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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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Rodríguez Durán et al.,STAR Protocols 2, 100703 September 17, 2021 © 2021 The Author(s).
https://doi.org/10.1016/j.xpro.2021.100703
In vitro differentiation of *Trypanosoma cruzi* epimastigotes into metacyclic trypomastigotes using a biphasic medium

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https://doi.org/10.1016/j.xpro.2021.100703

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 DTUII 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.
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 Na₂HPO₄ 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 × 10⁷ parasites from a seven days-old culture in fresh, complete LIT medium to a final density of 3 × 10⁵ cells/mL.
   b. On day 7, repeat the step 3.a.
   c. Use the culture at day 5 after the last weekly dilution to differentiate E forms, when the cell density is approximately 1 × 10⁶ 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 Hemothrapy Center “Fundación Hemoentro”, Buenos Aires, Argentina | N/A |
| Fetal bovine serum  | NATOCOR | Cat#NTC-HK31 |
| Fetal bovine serum, irradiated and heat-inactivated | NATOCOR | Cat#NTC-HS88 |
| 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 |
| Genetin             | InvivoGen | Cat#ant-gn-5 |
| Experimental models: organisms/strains |        |            |
| T. cruzi epimastigotes, Y strain, DTUI | This paper | N/A |
| T. cruzi epimastigotes, unnamed strain, DTUI | Potenza et al., 2012 | N/A |
| Recombinant DNA      |        |            |
| pH7.4 buffer 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 |
| NaHCO₃     | 2.0 g/L             | 2 g    |
| 1NaOH/ 1N HCl| n/a | Up to pH 7.1–7.2 |
| ddH₂O      | n/a                 | Up to 1000 mL |
| Total      | n/a                 | 1000 mL |

Dissolve RPMI powder and NaHCO₃ 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.
Upon preparation in sterility store the medium at 4°C for up to one month.

Alternatives: DMEM can be used instead of RPMI.

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 CO2.

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 ~ 2×10⁶ 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).
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 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 tubewith 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 5×10⁶ MT from an initial 2×10⁶ 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:

\[ MT \text{ forms} = \frac{(MT \text{ number} \times 100)}{(MT \text{ number} + E \text{ number})} \]
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
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 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.

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 DTUII 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 and 7).

Potential solution
Transfer the culture containing parasites together with the small pieces of blood-agar to a new conical tube. Centrifuge at 200 x 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 weak 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.

ACKNOWLEDGMENTS
This work was supported by grants from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, PIP 2015-0937) and Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT, PICT 2016-1028). M. P. and K. A. G. are members of the Research Career of CONICET. Authors thank Dr. Sergio Schenkman (Federal University of Sao Paulo, Brazil) for the anti-PFR antibody and Dr. Alejandro Schijman (INGEBI-CONICET) for typifying the T. cruzi strains. Healthy donor blood bag was
provided by Centro Regional de Hemoterapia Fundación Hemocentro Buenos Aires, according to Argentine Legislation (Blood Donation Law N° 22990, Res. N° 1409/15).

**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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