using *T. brucei rhodesiense* specific primers. The presence of PCR product of 308 bp indicates infection with one of the *T. brucei gambiense*. (Radwanska et al., 2002b)

6.2.4. Identification of *T. brucei rhodesiense*

- **Purpose**

This method is used to identify one of the *T. brucei rhodesiense* in DNA samples.

- **Scope**

This procedure is designed to identify *T. brucei rhodesiense* in the extracted DNA samples from tsetse or host blood.

- **Materials and equipment requirements**

The following materials and equipment are needed:

- DNA samples extracted in Section 3
- Clean room
- Air conditioning
- PCR work station
- PCR thermocycler
- Pipette (e.g. Pipetman set)
- Pipette tips (e.g. Pipetman tips)
- Refrigerator
- PCR master mix reagent
- *T. brucei rhodesiense* specific primers
- Agarose
- Gel electrophoresis apparatus
- DNA dye (Ethidium bromide, Safe green or etc.)
- DNA ladder
- Sterile distilled water
- Gloves

- **Required skills**

Research or laboratory technician with the required skills for molecular biology work, including PCR technology and controlling PCR results on agarose gel.

- **Procedure**

Warning: PCR detection method is very sensitive, and precautions should be taken to avoid cross contamination between samples (from instruments, equipment etc.).

1. Use 1.5 µL of the DNA extracted from each sample in a 25 µL PCR reaction.
2. Conduct PCR using *T. brucei rhodesiense* specific primers (TbrSRA-F: 5'-ATAGTGACAAGATGCGTACTCAACGC-3' and TbrSRA-R: 5'-AATGTGTTTCGAGTACTTCGGTCACGCTC-3') with negative (no DNA template) and positive DNA sample. Use the following PCR program for primers 5 min 95°C, 35 cycles of 94°C for 60 sec, 68°C for 60 sec and 72°C for 60 sec, then 72°C for 10 min as described by Radwanska *et al.*, 2002b. **Note:** that in case of using Hot Start DNA polymerase enzyme in the PCR reaction, the start denaturation should be extended to 15 min.
3. Separate PCR products by electrophoresis on 2% agarose gel (**Fig. 3**). DNA dye (Ethidium bromide, Safe green or etc.) can be used to stain the PCR product, although precautions should be taken to avoid any contact with this product as it is a powerful mutagenic.

4. Visualize the results of electrophoresis using gel documentation system (**Figure 4**).
5. Analyze the PCR results of each sample with the positive control and record the band size/length of each sample using the DNA ladder (**Figure 12**). The presence of band of 284 bp indicate the infection with one of the *T. brucei rhodesiense*.

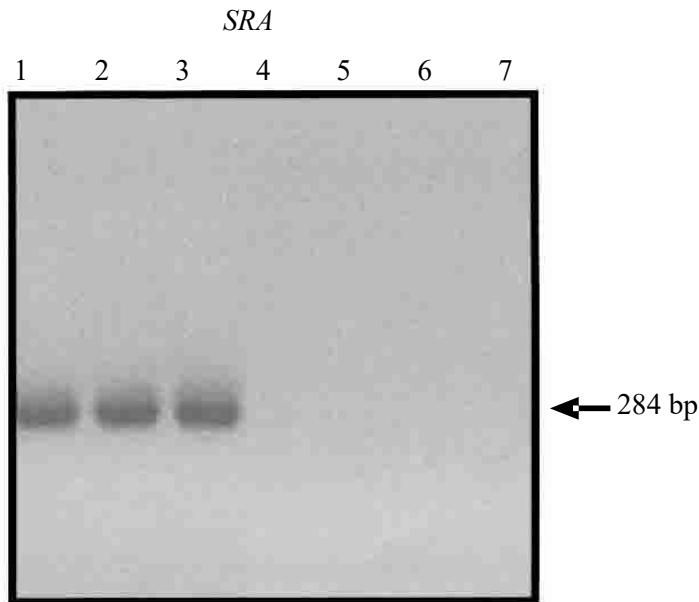


Figure 12. 2% agarose gel images showing the amplification of trypanosome genomic using *T. brucei rhodesiense* specific primers. The presence of PCR product of 284 bp indicates infection with one of the *T. brucei rhodesiense* (Radwanska et al., 2002a).

6.2.5. Identification of *T. congolense* Savannah and *T. congolense* Forest (PCR-RFLP).

- **Purpose**

This method can be used to distinguish between *T. congolense* Savannah and *T. congolense* Forest in DNA samples.

- **Scope**

This procedure is designed to distinguish between *T. congolense* Savannah and *T. congolense* Forest in the extracted DNA samples from tsetse or host blood.

- **Materials and equipment requirements**

The following materials and equipment are needed:

- DNA samples extracted in Section 3
- Clean room
- Air conditioning
- PCR work station
- PCR thermocycler
- Pipette (e.g. Pipetman set)
- Pipette tips (e.g. Pipetman tips)
- Refrigerator

- PCR master mix reagent
- *T. congolense* savannah and *T. congolense* forest specific primers (18S primers)
- *Msp* I restriction enzyme
- Incubator at 37 ° C
- Agarose
- Gel electrophoresis apparatus
- DNA dye (Ethidium bromide, Safe green or etc.)
- DNA ladder
- Sterile distilled water
- Gloves

- **Required skills**

Research or laboratory technician with the required skills for molecular biology work, including PCR technology and controlling PCR results on agarose gel.

- **Procedure**

Warning: PCR detection method is very sensitive, and precautions should be taken to avoid cross contamination between samples (from instruments, equipment etc.).

1. Use 1.5 µL of the DNA extracted from each sample in a 25 µL PCR reaction.
2. Conduct PCR using 18S Trypanosoma generic primers (18STnF2: 5'-CAACGATGACACCCATGAATTGGGGA-3' and 18STnR2: 5'-GTGTCTTGTCTCACTGACATTGTAGTG-3') with negative (no DNA template) and positive DNA samples. Use the following PCR program for primers 5 min 95°C, 40 cycles of 94°C for 30 sec, 58°C for 30 sec and 72°C for 30 sec, then 72°C for 10 min as described by Geysen *et al.*, 2003. Note 1: in case of using Hot Start DNA polymerase enzyme in the PCR reaction, the start denaturation should be extended to 15 min; Note 2: at this stage the PCR product can be visualized according to 5. and 6. The presence of a PCR product (non-digested) of 600-700 bp indicates the amplified trypanosome 18S sequence (**Figure 14**).
3. Digest PCR reactions (4 µL of PCR products) with *Msp* I restriction enzyme for at least 2 hrs at 37 ° C.
4. Separate the digested PCR product by electrophoresis on a 2 % agarose gel (**Figure 3**). DNA dye (Ethidium bromide, Safe green or etc.) can be used to stain the PCR product, although precautions should be taken to avoid any contact with this product as it is a powerful mutagenic.
5. Visualize the results of electrophoresis using gel documentation system (**Figure 4**).
6. Analyse the digested PCR results of each sample with the positive control and record band restriction pattern of each sample using the DNA ladder (**Figure 11**). Band size of digested PCR product determines trypanosome species as shown in **Figure 13**.

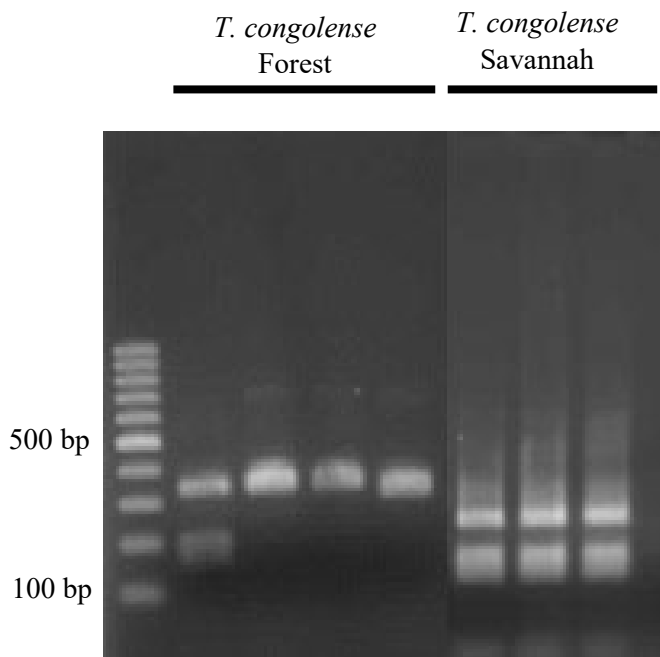


Figure 13. 2% agarose gel image showing the *Msp* I restriction pattern of the 18S amplified sequence for *T. congolense* Forest and *T. congolense* Savannah.

6.2.6. Identification of *Trypanosoma theileri* (RFLP)

- **Purpose**

This method can be used to distinguish *Trypanosoma theileri* from other species except *T. simiae* in DNA samples. It is important to note that *T. theileri* is a nonpathogenic trypanosome but in some area is more prevalent and it is important to include it in the screening. The presence of DNA of *T. theileri* in the tested vertebrate host sample will give positive results in the Pan-species PCR tests similar to other pathogenic trypanosomes.

- **Scope**

This procedure is designed to distinguish *Trypanosoma theileri* from other species except *T. simiae* in DNA samples from tsetse or host blood. This test is similar to the test in section 7.2.5 but the restriction profile is different from other *Trypanosoma* species except *T. simiae*.

- **Materials and equipment requirements**

The following materials and equipment are needed:

- DNA samples extracted in Section 3
- Clean room
- Air conditioning
- PCR work station
- PCR thermocycler
- Pipette (e.g. Pipetman set)
- Pipette tips (e.g. Pipetman tips)
- Refrigerator

- PCR master mix reagent
- *Trypanosoma* specific primers (18s primers)
- *Msp* I restriction enzyme
- Incubator at 37 ° C
- Agarose
- Gel electrophoresis apparatus
- DNA dye (Ethidium bromide, Safe green or etc.)
- DNA ladder
- Sterile distilled water
- Gloves

- **Required skills**

Research or laboratory technician with the required skills for molecular biology work, including PCR technology and controlling PCR results on agarose gel.

- **Procedure**

Warning: PCR detection method is very sensitive, and precautions should be taken to avoid cross contamination between samples (from instruments, equipment etc.).

1. Use 1.5 µL of the DNA extracted from each sample in a 25 µL PCR reaction.
2. Conduct PCR using 18S *Trypanosoma* generic primers (18STnF2: 5'-CAACGATGACACCCATGAATTGGGGA-3' and 18STnR2: 5'-GTGTCTTGTTCTCACTGACATTGTAGTG-3') with negative (no DNA template) and positive DNA samples. Use the following PCR program for primers 5 min 95°C, 40 cycles of 94°C for 30 sec, 58°C for 30 sec and 72°C for 30 sec, then 72°C for 10 min as described by Geysen *et al.*, 2003. Note 1: in case of using Hot Start DNA polymerase enzyme in the PCR reaction, the start denaturation should be extended to 15 min; Note 2: at this stage the PCR product can be visualized according to 5. and 6. The presence of a PCR product (non-digested) of 600-700 bp indicates the amplified trypanosome 18S sequence (**Figure 14**).
3. Separate digested PCR products by electrophoresis on 2% agarose gel (**Figure 2**). DNA dye (Ethidium bromide, Safe green or etc.) can be used to stain the PCR product, although precautions should be taken to avoid any contact with this product as it is a powerful mutagenic.
4. Visualize the results of electrophoresis using gel documentation system (**Figure 3**).
5. Analyse the digested PCR results of each sample with the positive control and record the band size/length of each sample using the DNA ladder (**Figure 14**).



Figure 14. 2% agarose gel images showing the amplification of trypanosome genomic DNA using *Trypanosoma* specific primers. The amplification PCR product (non-digested) with 700 bp indicates trypanosome infection. The presence of variable size of digested PCR prod

7. CONCLUSION

The following scheme (**Figure 15**) can be used as guidance for the steps required identifying or distinguishing trypanosome species.

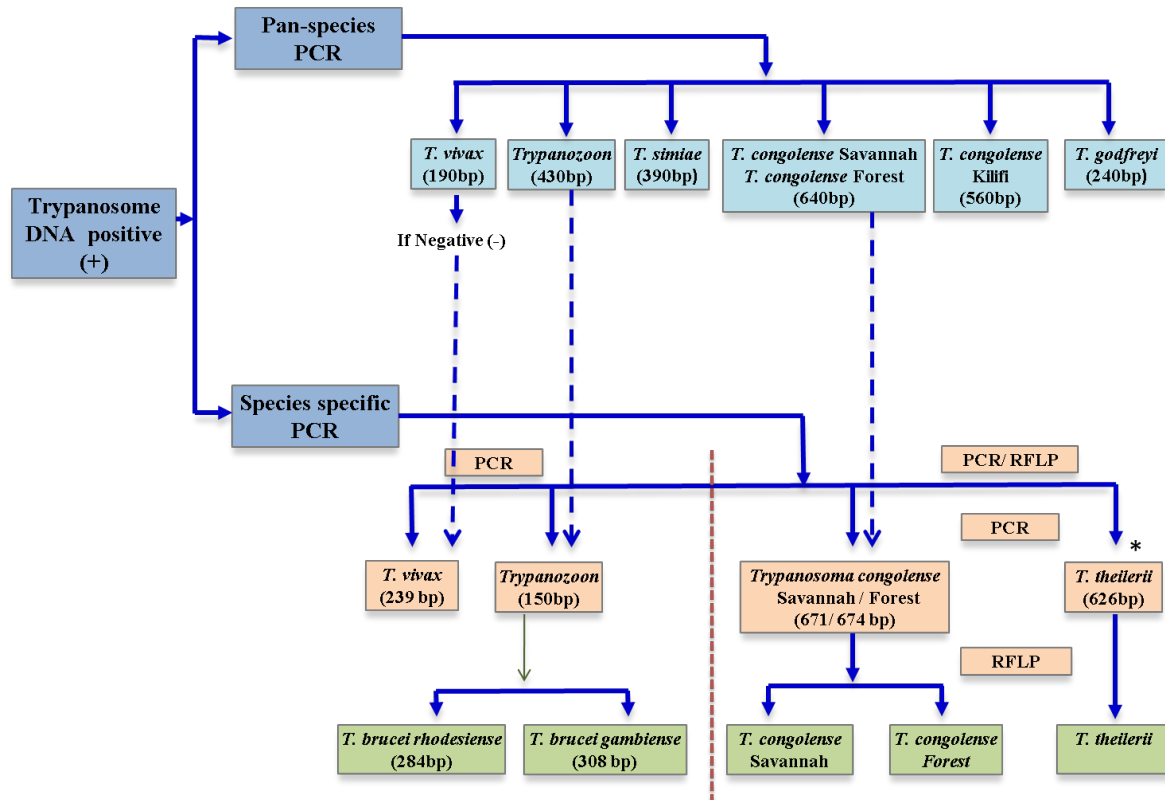


Figure 15. Schematic representation of step by step application of molecular tools for trypanosome species identification.

**T. theileri* is not a pathogenic trypanosome (see section 6.2.6).

8. REFERENCES

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9. DEFINITIONS

Nano-drop technology: A technology developed for micro-volume quantitation and analysis.

Gel documentation system: A gel image system or gel imager, is equipment used in molecular biology for the imaging and documentation of nucleic acid and protein suspended within polyacrylamide or agarose gels.

DNeasy®: This is the registered trade mark for a kit used for DNA extraction.

Pan-Species trypanosome PCR: Generic PCR-method utilizing primers that recognize a semi-conserved region of the trypanosome genome allowing the amplification of the target sequence in a broad range of trypanosomes. This method is less sensitive than species-specific PCR methods but allow for multiple trypanosome species to be identified with a single test.

APPENDIX 1: Special equipment and materials with specifications.

Item	Specification	Example of supplier and/or distributor
A. Sterile micropestles	Hand-operated or motor-driven grinders for disrupting tissue in microcentrifuge tubes. Pestle ends should be specially designed to mate with 0.5 ml or 1.5 ml microtubes.	Labsco or Sigma
B. Autoclave	An autoclave is a pressure chamber used to sterilize equipment and supplies by subjecting them to high pressure and saturated steam at 121 °C for around 15–20 minutes depending on the size of the load and the contents.	Labsco or Sigma
C. PCR work station	A self-contained work area that will help protect PCR runs against contamination. It should have HEPA H14 filter, stainless steel work surface, and a UV light with a timer. Dual UV bulbs irradiate the work area prior to use, reducing the possibility that any contaminating DNA will be amplified. Containment features reduce the chance of airborne contamination.	Labsco, Erlab or Sigma
D. PCR thermocycler	A device to conduct DNA amplification; it should be a compact 96-well thermal cycler with thermal gradient optimization and reliable performance.	Bio-Rad, Eppendorf, Labsco or VWR
E. Pipette (e.g. Pipetman set)	A set of pipette includes 6 adjustable-volume pipettes (0.1-2.5 µl, 0.5–10 µl, 2–20 µl, 10–100 µl, 20–200 µl, 100–1,000 µl).	Eppendorf or Gilson
F. Pipette tips (e.g. Pipetman tips)	Sterile tips with reload or filtered, which fit the different pipettes size.	Labsco or VWR
G. Water bath	Water bath with microprocessor controller for temperature selection, rapid heat-up and excellent stability. It should be equipped with an adjustable over-temperature safety cutout, which sounds an audible alarm and shuts the heater off to protect samples, and recessed control panel to protect against spills. Also, it should have a seamless passivated tank to prevent against rust and corrosion.	Labsco or VWR
H. Microcentrifuge. i.e. Centrifuge 5424 / 5424 R, Eppendorf.	Micro-centrifuge with the following specification as example: max. rcf: 20,238 x g,	Eppendorf, Labsco or VWR

	<p>max. speed: 14,680 rpm, max. rotor capacity: 24x 1.5/2.0 ml, no. of rotors: 4, acceleration time to max. speed: 16 s, braking time from max. speed: 18 s, SOFT ramp: adjustable, Noise level with rotor FA-45-24-11: <51 dB(A), dimensions in cm (W x D x H): 24 x 32 x 23, weight without rotor: 13.4 kg, power supply: 230 V/50–60Hz, power requirement max.: 250 W</p>	
I. DNeasy kit	Kit for DNA extraction	Qiagen or Labsco
J. PCR master Mix reagent	Ready-made mix for PCR reaction; it contains all the required components, including MgCl ₂ , buffer, dNTPs, DNA polymerase for PCR reaction, except primers and DNA template	Qiagen or Labsco
K. Gel electrophoresis apparatus	A horizontal electrophoresis chambers with a wide (15 cm) platform to provide higher capacity. It should include a buffer tank, safety lid with cables, and leveling bubble.	Bio-Rad, Or Labsco
L. DNA ladder	A mixture of DNA fragments with known length, which helps to determine the size of unknown DNA fragments using gel electrophoresis.	Qiagen or Invitrogen
M. Nano-drop spectrophotometer	A spectrophotometer that measures 1 µl nucleic acid samples with high accuracy and reproducibility. It should have a full spectrum (220nm-750nm) spectrophotometer. It requires a PC or laptop with suitable software to analyze and calculate the nucleic acid concentration.	Thermo Scientific or Labsco