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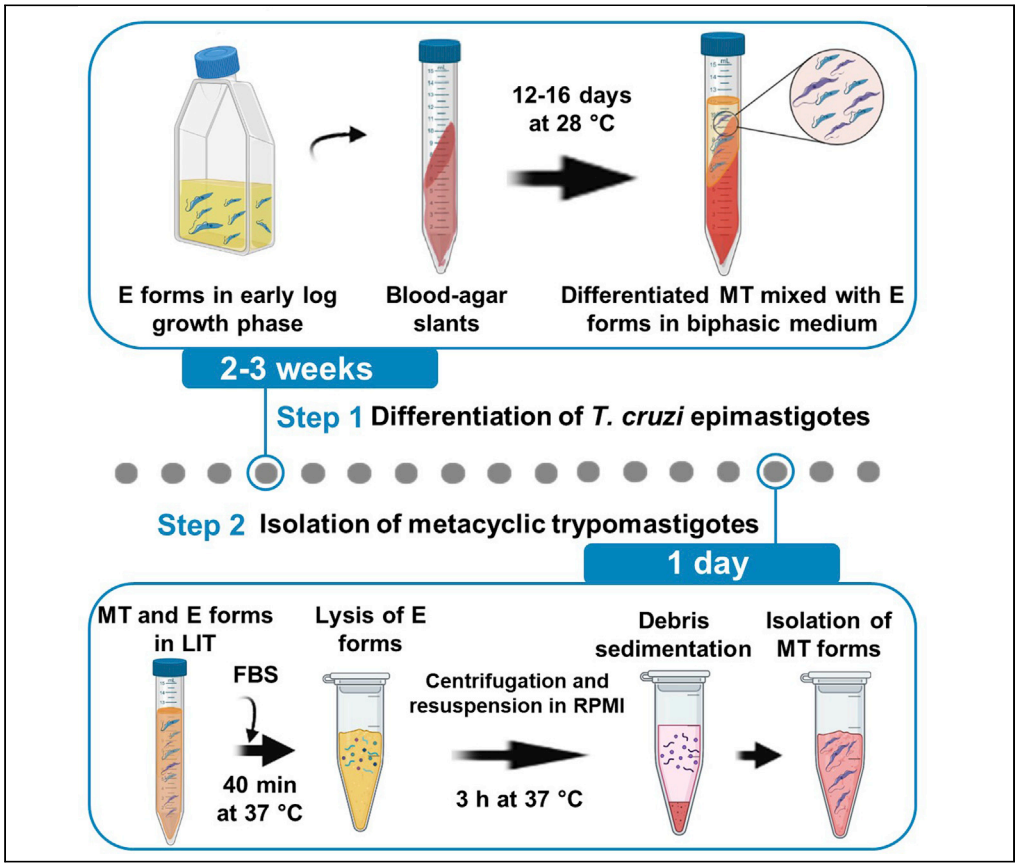
In vitro differentiation of *Trypanosoma cruzi* epimastigotes into metacyclic trypomastigotes using a biphasic medium



The pathogen *Trypanosoma cruzi* differentiates from epimastigotes (E) into infective metacyclic trypomastigotes (MTs) to invade the mammalian cell. This process, called metacyclogenesis, is mimicked in vitro by nutrient starvation or incubation with minimal media. Here, we describe an alternative protocol for metacyclogenesis by incubating E forms in a biphasic medium supplemented with human blood. Although time consuming, this procedure yields fully differentiated MTs without the presence of intermediate forms, even for cultures that have been maintained as E for years.

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Highlights

T. cruzi epimastigotes are differentiated into metacyclic forms in biphasic media

Fully differentiated metacyclic trypomastigotes are obtained without intermediate forms

Long-term cultured epimastigotes can be differentiated by this approach

Metacyclogenesis occurs in the presence of the widely used resistance marker Geneticin

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In vitro differentiation of *Trypanosoma cruzi* epimastigotes into metacyclic trypomastigotes using a biphasic medium

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SUMMARY

The pathogen *Trypanosoma cruzi* differentiates from epimastigotes (E) into infective metacyclic trypomastigotes (MTs) to invade the mammalian cell. This process, called metacyclogenesis, is mimicked in vitro by nutrient starvation or incubation with minimal media. Here, we describe an alternative protocol for metacyclogenesis by incubating E forms in a biphasic medium supplemented with human blood. Although time consuming, this procedure yields fully differentiated MTs without the presence of intermediate forms, even for cultures that have been maintained as E for years.

BEFORE YOU BEGIN

The protocol presented here describes the steps to differentiate E forms into MT using the Y strain of *T. cruzi*, typified as a Discrete Typing Unit II, DTUII (Zingales et al., 2009). However, we have success to differentiate E forms from a *T. cruzi* strain belonging to the DTUI lineage.

The *in vitro* metacyclogenesis procedure was performed in presence of Geneticin, using genetically modified E forms harboring an empty pTREX expression vector, which confers resistance to this antibiotic (Vazquez and Levin, 1999). Hence, this method may be useful to differentiate E forms overexpressing different proteins under study which requires this antibiotic as a selectable marker.

The procedure detailed in this protocol consists on the incubation of E forms at 28°C in a biphasic medium based on blood-agar slants modified from Neal and Miles, 1963. The preparation of both solid and liquid components of the biphasic medium, as well as the starting E culture, is depicted in this section.

Preparation of blood agar slants and liver infusion tryptose (LIT) media

Timing: 1 day

1. Blood-agar slants (solid phase)
   a. Dissolve 0.3 g beef-extract, 0.5 g peptone and 0.8 g NaCl in 80 mL of Milli-Q water.
   b. Add 1.5 g agar and fill up to 100 mL with Milli-Q water.
   c. Sterilize by autoclaving (120°C for 20 min).
   d. Cool the nutritive medium to 40°C using a thermostatic water bath.

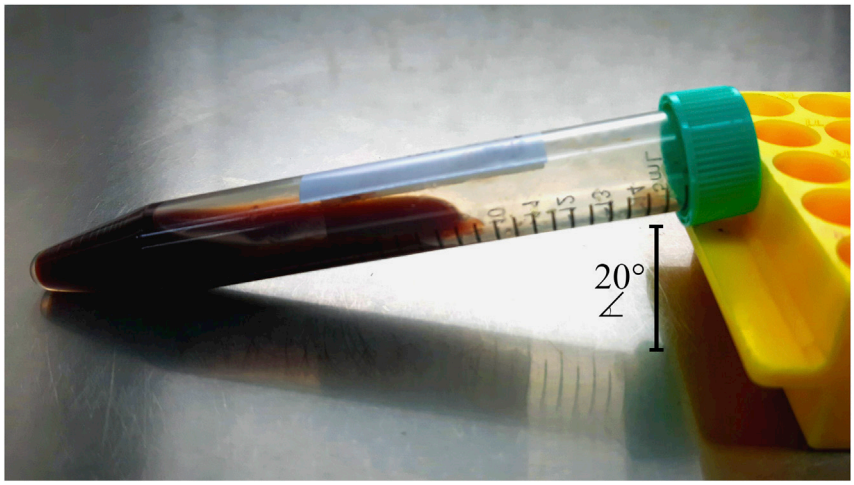

Figure 1. Placing the tubes containing the blood agar medium for solidification
Note the inclination of approximately 20° between the tube and the hood's work table to generate the maximal slant surface.

e. In a sterility hood, supplement the medium adding 10 mL of defibrinated human blood (from a donation bag), 10 U/mL penicillin and 10 mg/L streptomycin.
f. Dispense immediately 4 mL aliquots in 15 mL conical screw-cap centrifuge tubes and place them slanted to allow solidify (Figure 1).
g. Storage the solidified agar slants at 4°C until use.

⚠ CRITICAL: do not add the defibrinated human blood to the nutritive medium if it is still above 40°C to avoid protein denaturation, nor below 38°C to avoid too fast agar solidification.

2. Complete LIT medium (liquid phase)
   a. Dissolve 0.9 g Liver Infusion broth, 0.5 g Tryptose, 0.1 g NaCl, 0.8 g Na_2HPO_4 and 0.1 g glucose in 80 mL of Milli-Q water.
   b. Adjust pH to 7.4 with NaOH 1 N, if necessary.
   c. Sterilize by autoclaving at 120°C for 20 min.
   d. Supplement LIT medium with 10% v/v heat-inactivated Fetal Bovine Serum (FBS), 20 μM hemin and 500 μg/mL geneticin.
   e. Storage the complete LIT medium at 4°C protected from light until use.

Note: see the materials and equipment section for details to prepare the hemin stock solution.

Prepare *T. cruzi* epimastigotes growing at the exponential phase

Timing: 5–12 days

3. Grow *T. cruzi* E forms in complete LIT medium at 28°C as static culture in plastic tissue culture flasks with continuous dilution every week.
   a. On day 1, dilute 1×10^7 parasites from a seven days-old culture in fresh, complete LIT medium to a final density of 3×10^5 cells/mL.
   b. On day 7, repeat the step 3.a.
4. Use the culture at day 5 after the last weekly dilution to differentiate E forms, when the cell density is approximately 1×10^6 cells/mL.

⚠ CRITICAL: E forms cultures must be in the early logarithmic growth phase (~ 4–5 days after the weekly dilution), when parasites are actively dividing and the cell density is approximately one order of magnitude greater than the initial one at day 1. Metabolically stressed cultures will yield low metacyclogenesis rates.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Defibrinated human blood donation bag.	Regional Hemotherapy Center "Fundación Hemocentro", Buenos Aires, Argentina	N/A
Fetal bovine serum	NATOCOR	Cat#NTC-HK31
Fetal bovine serum, irradiated and heat-inactivated	NATOCOR	Cat#NTC-H588
Chemicals, peptides, and recombinant proteins		
Peptone from meat	Britania	Cat#B0100506
Penicillin/ Streptomycin	Serendipia Lab	Cat#DC1600
Tryptose	BD Biosciences	Cat#211713
Glucose	Sigma-Aldrich	Cat#G8270
Hemin	Sigma-Aldrich	Cat#H9039
Geneticin	InvivoGen	Cat#ant-gn-5
Experimental models: organisms/strains		
T. cruzi epimastigotes, Y strain, DTUII	This paper	N/A
T. cruzi epimastigotes, unnamed strain, DTUI	Potenza et al., 2012	N/A
Recombinant DNA		
pTREX vector	Vazquez and Levin, 1999	N/A
Software and algorithms		
GraphPad Prism v6	GraphPad Software, Inc	https://www.graphpad.com/ RRID:SCR_002798
Other		
Falcon tubes	Jet Biofil	Cat#CFT11150
Eppendorf centrifuge	Eppendorf	Cat#R-5425
Neubauer chamber	Boeco	Cat#BOE 13

Note: Human blood can also be purchased from different vendors or obtained from healthy donors by other sources appropriate for the researchers' country with ethics approval.

MATERIALS AND EQUIPMENT

RPMI medium	Final concentration	Amount
RPMI 1640, powder	n/a	10.4 g
$NaHCO_3$	2.0 g/L	2 g
1NaOH/ 1N HCl	n/a	Up to pH 7.1–7.2
ddH_2O	n/a	Up to 1000 mL
Total	**n/a**	**1000 mL**

Dissolve RPMI powder and $NaHCO_3$ in 900 mL of bi-distilled water in a clean glass beaker using a stir bar. Adjust pH to 0.2–0.3 units below the required value with 1 N HCl or 1 N NaOH. The pH usually will rise 0.2–0.3 units upon filtration. Add bi-distilled water up to 1 liter. Sterilize by filtration using 0.2-micron membrane filter and store at 4°C for up to one month.

Complete RPMI medium	Final concentration	Amount
RPMI medium	n/a	986 mL
L-Glutamine solution (200 mM)	2 mM	10 mL
Penicillin (500×)	100 U/mL	2 mL
Streptomycin (500×)	100 μg/mL	2 mL
Total	**n/a**	**1000 mL**

Upon preparation in sterility store the medium at 4°C for up to one month.

Alternatives: DMEM can be used instead of RPMI.

Hemin stock solution	Final concentration	Amount
NaOH	0.2 N	0.4 g
Hemin	40 mM	1.3 g
ddH_2O	n/a	Up to 50 mL
Total	**n/a**	**50 mL**

Dissolve 0.4 g of NaOH in 50 mL of bi-distilled water in a clean glass beaker using a stir bar. Add 1.3 g of hemin powder and stirring until its complete dissolution. Sterilize by autoclaving at 120°C for 20 min and store the solution at 4°C protected from light. It is stable for months.

Note: If a highly concentrated NaOH solution is used to dissolve the hemin (eg. 1 N), the pH of the complete LIT medium must be readjusted to 7.4 after addition of hemin stock solution.

A water-jacketed incubator set at 28°C from Thermo Forma Scientific Series II was used in this protocol. The cultivation of E forms does not require the supply of injected CO_2.

An inverted light microscope from Leica DM IL LED equipped with a 40× objective was used for counting E and MT forms.

STEP-BY-STEP METHOD DETAILS

Differentiation of *T. cruzi* epimastigotes

Timing: 2–3 weeks

In this step, metacyclogenesis is achieved incubating E forms in a biphasic medium formed by liquid LIT medium and solid blood-agar slant.

1. Dispose of as many screw-cap tubes containing blood-agar slants as E cultures to be differentiated already tempered at 28°C.
2. Dilute ~ 2×10^6 E forms from a culture at the early logarithmic growth phase (day 5 after the weekly dilution) in 3–4 mL complete LIT medium.
3. Place the diluted E forms in the tube containing the blood-agar slant.
4. Incubate the tube with the biphasic medium containing E forms at 28°C.

⚠ CRITICAL: In this point, the volume of complete LIT medium used to dilute the E forms should be enough to be in contact with the whole area formed by the agar slant (see Figure 2).

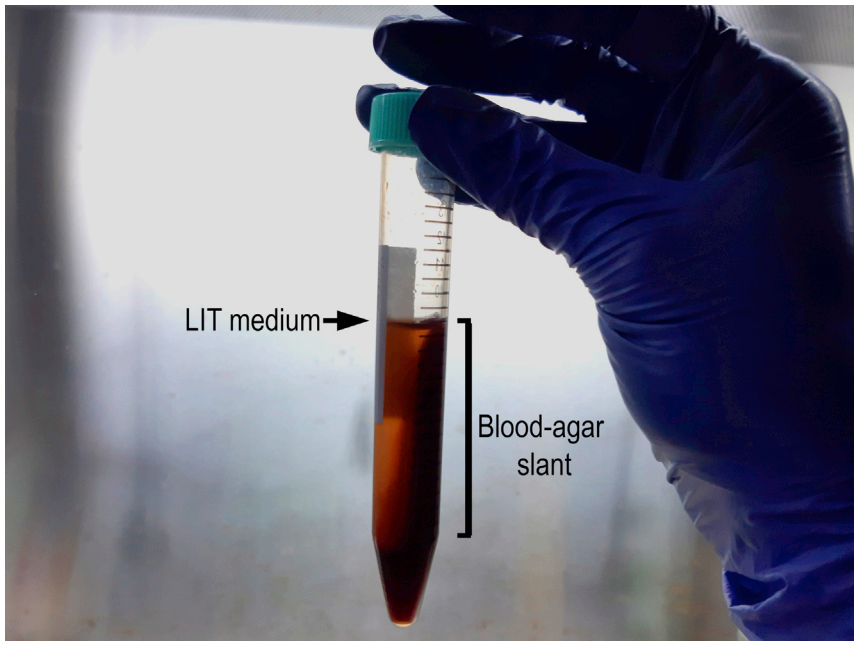

Figure 2. Disposition of LIT medium containing the E forms overlaying the blood-agar slant
Note the level of liquid medium matching the top of the slant formed by the solid phase.

5. Check the presence of differentiated MT forms since day 12–14 post-incubation of the E forms on biphasic medium.
   a. Load 10 μL of culture from the liquid phase into a Neubauer chamber.
   b. Count E and MT forms using a microscope equipped with a 40× objective.
   c. Calculate the percentage of MT forms in the sample (see quantification and statistical analysis section).
6. Harvest the culture for downstream applications when the number of MT forms in the liquid phase of the biphasic medium reaches its maximum (see problem x in troubleshooting section).

Note: Along the incubation on biphasic medium, the metacyclogenesis rate increases over time until it reaches a maximum. For convenience, an initial metacyclogenesis procedure should be carried out to monitor the differentiation kinetics for each strain, which allows setting up the time to yield the maximal percentage of the MT forms (See expected outcomes).

Isolation of metacyclic trypomastigotes

Timing: 1 day

MT can be isolated from non-differentiated E forms for downstream applications, such as cell invasion assays or further differentiation to axenic amastigotes (the *in vitro* representative form of the *T. cruzi* intracellular amastigote). This can be achieved by the clearance of the remaining E forms in the differentiated culture by complement-mediated parasite lysis, following the steps detailed here. E forms are highly susceptible to complement mediated-lysis, meanwhile MT are resistant (Lidani et al., 2017).

7. Transfer the liquid phase of the biphasic medium containing the MT and the non-differentiated E forms into a new centrifugal tube.
8. Centrifuge the culture at 3000 × *g* for 10 min.

9. Discard the supernatant and resuspend the pellet in 200 μL of RPMI.
10. Add 800 μL of FBS and mix thoroughly up and down with a pipette tip.

⚠ CRITICAL: Do not use heat-inactivated nor irradiated FBS because it will not lyse the E forms

11. Incubate the mixture at 37°C for 40 min to allow the lysis of E forms by the complement system contained in the FBS.
12. Load 10 μL of the mixture into a Neubauer chamber to check the absence of E forms in the sample.

Note: At this point, the mixture is composed of cellular debris and highly motile MT forms (see Methods video S1). Nevertheless, if motile E forms are still observed in the sample, incubate the mixture at 37°C for an additional 15 min or as long as it is necessary.

13. Centrifuge the mixture at 3000 × *g* for 10 min at 20°C–22°C and discard the supernatant, after the complete lysis of E forms.
14. Resuspend the pellet in 500 μL of the complete RPMI medium supplemented with 5% FBS (heat-inactivated).
15. Centrifuge the suspension at 3000 × *g* for 10 min to pellet both the MT forms as well as the cellular debris generated by the E lysis.
16. Incubate the pelleted cells and debris at 37°C for at least 3 h to allow the motile MT forms swim back again to the supernatant.
17. Transfer the supernatant carefully to a new tube with a pipette tip, avoiding disturbing the pellet which contains the lysed debris.

Optional: Alternatively to the protocol based on E lysis, the MT forms can be purified by ion exchange chromatography based on DEAE matrix (Cruz-Saavedra et al., 2017).

EXPECTED OUTCOMES

Upon the completion of this protocol, viable, fully differentiated MT forms ready for downstream applications should be obtained (Figure 3). Typically, following the detailed steps for *T. cruzi* Y strain belonging to the DTUII lineage, we have obtained 5×10^6 MT from an initial 2×10^6 E forms placed onto biphasic medium at day 1. The highest metacyclogenesis rate incubating these parasites in the blood-agar medium was achieved at day 16, when ~ 40% of parasites differentiated into the MT forms (Figure 4). This resulted in an improvement of the differentiation yield in comparison with the very low percentage of MT forms obtained for this strain using the Triatomine Artificial Urine (TAU) medium (Contreras et al., 1985). Also, the blood-agar method was suitable to obtain MT forms from E isolates that exhibit low infectivity or that have been cultivated *in vitro* for long periods. The impaired *in vitro* metacyclogenesis is a common feature for those isolates that have been cultivated repeatedly as E forms without any passage through mammalian cells or experimental infection (Contreras et al., 1998). However, following the blood-agar method, up to 18% of E forms culture from the *T. cruzi* strain typified as DTUI was differentiated into MT forms (Figure 5). This strain exhibits low infectivity; it has been maintained as E stage for years in our laboratory and does not differentiate into MT forms by incubating them in chemically defined media, such as TAU or Grace's Insect Tissue Culture medium (Sullivan, 1982).

QUANTIFICATION AND STATISTICAL ANALYSIS

To calculate the percentage of metacyclogenesis over time, count the number of E and MT forms using a Neubauer chamber loaded with 10 μL of culture sample and use the formula:

$$MT\ forms = \frac{(MT\ number \times 100)}{(MT\ number + E\ number)}$$

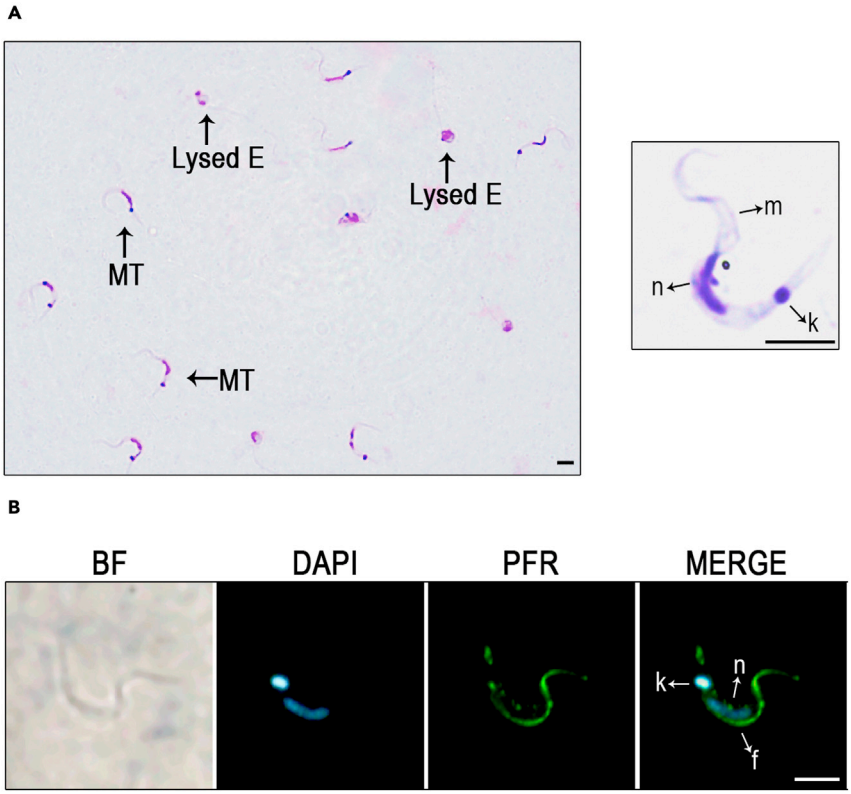

Figure 3. Morphology of metacyclic trypomastigotes by using biphasic medium

In the A panel, MT and lysed E fixed for 10 min with ice-cold methanol, were subjected to Giemsa staining (0.1% w/v in phosphate-buffered saline, PBS, pH 6.8) and observed at the optical microscope. Giemsa staining revealed the presence of an undulating membrane and a flagellum attached to the cell body; being both characteristic of trypomastigote morphology (left panel). In the B panel, a representative immunofluorescence microscopy is shown, which was performed placing MT forms onto poly-lysine microscope slides, fixed for 10 min with 4% paraformaldehyde in PBS, permeabilized with 0.1% Triton X-100, and washed twice with PBS. After blocking with 2% bovine serum albumin (PBS-BSA), slides were incubated with a mouse monoclonal anti-PFR antibody (diluted 1:2 in 1% PBS-BSA) followed by Alexa Fluor 488-conjugated goat anti-mouse secondary antibody (1:500 in 1% PBS-BSA) to reveal the flagellum; both incubations were carried out for 1 h each at 20°C–24°C. Slides were mounted in ProLong Gold antifade reagent (Molecular Probes), containing 10 μg/mL of DAPI for nuclei and kinetoplast staining. This staining revealed the position of the flagellum in more detail, which emerges near the posterior end of the parasite and edgings the cell body. The staining with 4′,6-diamidino-2-phenylindole (DAPI) also showed the position and morphology of DNA-containing structures: a rounded mitochondrial DNA (the kinetoplast) located posterior to the elongated, and the nuclear DNA positioned at the central portion of the cell. Cells were observed in an Olympus BX-61 fluorescence microscope. BF: bright field, PFR: anti-PFR, MERGE: images from DAPI and anti-PFR merged, m: undulating membrane, k: kinetoplast, n: nuclear DNA, f: flagellum. Scale bars: 5 μm.

Each experiment can be performed in triplicate and the percentage values obtained analyzed using the GraphPad Prism software for the calculation of the standard deviation (SD).

LIMITATIONS

The protocol described here was the solution we find in our lab to obtain fully differentiated MT forms from a *T. cruzi* strain which has very low metacyclogenesis rate using the TAU method (less than 8%) and no metacyclogenesis at all by incubation with Grace Medium. However, one limitation of the method presented here is that is time-consuming, because the incubation times for metacyclogenesis takes at least more than two weeks, in comparison with the 72–96 h of incubation used in TAU or Grace medium (albeit the latter two are strain-dependent). Finally, as this method does not

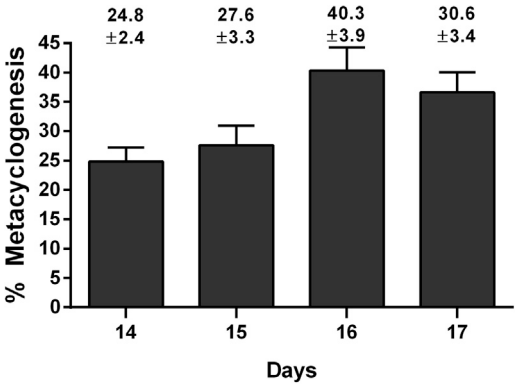

Figure 4. *In vitro* metacyclogenesis yield

The number of MT obtained from the incubation of the E forms in the biphasic medium was counting with a Neubauer chamber in triplicate. The percentages of the MT forms were calculated over time using the formula indicated in the section "quantification and statistical analysis". Data are representative of three independent experiments. The numbers above columns indicate the media ± SD.

produce partially differentiated parasites, it is not suitable for those studies focused on the characterization of E to MT intermediate forms.

TROUBLESHOOTING

Problem 1

Early solidification of the blood-agar medium when it is still contained in the bottle, while it is being dispensing into the centrifuge tubes or before they are placed slanted (step 1.e-f at before you begin section).

Potential solution

This may be a common problem when a large number of agar-slanted tubes are being prepared. If blood-agar medium starts to solidify early than desired, it will interfere with the correct formation of the agar slant, generating irregular and/or dissimilar slant surface areas among tubes. To avoid this, a potential solution is to keep several bottles, each containing unsolidified blood-agar volume just enough to prepare a limited number of tubes (e.g., 10) in the water bath at 40°C. Place each bottle in the sterility hood for dispensing by one, keeping the rest of the medium above de solidification temperature in the water bath. Once the dispensing of the first set of tubes is finished, continue with another bottle and so on, until the required number of slants is obtained.

Problem 2

Epimastigote forms do not differentiate into metacyclic trypomastigote after two weeks of incubation in biphasic medium (step 5).

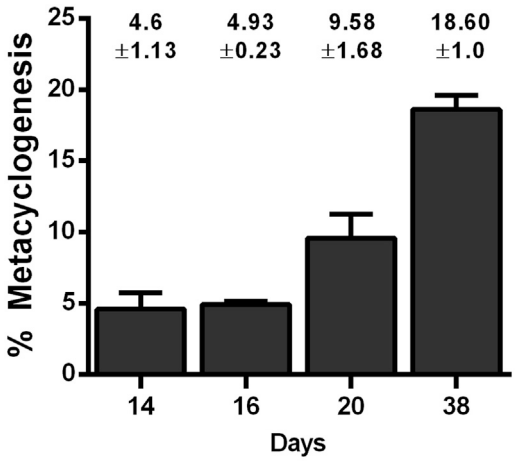

Figure 5. *In vitro* metacyclogenesis of the strain belonging to DTUI lineage

The E forms growing in LIT medium were overlaid onto blood-agar slants and incubated at 28°C up to 6 weeks. Metacyclogenesis rates were calculated using the formula indicated in the section "quantification and statistical analysis". The figure shows a representative graphic of three independent experiments. The numbers above columns indicate the media ± SD.

Potential solution

Continue with the incubation of parasites in the biphasic medium for longer periods, checking the presence of differentiated MT each 24–48 h. Depending on the *T. cruzi* strain and culture conditions, metacyclogenesis may require longer incubation times on the biphasic medium. Regarding this and as an example, the metacyclogenesis of E forms from the *T. cruzi* strain belonging to DTUI lineage usually takes twice the incubation time in the biphasic medium as for the Y strain (DTUII) (Figure 5).

Problem 3

Small blood-agar pieces detach from the slant into the liquid medium when transferring the differentiated culture for downstream applications (steps 6 and7).

Potential solution

Transfer the culture containing parasites together with the small pieces of blood-agar to a new conical tube. Centrifuge at 200 × *g* for 5 min. Transfer the supernatant carefully into a new conical tube containing intact blood-agar slant and continue with the isolation of MT forms.

Problem 4

E forms are not completely lysed by the treatment of non-heat inactivated BFS, even with an additional incubation of 15 min (steps 10–11).

Potential solution

Use a different batch of FBS or change the sera supplier. Some FBS exhibit week complement activity, even being non-heat inactivated, due to a minor and/or undeclared irradiation treatment.

Problem 5

MT forms contaminated with cellular debris after 3 h of incubation at 37°C (steps 16 and 17).

Potential solution

Repeat step 15 centrifuging mixture at 3000 g for longer times (30 min). Then incubate the pelleted MT forms and cell debris at 37°C 6–12 h. This will allow the motile MT forms swim back again to the supernatant. Continue with the protocol.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Mariana Potenza, marian.potenza@gmail.com or potenza@dna.uba.ar

Materials availability

This study did not generate new unique reagents.

Data and code availability

This protocol generates no datasets or code.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2021.100703.

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AUTHOR CONTRIBUTIONS

Conceptualization, J.R.D. and M.P.; methodology, J.R.D. and A.M.-C.; formal analysis, J.R.D.; writing – original draft, J.R.D. and M.P.; writing – review, editing & additional text, J.R.D., A.M.-C., K.A.G., and M.P.; funding acquisition, M.P.; supervision, M.P., project administration, M.P.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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