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# Purification of trypanosomes from blood by Anion-exchangers (Diethylaminoethyl-cellulose columns)

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**TITLE:**

# Purification of Extracellular Trypanosomes, including African, from Blood by Anion-Exchangers (Diethylaminoethyl-cellulose columns)

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**KEYWORDS:**

Trypanosomes, purification, anion-exchangers, DEAE-cellulose, chromatography, mini-column, parasite detection, pharmaceutical tests, immunological analysis, antigens, cell biology, molecular biology

**SUMMARY:**

This method of trypanosome separation from blood depends on their surface charge being less negative than mammalian blood cells. Infected blood is placed and treated on an anion-exchanger column. This method, the most fitting diagnostic for African trypanosomiasis, provides purified parasites for immunological, biological, biochemical, pharmaceutical and molecular biology investigations.

**ABSTRACT:**

This method allows the separation of trypanosomes, parasites responsible for animal and human African trypanosomiasis (HAT), from infected blood. This is the best method for diagnosis of first stage HAT and furthermore this parasite purification method permits

serological and research investigations.

HAT is caused by Tsetse fly transmitted *Trypanosoma brucei gambiense* and *T. b. rhodesiense.* Related trypanosomes are the causative agents of animal trypanosomiasis. Trypanosome detection is essential for HAT diagnosis, treatment and follow-up. The technique described here is the most sensitive parasite detection technique, adapted to field conditions for the diagnosis of *T. gambiense* HAT and can be completed within one hour. Blood is layered onto an anion-exchanger column (DEAE cellulose) previously adjusted to pH 8, and elution buffer is added. Highly negatively charged blood cells are adsorbed onto the column whereas the less negatively charged trypanosomes pass through. Collected trypanosomes are pelleted by centrifugation and observed by microscopy. Moreover, parasites are prepared without cellular damage whilst maintaining their infectivity.

Purified trypanosomes are required for immunological testing; they are used in the trypanolysis assay, the gold standard in HAT serology. Stained parasites are utilized in the card agglutination test (CATT) for field serology. Antigens from purified trypanosomes, such as variant surface glycoprotein, exoantigens, are also used in various immunoassays. The procedure described here is designed for African trypanosomes; consequently, chromatography conditions have to be adapted to each trypanosome strain, and more generally, to the blood of each species of host mammal.

These fascinating pathogens are easily purified and available to use in biochemical, molecular and cell biology studies including co-culture with host cells to investigate host-parasite relationships at the level of membrane receptors, signaling, and gene expression; drug testing *in vitro*; investigation of gene deletion, mutation, or overexpression on metabolic processes, cytoskeletal biogenesis and parasite survival.

**INTRODUCTION:**

The method presented described here allows the separation of trypanosomes, parasites responsible for animal and human African trypanosomiasis (HAT), from blood. This is the best method for diagnosis of first stage HAT and furthermore this parasite purification method permits robust serological and research investigation.

HAT is caused by Tsetse fly transmitted *Trypanosoma brucei gambiense* and *T. b. rhodesiense*[1]. These protozoan parasites multiply extracellularly in the bloodstream, lymph, and interstitial fluids during the first stage of the disease (hemolymphatic stage). The second stage (meningoencephalitic stage) begins when parasites cross the blood brain barrier; neurological signs, including a sleep disorder, which has given its name “sleeping sickness” to this disease, are typical of this second-stage[2]. Related trypanosomes (*T. evansi, T. congolense, T. vivax, T. b. brucei*) are the causative agents of animal African trypanosomosis (AAT)[3].

The World Health Organization (WHO) aims to eliminate HAT as a public health problem by 2020 and to stop transmission by 2030[4]. The recent introduction of rapid diagnosis tests has improved serological diagnosis[1,4,5]. Several molecular diagnostic tests have been developed but

their role in field diagnostics has not yet been established[5]. They are used to identify the sub-species of the *brucei* group and atypical trypanosomiasis caused by parasites responsible for animal trypanosomosis[6].

The detection of the parasite is essential for the diagnosis, treatment and follow-up, as serology can give false positive and unfortunately false negative results[1]. The direct microscopical observation of these hemoflagellate protists is difficult in HAT cases that are caused by *T. b. gambiense*, (more than 95% of cases) as low parasitemias are the rule, whereas for HAT caused by *T. b. rhodesiense*, a large number of parasites are frequently present in the blood. Various concentration techniques have been used, such as thick drop and capillary tube centrifugation (CTC), but the separation of parasites from blood by a column of anion-exchanger (DEAE cellulose) followed by centrifugation and microscopic observation of the pellet, is the most sensitive method (around 50 parasites/mL of blood can be detected)[1,7]. Consequently, the purification of trypanosomes from by anion-exchangers (DEAE cellulose) method is the best and, to date, the reference method for visualizing and isolating parasites from blood for HAT diagnosis. In field conditions, a mini-column of DEAE cellulose has been successfully used and several improvements have facilitated microscopical observation[7,8].

The method of trypanosome separation from blood, described below, depends on parasite surface charge, which is less negative than mammalian blood cells[9]. Interestingly, this method was developed 50 years ago, in 1968 by Dr. Sheila Lanham, and remains the gold standard for detection and preparation of bloodstream trypanosomes. It is fast and reproducible for salivarian trypanosomes from a wide range of mammals, permitting the diagnosis of both animal and human trypanosomiasis[10].

To obtain living, purified parasites, infected blood is added onto an anion-exchanger column. Chromatography conditions (mainly pH, ionic strength of buffers/media) have to be adapted to each trypanosome species, and more generally, to each mix of mammalian blood cells and trypanosomes[10]. Elution buffer is precisely adjusted to pH 8 for most African trypanosomes[10]. This method favors the concentration of parasites found in the blood of patients, because parasitemias can be too low to be detected by microscopic observation alone, and it also enables laboratory investigations. Working with freshly isolated trypanosomes and on blood from infected animals is more pertinent using this technique for various investigations than studies with parasites that have been cultured in axenic conditions in the laboratory for an indefinite period.

Host-parasite relationships are best studied with a parasite infecting its natural host, therefore, *T. musculi*, a natural murine parasite, which is representative of extracellular trypanosome, has many advantages as murine infection involves in a laboratory small animal and does not require biohazard safety level (BSL) conditions. *T. musculi* does not kill immunocompetent mice, unlike many other *Trypanosoma* species, including human pathogens. *T. musculi* are not eliminated in T cell-deprived mice and parasitemias can be increased in infected mice by modifying food and nutrient intake[11]. This parasite modulates the immune response in co-infections with other pathogens[12]. *T. musculi* from infected mice exhibit differences from cultured *T. musculi,* for

example, the expression of membrane Fc receptors is lost in *T. musculi* axenic cultures, compared to parasites purified from infected mice[13,14]. Excreted-secreted factors (ESF) are also qualitatively and quantitatively less expressed in axenic trypanosome cultures and differ between strains isolated in endemic areas[15]. ESF are the first antigens to be displayed to the host immune system and so play an important role in the initial host immune response[16].

In experimentally infected animals for laboratory investigations, this protocol facilitates experimentation on a greater number of parasites, minimizing the number of mice required especially when using immunosuppressed animals. The variant surface glycoproteins (VSGs) that are used in the Card Agglutination Test for Trypanosomiasis (CATT) in mass screening are still purified from trypanosomes that are propagated in rats. The two rapid diagnostic tests (individually wrapped cassettes) that are now available for use in the field, are still using an infective model source of native VSGs and not *in vitro* cultured trypanosomes[1,4,5]. The advancement in the study of trypanosome immunology and biology have been facilitated since these DEAE cellulose purified parasites can be easily obtained in large quantities from naturally or experimentally infected hosts, and in particular, rodents.

**PROTOCOL:**

Investigations conformed to the Guidelines for the Care and Use of Laboratory Animals (NIH Publication No. 85±23, revised 1996). Protocols were approved by our local ethics committee.

**1. Animals**

1.1. Keep female Swiss (OF-1) mice aged eight to ten weeks old, 20-25 g in an animal housing facility fifteen days before each experiment. House them in ventilated boxes that are kept in a protected, temperature (22 °C) and humidity (50%) controlled room, with 12 hours on/off light cycle.

1.2. Give animals free access to food and water. Minimize pain, suffering and distress and provide enrichment of the environment.

1.3. For housing, use clear-walled cages, enrichment with wooden sticks and cardboard tunnels. Gently draw an animal into a tunnel to transfer it from the cage to the palm of the hand.

1.4. Perform daily monitoring to assess signs of prostration, social isolation, body injury, ruffled hair, lack of grooming.

1.5. Weigh each animal once per week. Perform regular inspections by a veterinarian.

1.6. For natural parasites, collect blood at the peak of parasitemia and for parasites causing animal death, collect blood the day before presumed death.

NOTE: All experiments with infectious agents are performed in dedicated rooms, according to university approved guidelines.

## 2. Buffers, media preparations

2.1. Weigh out each substance and add distilled water for the following buffers:

2.1.1. Prepare Concentrated Phosphate-Buffered Saline (2x),

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|---|---|
| $Na_2HPO_4$ (anhydrous) (MW 141.96 g) | 10.14 g |
| $NaH_2PO_4 \cdot 2H_2O$ (MW 156.01 g) | 0.62 g |
| NaCl (MW 58.44 g) | 2.55 g |
| Distilled $H_2O$ to | 1 L |

2.1.2. Prepare Phosphate-Buffered Saline-Glucose:

| | |
|---|---|
| $Na_2HPO_4$ (anhydrous) (MW 141.96 g) | 5.39 g |
| $NaH_2PO_4 \cdot 2H_2O$ (MW 156.01 g) | 0.31 g |
| NaCl (MW 58.44 g) | 1.70 g |
| Glucose (MW 180 g) | 10 g |
| Distilled $H_2O$ to | 1 L |

2.1.3. Prepare 1 M $KH_2PO_4$.

2.1.4. Prepare Elution Buffer: Supplement Phosphate-Buffered Saline-Glucose with Penicillin (100 U/mL), Streptomycin (100 µg/mL), and Phenol red (5 µg/mL).

## 3. Preparation of DEAE-cellulose

3.1. Plan around 5 hours for the DEAE-cellulose preparation.

3.2. Wash 100 g of DEAE-cellulose with distilled water in a flask with a narrow neck and allow to settle then discard fine particles. Repeat washes until the supernatant is clear.

3.3. Add 3 L of concentrated 2x Phosphate-Buffered Saline and stir.

3.4. Adjust pH to 8.0 with 1 M $KH_2PO_4$ and discard supernatant.

3.5. Wash twice with distilled water and leave to settle.

3.6. Wash and allow to settle twice with 3 liters Phosphate-Buffered Saline-Glucose and discard the supernatants.

3.7. Measure the volume of cellulose and add an equal volume of Phosphate-Buffered Saline-Glucose, distribute into plastic bottles and store at -20 °C.

## 4. Parasites

4.1. Collect trypanosome strains in areas endemic for human and animal trypanosomiasis. Keep parasites frozen in liquid nitrogen.

NOTE: *Trypanosoma musculi* is non-pathogenic to humans and is an extracellular trypanosome used to safely replace pathogenic trypanosomes in various laboratory experiments.

4.2. In the case of laboratory investigations requiring human pathogens, handle experiments with care in dedicated appropriate biohazard safety level (BSL) conditions (precautions), BSL2 for *T. b. gambiense* and BSL3 for *T. b. rhodesiense*. In field conditions, standard microbiological practice is established: secure sampling, mechanical pipetting, frequent decontamination of surfaces, and sterilization of waste.

## 5. Mouse infection

5.1. Quickly thaw parasites in a water bath at 37 °C.

5.2. Observe a drop of thawed infected blood with a microscope. Assess parasite viability by measuring the percentage of motile forms[17].

5.3. Inject parasites intraperitoneally into mice (0.5 mL per mouse). Each day, post-infection, collect 20 µL of tail blood by needle puncture and observe under a microscope. Evaluate parasitemia according to Herbert and Lumsden[18] by counting parasite in several microscope fields, or by using an haemocytometer.

5.4. When the parasitemia reaches a threshold that is defined for each strain, collect the blood (1 mL/mouse) in the elution buffer (5 mL/mouse) containing heparin (10 U/mL).

NOTE: The original and novel work of Lanham and Godfrey reported the optimal ionic strength of phosphate buffered saline glucose for several host/parasite species from a pH 8.0 stock solution.

## 6. Parasite separation

NOTE: All experiments from this point onwards must be done in a tissue culture hood wearing gloves. The room temperature and humidity in the laboratories used were 22 °C and 45% respectively. In field conditions, parasite separation has been successfully performed at 34 °C.

6.1. Place a 10 mL syringe in a vertical support and add a previously cut circular, piece of filter paper, glass wool or cellulose sponge.

6.2. Pour the DEAE cellulose into the syringe until the 8 mL level is reached, and then wash with 25 mL of elution buffer.

6.3. Carefully add 2 mL of diluted blood at the top of the column and then add elution medium. Regularly add elution buffer according to the transit of the trypanosomes.

6.4. Collect effluent drops from the columns in a centrifuge tube and check regularly for the presence of parasites with a microscope.

6.5. When parasites are no longer detected in the column effluent, centrifuge the tube (1,800 x *g*, 10 minutes, at 4 °C in the laboratory and at ambient temperature in field conditions).

6.6. Remove the supernatant and suspend the parasites in 1 mL of the relevant medium required for the next investigative step.

6.7. Count parasites with a hemocytometer and dilute them in appropriate medium if necessary.

**REPRESENTATIVE RESULTS:**

Purified trypanosomes have been used in pharmaceutical tests. Parasites are transferred into culture wells containing serial dilutions of specific drugs, either alone or mixed[19]. Microscopic observations, evaluating motility is a marker of viability, can be performed when only a few dugs are being tested, whereas AlamarBlue cell viability assay is an excellent method for large motility assays during drug screening[20]. The effects of pentamidine, a reference drug used in HAT therapy, is displayed in **Figure 1**.

Macrophages are very useful in cultures as feeder cells. They allow, in vitro, the initiation and development of trypanosome cultures[21]. We have reported that the numbers of alternatively activated macrophages are increased in trypanosome-infected mammals in which they favor parasite growth[22]. This alternative macrophage activation supplies L-ornithine, which is essential for parasite growth. In vitro macrophage-parasite co-cultures have shown that trypanosomes induce macrophage alternative activation via secreted factors. Extracellular trypanosomes secrete a kinesin which binds macrophage mannose binding receptors inducing arginase expression providing L-ornithine production favoring parasite differentiation and multiplication[23,24] (**Figure 2**). Mannose inhibits kinesin binding and arginase induction, and mannose receptor-deficient mice elucidate the target of kinesin on macrophages (**Figure 3**).

Since numerous trypanosome genomes have now been sequenced and annotated, we have been able to take advantage of these large data sets. Subsequently, we have been able to carry out forward and reverse genetics on these parasites. Using these data, purified bloodstream form trypanosomes have been used to characterize and analyze important structures such as the flagellar pocket (FP) and its associated cytoskeleton. The FP is the sole site of endo and exocytosis in trypanosomes and is also where the variable surface glycoproteins are trafficked from the endomembrane system[25]. The flagellar pocket collar (FPC) is an annular shaped structure that is attached to the flagellum at the point where it exits the FP, but until recently

little was known about the protein constituents of the FPC. We have identified a major protein of the FPC, *Tb*BILBO1, and have shown that it is essential for parasite survival both in the cultured insect tsetse fly form and in the bloodstream form[26]. Knockdown of *Tb*BILBO1 by RNAi prevents FP formation and inhibits the biogenesis of many other important cytoskeletal structures, making the FPC and the FP important targets for intervention in all pathogenic trypanosomes. Probing purified or cultured parasite cells with antibodies to *Tb*BILBO1 indicates that in bloodstream form and procyclic (insect form), it creates a ring-shaped structure that circumvents the flagellum. Such labelling, on a bloodstream form, is shown in **Figure 4**.

Purified bloodstream form trypanosomes have allowed the characterization of many unusual biochemical and metabolic peculiarities, including metabolism of glucose, which take place in peroxisome-like organelles called glycosomes (see **Figure 5**). It was generally accepted that pyruvate is the major end-product excreted from glucose metabolism by the bloodstream trypanosomes, with virtually no production of succinate and acetate inside the mitochondrion. In contrast, the procyclic trypanosomes convert threonine into acetate and glucose into succinate and acetate[27,28]. Energy metabolism of trypanosomatids can be evaluated by adaptation to available carbon sources. Combining reverse genetics and metabolomic analyses confirmed production in the mitochondrion of bloodstream trypanosomes of acetate from glucose-derived pyruvate and threonine, as well as production of succinate from glucose[19,20] (**Figure 5**). For instance, $^{1}$H-NMR analysis of end products excreted by bloodstream form trypanosomes incubated in PBS containing 4 mM glucose revealed that glucose is mainly converted into pyruvate (85.1% of the excreted end products), with minor production of alanine (9.2%), acetate (4.9%) and succinate (0.8%)[29]. These pathways, which are minor in terms of metabolic flux compared to pyruvate production from glucose, are essential for growth of the parasite. The succinate production pathway can thus be considered as a potential good target for the development of new trypanocidal drugs.

**FIGURE AND TABLE LEGENDS:**

**Figure 1: In vitro effect of pentamidine on *T. b. brucei*.** Dilutions of pentamidine were added to 2 x $10^5$ parasites to determine the concentration inhibiting parasite growth by 50% (IC50). Dose-effect curves at 24 hours of culture. Error bars represent the standard error of the mean from 5 independent experiments.

**Figure 2: Trypanosome-mediated arginase induction.** A kinesin released by trypanosomes binds to C-type lectin receptors leading to arginase induction. This results in increased production of L-ornithine and polyamines, essential for parasite growth and differentiation, and in L-arginine depletion, resulting in lower production of cytotoxic NO by macrophage NOS II.

**Figure 3: Macrophage arginase activity.** Arginase activity in macrophages from control mice and Mannose receptor knock out (KO) mice cultured *in vitro* in medium for 48 hours, with or without kinesin or mannose. Error bars represent the standard error of the mean from 5 independent experiments.

**Figure 4: *Tb*BILBO1 labeling of a bloodstream form *Trypanosoma brucei brucei* cell** (**A**) Immunofluorescence labeling of a bloodstream, culture form, 427 90–13 cell that has been probed with anti-BILBO1 monoclonal antibody followed by a FITC-labelled anti-mouse antibody and visualized using ultraviolet light, (*Tb*BILBO1 are the green annular signals) and the DNA binding dye DAPI (blue signals) (**B**) A merge of DAPI, anti-BILBO1 and phase contrast images of A. Scale bar equals 10 µm. Anti-BILBO1 mouse monoclonal was diluted 1:10 in PBS and the secondary antibody (anti-mouse IgM FITC) was diluted 1:100. Images were taken on a microscope fitted with a digital camera.

**Figure 5: Schematic representation of glucose and threonine metabolism in the bloodstream form trypanosomes**. Excreted end-products from glucose and threonine metabolism are boxed. The thick blue arrows indicate enzymatic steps of glucose metabolism leading to pyruvate production, which is the main end-product excreted from glycolysis. Black arrows represent overlooked minor metabolic pathways from glucose and threonine degradation, which are essential for growth of the parasite. The contribution of the indicated enzymes has been experimentally validated: ACH, acetyl-CoA thioesterase (EC 3.1.2.1); ASCT, acetate:succinate CoA-transferase (EC 2.8.3.18); AKCT, 2-amino-3-ketobutyrate coenzyme A ligase (EC 2.3.1.29); PEPCK, phosphoenolpyruvate carboxykinase (EC 4.1.1.49); PDH, pyruvate dehydrogenase complex (EC 1.2.4.1); TDH , threonine 3-dehydrogenase (EC 1.1.1.103). Abbreviations: AcCoA, acetyl-CoA; AOB, amino oxobutyrate; DHAP, dihydroxyacetone phosphate; G3P, glyceraldehyde 3-phosphate; MAL, malate; OA, oxaloacetate; PEP, phosphoenolpyruvate; PYR, pyruvate.

**DISCUSSION:**

Purified trypanosomes represent a powerful means to study immunology, biochemistry, cell and molecular biology. Large expanses of data and results have been obtained from trypanosomes, which has then helped to obtain information from other eukaryotic cells[30]. Trypanosomes are also the subject of important and interesting research because they have devised numerous mechanisms that permit them to survive and grow in two very different environments: the tsetse fly vector and the mammalian host[23,31]. Various techniques to isolate trypanosomes have been reported, and a review on microfluidics-based approaches has recently been published[32]. Hence, a reproducible and robust means of parasite separation is essential.

DEAE cellulose preparation is an indispensable step in this parasite preparation protocol. Washing conditions have to be performed cautiously to eliminate the fine particles, and equilibrate the resin, and the pH must be precisely adjusted (pH 8.0 is apt for most trypanosome species). All steps have to be adjusted to improve parasite purification and yield while maintaining parasite viability and cellular properties. Importantly, parasite viability and infectivity have been maintained after purification through a DEAE-cellulose column. However, some strains are more fragile than others and might be less infective after purification[33]. Therefore, the impact of separation conditions on pellicular membrane components, parasite metabolism, signaling, nucleic acid functions, and animal infectivity, have to be assessed and separation conditions have to be adapted accordingly.

Limitations to this technique are that this procedure has to be adapted to each trypanosome species in a given host and is also time consuming. Moreover, DEAE cellulose is now expensive. Preliminary assays are necessary to optimize separation conditions, notably the media, which may have varying ionic strengths and a precise pH. Pre-column steps, including anticoagulant choice, prior centrifugation to remove the majority of erythrocytes, buffy coat use, and erythrocyte lysis, are chosen according to each experimentation. Precise changes on a single parameter (buffers, temperature throughout the protocol, centrifugation parameters) might greatly increase the number, degree of purification and viability of parasites obtained[33]. Developing new separation parameters according to parasite species and the mammalian blood cells to be separated, might be necessary. Adjustments to the initial Lanham and Godfrey's protocol has allowed the purification of biologically and antigenically preserved *T. cruzi* from blood[34]. New resins can also be tested and used with appropriate conditions for different species[35].

The major role of excreted/secreted factors (ES) by trypanosomatids has recently been emphasized[16]. ES contain molecules involved in pathology and immunomodulation, such as kinesin, which is conserved among trypanosomes[24]. ES preparation from purified parasites requires particular care to avoid contamination by elution media components and lysed parasites.

An ES-based vaccine effective against *Leishmania*, a related parasite, already exists and is available (CaniLeish Virbac)[36]. Association of conserved molecules playing essential roles in parasite survival and growth might represent the basis for a future vaccine against trypanosomes, for both humans and animals, in a one-health approach. Purification of African trypanosomes from blood by DEAE-cellulose columns, with improvements, remains the gold standard for trypanosome detection in natural hosts with low parasitemias in endemic areas and for the need for parasites in large numbers for experimental investigations.

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**DISCLOSURES:**

The authors have nothing to disclose.

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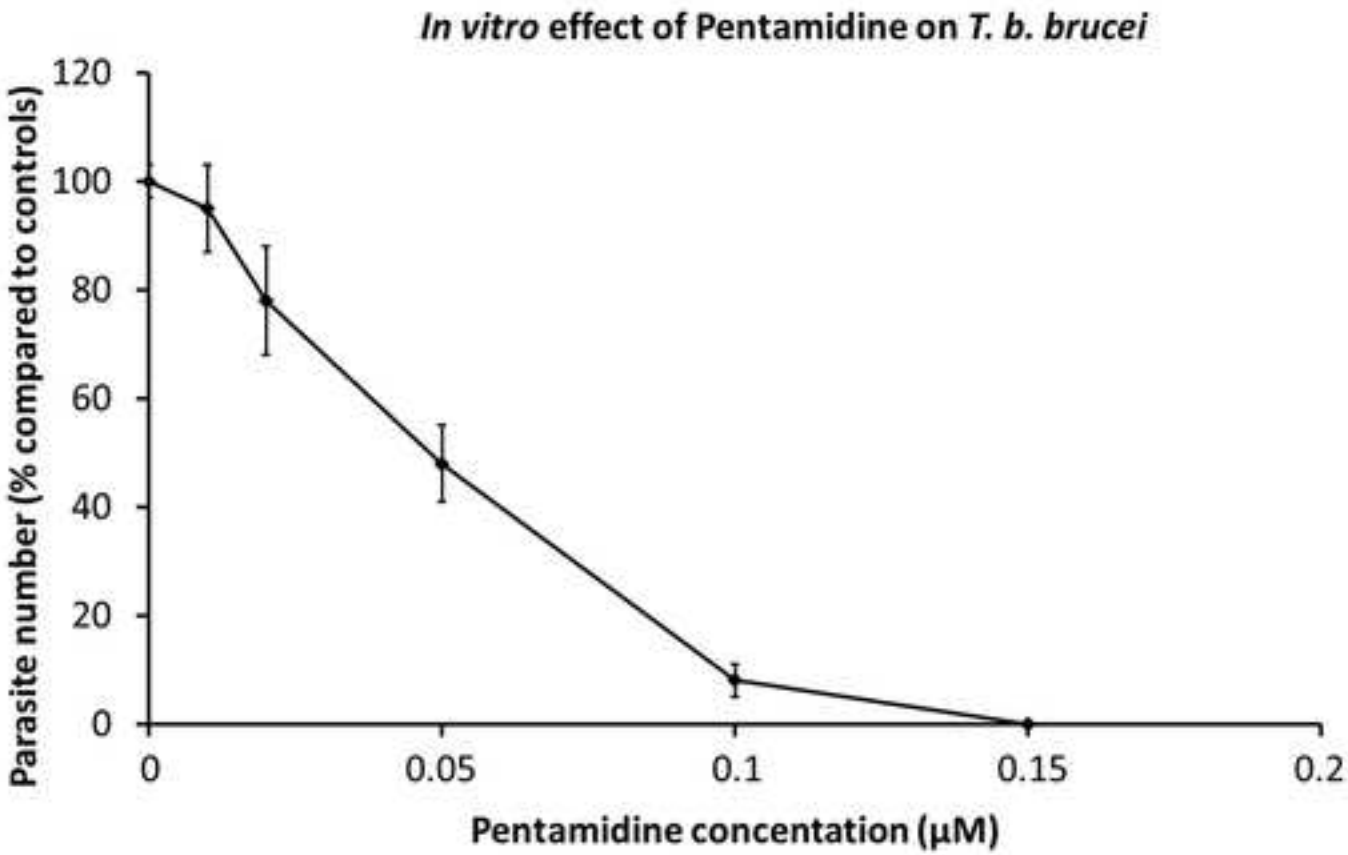
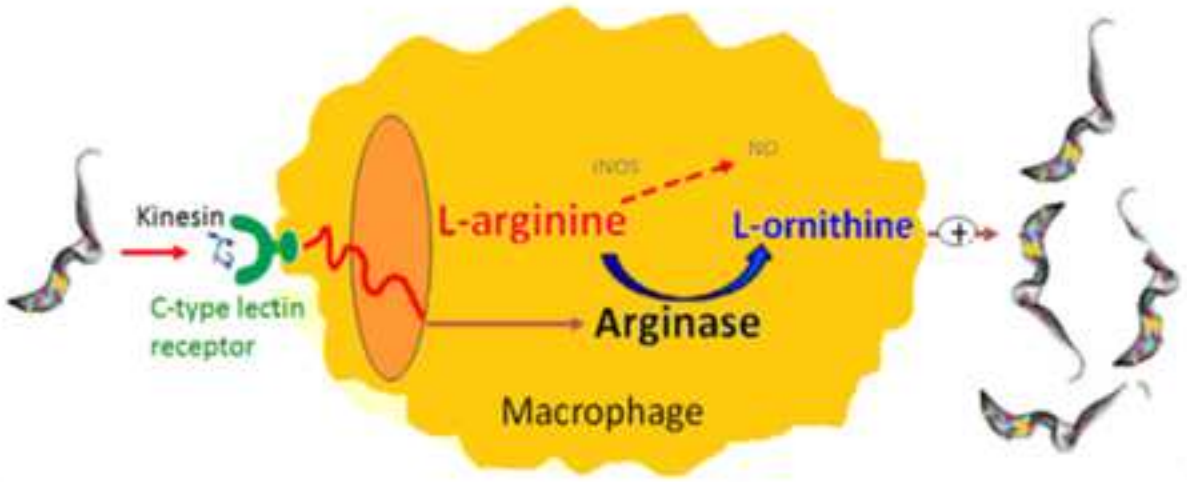
Figure





Figure

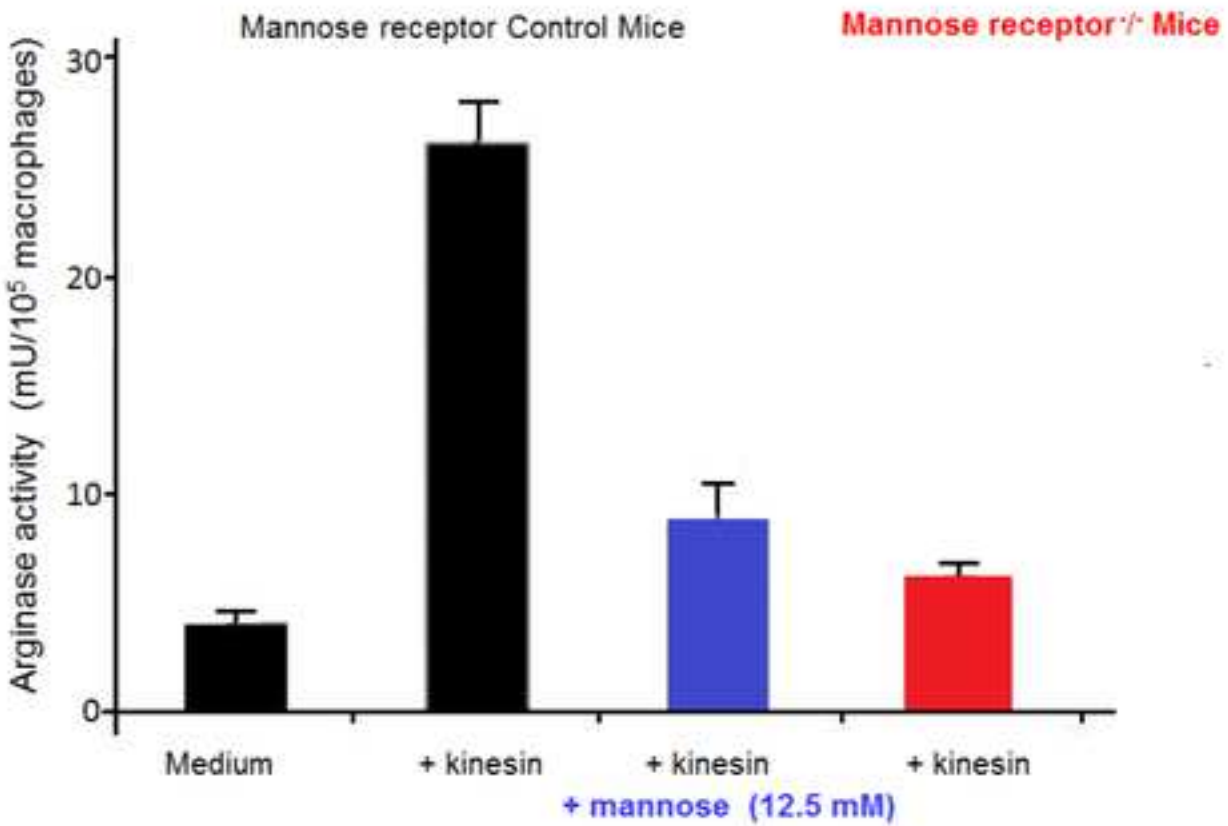





The majority of L-arginine is hydrolysed into L-ornithine, a precursor of polyamines and trypanothione, essential for parasite survival and multiplication (growth).
Morover, L-arginine depletion decreases the synthesis of microbicidal NO and its derivatives

Figure

Click here to access/download;Figure;Fig 3.TIF



Figure

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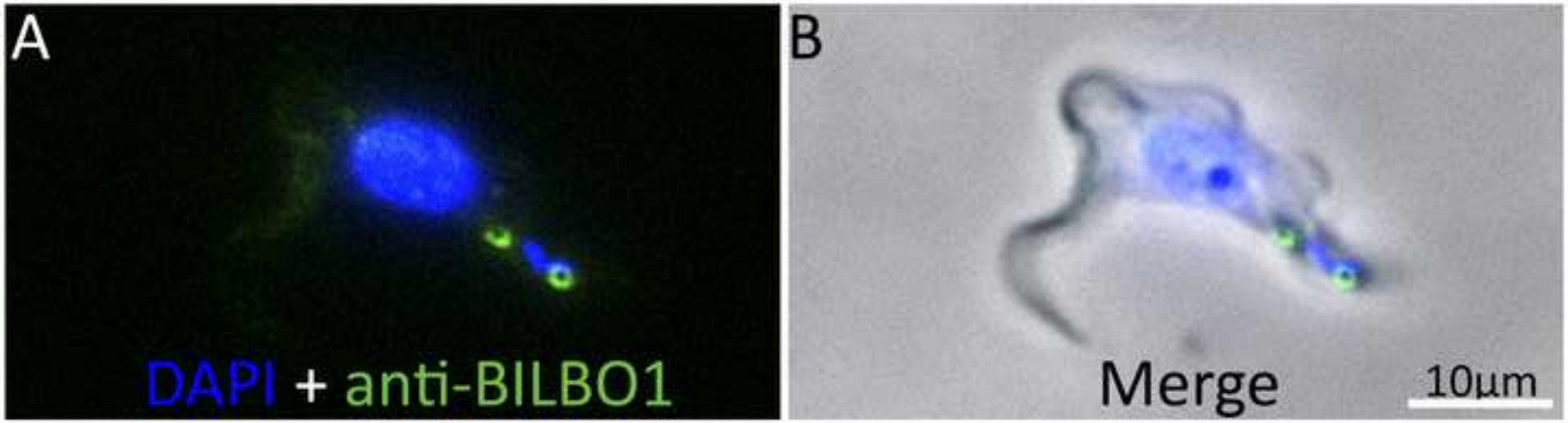
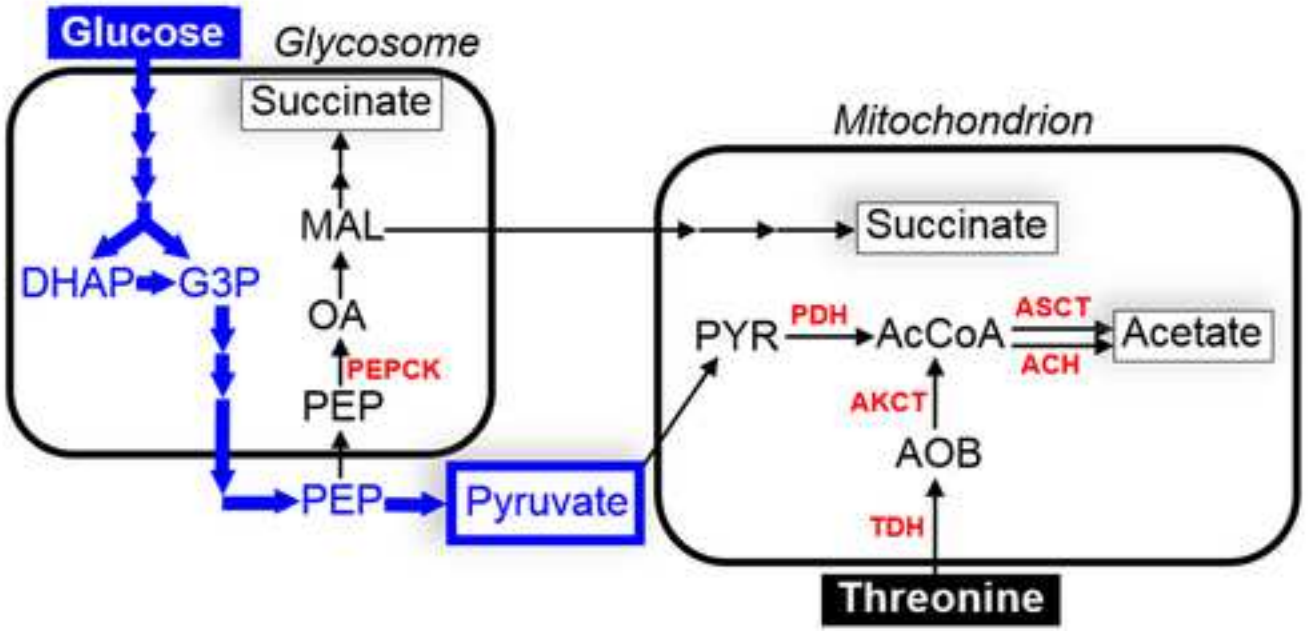


Figure





| Name of Material/ Equipment | Company | Catalog Number |
|---|---|---|
| 10 mL Pipettes | Falcon | 357,551 |
| 2 mL Pipettes | Falcon | 352,507 |
| Centrifugation tube 50 mL | Falcon | 352,070 |
| Centrifuge | Sigma Aldrich | 4K15 |
| DEAE cellulose | Santa Cruz | s/c- 211213 |
| filter paper | Whatman | 1,001,125 |
| Flat bottom flask narrow neck | Duran | 21 711 76 |
| Glucose | VWR | 101174Y |
| Heparin | Sigma Aldrich | H3149-50KU |
| $KH_2PO_4$ | VWR | 120 26936.260 |
| Microscope | Olympus | CH-20 |
| Microscope coverslips | Thermofisher scientific | CB00100RA020MNT0 |
| Microscope slides | Thermofisher scientific | AGAA000001 |
| $Na_2HPO_4$ | VWR | 100 28026;260 |
| NaCl | VWR | 27800.291 |
| $NaH_2PO_4$ | VWR | 110 33616;262 |
| Nalgene Plastic Media Bottles size 125 mL | Thermofisher scientific | 342024-0125 |
| Nalgene Plastic Media Bottles size 500 mL | Thermofisher scientific | 342024-0500 |
| Pasteur Pipette | VWR | BRND125400 |
| Penicillin 10,000 UI/Streptomycin 10,000 µg | EUROBIO | CABPES01 OU |
| Phenol red | Sigma Aldrich | P0290 |
| Syringue | Dutscher | SS+10S21381 |
| Tissue culture hood | Thermoelectro Corporation | MSC-12 |
| *Trypanosoma brucei brucei* | Institute of Tropical Medicine (Antwerp, Belgium). | |
| *Trypanosoma brucei gambiense* | Institute of Tropical Medicine (Antwerp, Belgium). | |
| *Trypanosoma musculi* | London School of Hygiene and Tropical Medicine (UK) | |

| Comments/Description |
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Author(s): Pierrette Courtois, Patricia Nabos, Romaric Nzoumbou-Boko, Christine Reix, Frédéric-Antoine Dauchy, Sylvie Daulouede, Frédéric Bringaud, Derrick R. Robinson, Philippe Vincendeau

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Changes to be made by the Author(s) regarding the written manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Manuscript has been proofread
2. Please revise lines 45-46, 75-77, and 134-140 to avoid previously published text. Done
3. Please label/number the institutional affiliation of each author sequentially. For example, the affiliations labels for Christine Reix should be 3,4 instead of 4,5. Done
4. Please rephrase the Abstract (in 150-300 words) to more clearly state the goal of the protocol. Done
5. Please rephrase the Introduction to include a clear statement of the overall goal of this method. Done
6. Please use the period symbol (.) for the decimal separator. For example, 10.14 g, 0.62 g, etc. Corrected
7. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.). Corrected
8. Please revise the protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Done
9. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion. Done
10. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Some examples:

1.1: Please specify the temperature and humidity of the room. Done

2.1-2.4: Please write the text in the imperative tense in complete sentences. Is water used here distilled or deionized? Done

3.1: Please specify the amount/volume of DEAE-cellulose used. Done

3.2: What container is used here? Done

3.4: Is the PBS solution discarded before washing? Please specify. Done

3.6: What volume of Phosphate-Buffered Saline-Glucose is added? Added

5.2: Please describe how to evaluate parasite viability. Added

5.3, 5.4: How much blood is collected? Added

5.4: What is used to adjust the pH? Added

11. Lines 172-180: Please move the material information to the Materials Table. Removed
12. Figures: The uploaded figures seem to be mislabeled. For instance, Figure 2 legend corresponds to Figure 3, while Figure 3 legend corresponds to Figure 2. Corrected
13. Figure 3: Please define the scale bar in the figure or its figure legend. Added
14. Discussion: Please discuss any limitations of the technique. Added

Changes to be made by the Author(s) regarding the video:

1. Please increase the homogeneity between the written protocol and the narration in the video. It

would be best if the narration is a word for word from the written protocol text.
2. Titles of the written manuscript and the video do not match. OK
3. Please use the same subtitle in the video as in the written protocol so that the protocol is easier to follow. For example, buffer preparation is in step 2 of the written protocol while it is combined with DEAE-cellulose preparation in the video. OK
4. 1:29-2:20 - We recommend removing this part of the video. The only visual during this time is text, so, rather than having the narrator read through the text, narration or a brief text overlay can be added later in the video that directs the viewer to the text protocol for information on DEAE-cellulose preparation. Alternatively, from 01:30-02:40, please show the actual process of preparing the solutions and infection process instead of showing a slide. OK
5. 01:30: Please use the period symbol (.) for the decimal separator. For example, 10.14 g, 0.62 g, 2.55 g, etc. Please include a space between the numerical value and the temperature unit (4 °C, 20 °C). Corrected
6. The details in the video are not the same as the details in the written manuscript. Please cross-reference the video narration with the protocol text. For example:
03:25: Please specify the volume of parasites injected into mice in the video (2 mice per 1 mL is indicated in the written protocol. Corrected
04:26: There is not wash step in the video while the written protocol (6.2) states that “wash with 25 mL of supplemented RPMI medium.” Corrected
7. 3:30, 4:23, 4:40 - These edits feel jarring. Crossfades should be used to smooth them out.
8. 5:04, 5:07 - These shots are not on screen long enough, resulting in too many edits in too short a span of time. Currently, the pacing at these points feels too quick. The shots need to either be extended or cut out.
9. Please use the same figures in the written manuscript and in the video in the Results section.
10. As a general note on audio quality, we recommend removing the audio from the camera for the protocol portion of the video. The camera audio tends to distract from the narration while not adding anything to the understanding of the protocol.
11. Please upload a revised high-resolution video here:
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**Reviewers' comments:**

**Reviewer #1:**
Manuscript Summary:
Purification of trypanosomes from blood by anion-exchange is a very useful method for both diagnosis of T. b. gambiense HAT and for isolation of trypanosomes for numerous research purposes. The text and video are well put togther. Most importantly they will allow others to reproduce this method accurately.

Minor Concerns:
1. Abstract Lines 57-59- 'this method is fast and reproducible'. It would be useful for people who want to carry out this method to know how long it actually takes to prepare and carry out, especially for those who are involved in active HAT screening for example. Added
2. Fig 2 is a bit pixelated Corrected
3. In the video it mentions that collected trypanosomes are centrifuged at 1200g for 12mins at 4oC. From a practical point of view it might be worth mentioning whether this could be done without a

refrigerated centrifuge which may not be available in the field. Added : at ambient temperature in field conditions

**Reviewer #2:**

Manuscript Summary:

The manuscript clearly describes a simple, yet profoundly useful protocol for the purification of trypanosomes and provides some examples as to the application of this method. The manuscript is accompanied by a good quality video with clear narration that demonstrates the method well enough to be repeated by any scientist.

Major Concerns:

1. While the description of the protocol is clear, both in the manuscript and in the video, there appears to be a disconnect between the explanatory figures in the video and the manuscript itself. This could be remedied if the figures that only appear in the video (at 7:55 and the 2D gel at 8:02) also appeared in the manuscript, with detailed figure legends. 2D gel has been removed
2. Glucose metabolism is discussed in the representative results section but no specific data are presented. A single experiment, demonstrating how purified parasites can be used to investigate glucose metabolism in trypanosomes, would make the results section much stronger. These data need not appear in the video so, re-shooting is unnecessary. Added

Minor Concerns:

1. As this work focuses on the purification of African trypansomes, the word "African" should appear in the title. Added
2. Line 53: "Adsorption" is probably a better term here than "absorption". We agree
3. Line 84: Please provide some examples for the recently developed diagnostic tests. added
4. Lines 110-112: The statement "conditions...have to be adapted to...each mix of mammalian blood cells and trypanosomes" suggests that the applicability of this method is greatly limited in the field. Does the author mean human vs. other animal blood/parasites or interindividual differences? (The latter would make field diagnosis difficult.) I assume gambiense and rhodesiense need somewhat different conditions. Is adaptation of the protocol necessary for optimization or is it an absolute requirement for parasite concentration? It is not interindividual difference. Conditions for T. b. gambiense diagnostic -are well established. Patients with rhodesiense HAT usually have high levels of parasitemia
5. Line 112: "This method favors the concentration of parasites found in the field". How so? Corrected
6. Line 117-119: Please avoid verbatim repetition of that sentence that also appears in line 39.OK
7. Please copy-edit the protocol. Line 132: mice 12-25g, Line 142: "2X" is missing from the description of the concentrated buffer (stated in the video), Line 154: MW of glucose is missing. Added
8. Lines 218-219: How was motility quantified? Microscopic observation of moving parasites. Added Assess parasite viability by measuring the percentage of motile forms.
9. Line 300: DEAE cellulose preparation is only an indispensable step in parasite preparation using THIS method. (Other purification methods exist). Please clarify. OK
10. Lines 267-271. Legend for Figure 2 (which is actually Figure 3 in the pdf). Please expand on the description (dilution of antibodies, type of microscope used). Corrected

11. Figure 3: Scale bar size is missing. Corrected

The video is of good quality in general but please include further explanations in the text.
The figure at 7:54 should be included in the manuscript with a detailed figure legend. Please explain in the text why SIGN-R1 KO mice are important, as this is not specifically explained in the video.
At 8:07, there is a gel in the middle of the slide with no explanation. Please include these data in the text and a detailed figure legend. The use of KO mice has been added. 2D gel has been removed, SIGN-R1 KO and 2D gel removed

**Reviewer #3:**
Manuscript Summary:
This article and video demonstrates a method of purification of African trypanosomes using anion exchange chromatography. The original separation method developed by Sheila Lanham and David Godfrey (refs 9 and 10) has undergone numerous changes as described here, some of which are of questionable value and are likely to involve major revision to the text and the video demonstration to make them suitable for publication. The original method was able to purify trypanosomes in substantial amounts from rat blood required for biochemical experiments. The current method is certainly useful for diagnostic work (but there are videos already on the internet, so not novel) and rather limited in its utility in terms of yields (e.g. ~109 trypanosomes per 1 ml mouse blood). If used to obtain such small amounts of parasite material, then the authors need to justify why they are not applying the 3Rs and using non-animal alternatives such as axenic cell culture.

The 3Rs have been addressed by the following; (1) Replacement - Mice have been replaced in many cases but cannot be replaced in some experiments due to loss of parasite pathogenicity that is routinely observed. Host-parasite relationships are the best studied with a parasite infecting its natural host.(2) Reduction - We have been able to reduce the number of mice used by improving methods. (3) Refinement - The technique has been refined by providing the best housing conditions, care, treatment and reduction of stress and pain for the mice.

Added in the text: Pain, suffering and distress are minimized and enrichment of the environment is provided. Housing conditions improvements are: clear-walled cages, enrichment with wooden sticks and cardboard tunnels. An animal is gently drawn into a tunnel to transfer it from the cage to the palm of the hand. Daily monitoring is performed to assess signs of prostration, social isolation, body injury, ruffled hair, lack of grooming. Each animal is weighed once per week. Regular inspections are performed by a veterinarian. For natural parasites, blood is collected at the peak of parasitemia and for parasites causing animal death, blood is collected the day before presumed death. All experiments with infectious agents are performed in dedicated rooms, according to university approved guidelines.

These sentences have been added

Host-parasite relationships are best studied with a parasite infecting its natural host, therefore, T. musculi, a natural murine extracellular trypanosome, which is representative of extracellular trypanosome, has many advantages as murine infection evolve in a laboratory small animal and does not require biohazard safety level (BSL) conditions. T. musculi does not kill immunocompetent mice, unlike many other Trypanosoma species, including human pathogens. T. musculi are not eliminated

in T cell-deprived mice and parasitemias can be increased in infected mice by modifying food and nutrient intake. This parasite modulates the immune response in co-infections with other pathogens. T. musculi from infected mice exhibit differences from cultured T. musculi, for example, the expression of membrane Fc receptors is lost in T. musculi axenic cultures, compared to parasites purified from infected mice. Excreted-secreted factors (ESF) are also qualitatively and quantitatively less expressed in axenic trypanosome cultures and differ between strains isolated in endemic areas. ESF are the first antigens to be displayed to the host immune system and so play an important role in the initial host immune response.

The variant surface glycoproteins (VSGs) that are used in the Card Agglutination Test for Trypanosomiasis (CATT) in mass screening are still purified from trypanosomes that are propagated in rats. The two rapid diagnostic tests (individually wrapped cassettes) that are now available for use in the field, are still using an infective model source of native VSGs and not in vitro cultured trypanosomes.

Major Concerns:

1. Line 110-112. The authors mention that the pH and ionic strength of the media have to be adapted for each trypanosome strain (I think they mean species) and for each type of animal blood. If the DEAE cellulose is not correctly equilibrated both in terms of pH (within 0.05 units of pH 8.0) and ionic strength (i=0.22) then the method does not work optimally. (I believe that the original method (ref 10) recommended PSG diluted 6:4 for rat blood, but somewhere else it was published that mouse blood separations were optimal with PSG 3:7). There is no mention of this critical point in their methodology. Indeed, they seriously deviate from the initial published protocol by eluting the column with RPMI media, adjusted to pH 8.0, not phosphate saline-glucose that the column was pre-equilibrated with! The original method notes that the 1g of pre-swollen DE52 cellulose (approximately 4 ml) absorbs 1.0 - 1.6 ml of whole blood, yet in the video 8 ml of resin fails to retain 2 ml of mouse blood. Worse, the video at 4 min 35 sec shows a marked irregularity in the erythrocyte front such that by 4 min 38s erythrocytes are eluting from the column. This does not happen when the correct eluting buffer (PSG) is used.

1-Line 110-112, We agree that effectively, species is the correct word. Precisely was added to pH 8.0 The following sentence have also been added: The original work of Lanham and Godfrey reported the optimal ionic strength of phosphate buffered saline glucose for several host/parasite species from a pH 8 stock solution. The eluted medium has been precised. Details have been added in the text. The first incorrect video has been amended. An 8 mL column is prepared before collecting blood from two infected mice.

2. The choice of representative results are not ideal. Lines 217 - 219 suggest that pharmaceutical testing for drug potency is measured microscopically by observing viable motile parasites. This method is low throughput and mention of more robust methods such as Alamar Blue / resazurin should be cited (Raz, B., etal . (1997) The Alamar Blue assay to determine drug sensitivity of African trypanosomes (T.b.rhodesiense and T.b.gambiense) in vitro. Acta Tropica 68, 139-147. The choice of posaconazole for a representative drug test is a bizarre example to choose. The IC50 potency against the South American trypanosome T. cruzi is less than 10 nM, whereas the IC50 ~8 µM for T. brucei is > 1,000-fold less potent. Indeed, the observed trypanocidal effect is likely to be due to off-target

effects not involving inhibition of CYP51. The authors would be better advised to illustrate this method using an existing treatment for HAT such as melarsoprol or pentamidine.
Posaconazole has been replaced by Pentamidine and Ref on Alamar Bue added

However, Treatment for second stage gambiense HAT relies principally on nifurtimox + eflornithine combination therapy (NECT) since 2009 with eflornithine 400 mg/kg. In mice, 20 mg/kg posaconazole has been used in various mycology investigations. In humans, high doses of posaconazole (600-800 mg per day) have been administered for one month and is well tolerated.

3. Macrophages as feeder cells is another strange choice of representative results. It is well established that trypanosomes can grow axenically in vitro at rates approaching those in vivo in media that is not supplemented with ornithine. Ornithine is not a rate-limiting nutrient because animal and human serum contain arginase, which converts arginine in the medium into ornithine for uptake by the parasite. A better example would be feeder cells maintaining cystine as cysteine in media (now circumvented by the addition of bathocupronine and 2-mercaptoethanol to axenic media).

Macrophages and others cells have been widely used for trypanosome growth in vitro. A feeder layer is most of the times required to adapt trypanosomes from hosts to in vitro cultures.

The inducible metabolism of L-arginine in macrophages plays an essential role. Trypanosome growth was inhibited when IFN-$\gamma$ is added to macrophage-trypanosome co-culture. Arginase induction by trypanosome is required to promote parasite growth and settlement in the host. Arginase inducing gene is conserved in trypanosome species. Antibodies directed to this factor inhibited *T. musculi* growth both in vitro and in vivo.

A Trypanosoma brucei kinesin heavy chain (TbKHC1) promotes parasite growth by triggering host arginase activity. In vitro, the growth of TbKHC1 KO parasites is identical to WT and TbKHC1 rescued parasites. The parasite load in the first peak of parasitaemia in TbKHC1 KO-infected mice was reduced by >70% as compared to WT parasites. Reinsertion of TbKHC1 in TbKHC1 KO parasites reverted early parasitaemia to WT levels. In mice lacking arginase-1 in myeloid cells/macrophages following a cross between Arg1 loxP-targeted mice and LysM Cre or Tie2 Cre deleter mice, WT parasitaemia dropped to that of TbKHC1 KO parasitaemia. Moreover, treatment of mice with L-ornithine increased the cumulative parasite load to a greater extent in TbKHC1 KO- than in WT-infected mice.

Arginase induction was firstly evidenced with trypanosomes to promote parasite growth and favor parasite settlement. In the same way, arginase induction is also used by others trypanosomatids (Leishmania ssp, Trypanosoma cruzi).

4. Lines 255 - 258. Threonine catabolism may have been "overlooked" by the authors of this article, but is well known in the literature since the 1970s (Linstead, D. J., Klein, R. A., and Cross, G. A. M. (1977) Threonine catabolism in Trypanosoma brucei. J. Gen. Microbiol. 101, 243-251; and Cross, G. A. M., Klein, R. A., and Linstead, D. J. (1975) Utilization of amino acids by Trypanosoma brucei in culture: L-threonine as a precursor for acetate. Parasitology 71, 311-326) and was not discovered using reverse genetics and metabolomics as implied here. This contentious statement should be withdrawn and appropriate reference to the above authors included. It is naïve to suggest formation

of acetyl-coenzyme A is a good drug target since reference 19 shows that simultaneous blockage of both the pyruvate and threonine pathways is necessary to inhibit growth.

lines 255-258 The corresponding paragraph has been rewritten.

"It was generally accepted that pyruvate is the major end-product excreted from glucose metabolism by the bloodstream trypanosomes, with virtually no production of succinate and acetate inside the mitochondrion. In contrast, the procyclic trypanosomes convert threonine into acetate and glucose into succinate and acetate (Cross, G. A. M., Klein, R. A., and Linstead, D. J. (1975) Utilization of amino acids by Trypanosoma brucei in culture: L-threonine as a precursor for acetate. Parasitology 71, 311-326; Bringaud F., L. Rivière & V. Coustou (2006) Energy metabolism of trypanosomatids: adaptation to available carbon sources. Mol. Biochem. Parasitol. 149:1-9.). Combining reverse genetics and metabolomic analyses confirmed production in the mitochondrion of bloodstream trypanosomes of acetate from glucose-derived pyruvate and threonine, as well as production of succinate from glucose19,20 (figure 4). These pathways, which are minor in terms of metabolic flux compared to pyruvate production from glucose, are essential for growth of the parasite. The succinate production pathway can thus be considered as a potential good target for the development of new trypanocidal drugs."

Minor Concerns:

1. There is no mention of the biohazard safety level required to safely handle BSL2 (T. gambiense) and BSL3 (T. rhodesiense) parasites. This should be included. Included
2. Line 137-138. It is completely unacceptable to make such vague statements such as "efforts are made to minimize the suffering of animals used". Please be specific about frequency of cage inspection depending on likelihood of achieving terminal parasitaemia, physical appearance (starey coat, prostration, etc) and behaviour of infected animals. Added
3. Lines 150 - 155. Specify the final pH (and acceptable range) and ionic strength or conductivity of this buffer.pH added conductivity 2060 µS
4. Line 160. Is it solid or liquid (what molarity?) sodium bicarbonate used to adjust pH? Table of materials mentions foetal calf serum. Is this used here and, if not, where in the article? Removed
5. Line 188. This reference is not readily available. Please provide details or substitute use of a haemocytometer for counting viable cells. Added
6. Line 196. Mention glass wool or cellulose sponges are also suitable alternatives to filter paper? Added
7. Lines 267 - 275. The figure legends are in reverse order. corrected
8. Line 281. Threonine as a source of acetate is not overlooked! overlooked" has been removed.
9. Line 284. "ligase" missing from AKCT. Add EC numbers to enzyme names for clarity. OK
10. Figure 4. The pathway from threonine is incorrect. TDH (EC 1.1.1.103) precedes AKCT (EC 2.3.1.29). corrected "ligase" has been included and EC numbers have been included for each enzyme in Figure
11. The video will need adjusting to take into account the above criticisms. The English pronunciation of kinesin is not standard in terms of the length of the letter "i" and emphasis on the last syllable. Should sound like "ki" (as in sky) "nes" (as in knees) and "in". O