A detailed procedure to maximize IgM-induced *Leishmania major* mating *in vitro*

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Method Article

**Keywords:** Microbiology, Parasitology, Parasite biology

**Posted Date:** August 25th, 2023

**DOI:** https://doi.org/10.21203/rs.3.pex-2345/v1

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Abstract

The steps presented here were developed to maximize the production of *Leishmania* hybrids in vitro. Our protocol was developed by mimicking as closely as possible the environment inside the gut of the sand fly where we successfully and consistently produced hybrids in vivo. It is important to note that producing hybrids in vitro is less optimal than producing them in vivo. Following this step-by-step protocol faithfully and carefully will significantly improve the success of producing hybrids in vitro.

Introduction

Reagents

IgM from bovine serum (MilliporeSigma, cat no. I8135)

Schneider’s Insect Medium (Lonza / Sigma, cat no. 04-351Q / S0146-1L)

Fetal Bovine Serum (Gibco, cat no. 16-140-071)

Adenosine (Sigma, cat no. A9251)

IgM (Sigma, cat no. I8135)

Peanut agglutinin (PNA) (Vector Laboratories, cat no. L-1070)

DreamTaq Hot Start Green PCR Master Mix (Thermo-Scientific, cat no. K9021)

Water, nuclease free (Thermo-Scientific, cat no. 10977023)

Phosphate Buffer Saline (1X) (Lonza, cat no. 17516F)

Selective drugs

Hygromycin B (HYG) (Gold Biotechnology, cat no. H-270-1)

Blasticidin (BSD) (Gold Biotechnology, cat no. B-800-500)

Nourseothricin (SAT) (Gold Biotechnology, cat no. N-500-1)

Primers

HYG-F (Eurofins, 5’-GGTAACGGTGCGGCTGACGCTGCACTGAAAGGCTGAACTC-3’)

HYG-R (Eurofins, 5’-CGAGATCCACGTAAGGTGCCTATCCCTTGTGGCTCG-3’)

BSD-F (Eurofins, 5’-ATGCCTTTGTCTCAAGAAGAATC-3’)

BSD-R (Eurofins, 5’-TTAGCCCTCCCACACATAAC-3’)
SAT-F (Eurofins, 5’-ACCTATCCGACCAAGGCTTT-3’)
SAT-R (Eurofins, 5’-CGCTGTTCGTTGACGACTT-3’)

**Equipment**

Sephacryl-S-200 gel filtration column (Cytiva)
100 kDa amicon filters (MilliporeSigma)
0.22µm membrane syringe filter
Low protein binding tubes
24-well plate (Costar, cat no. 3526)
25 cm² culture flasks (Nest Biotechnology, cat no. 707001)
96-well plate (for cloning only) (Costar, cat no. 3596)
0.22µm PES media filter (Nalgene, cat no. 565-0020)
Vortex
Centrifuge
27°C incubator without CO₂
4°C fridge
20°C freezer

**Procedure**

Inside the sand fly gut, IgM is digested after 24 hours, a critical step that allows the parasite clump to form during the first 24 hours, and then dissociate to free hybrids for further development. Having an excess of IgM or always having IgM in culture (in vitro) will prevent the parasite clump from dissociating leading to death of the parasites and absence of hybrid recovery in vitro. The use of controlled amounts of purified IgM in culture and the transfer of the parasite clump to a culture without IgM at 24 hours after mating, prevents the constant formation of the parasite clump and mimics in vivo conditions, mainly IgM digestion and parasite clump dissociation in the gut of the sand fly. To optimize hybrid recovery, we
recommend the use of purified IgM in initial in vitro hybridization attempts. Of note, commercially available purified IgM is usually stored with preservatives such as azide, then it is important to follow the steps provided to remove the azide that damages *Leishmania* parasites in culture.

**IgM preparation for in vitro use:**

Commercial purified IgM antibodies usually contain preservatives such as sodium azide. To avoid any toxicity to the parasites, removal of preservatives is essential. IgM from bovine serum (MilliporeSigma, I8135) should be purified via passage through a Sephacryl-S-200 gel filtration column (Cytiva) – or similar - equilibrated with PBS. Protein-containing fractions eluted with PBS can then be pooled together and the volume reduced to the desired concentration by ultrafiltration.

Concentrate the eluate if needed using 100 kDa amicon filters. A concentration of 3 to 5 mg/mL is a good range of stock concentration that will allow for the addition of small volumes to culture media. Sterilize the purified IgM solution by filtration (0.22µm membrane syringe filter), and store aliquots in low binding protein tubes at -20°C. Avoid cycles of freeze and thaw to the IgM solution as this can break apart its pentameric structure. Once the aliquot is defrosted keep it at 4°C. IgM will stay stable if not contaminated.

**Complete media formulation:**

Grow cultures in Schneider's supplemented with 20% heat-inactivated fetal bovine serum (FBS).

For general maintenance (C-SM):

A. 400 mL Schneider's
B. 100 mL FBS
C. Sterilize by filtration.

For mating assembly and hybrid recovery (C-SMA):

A. 400 mL Schneider's
B. 100 mL FBS
C. 33.4 mg of adenosine.

o You can add the powder directly to the media and homogenize to solubilize (preferable) or prepare a stock concentration at 20 mM (5.35 mg/mL) and add 6.25 mL per 500 mL of complete media.
D. Sterilize by filtration.

PNA solution for metacyclics enrichment:

Reconstitute the lectin with Phosphate Buffer Saline at 2mg/mL and sterilize by filtration. Store at 4°C.

Selective drugs:

All drugs are solubilized in molecular biology grade water and filter sterilized. HYG (100 mg/mL), BSD (10 mg/mL), SAT (100 mg/mL). Aliquots are made and stored for long term at -20°C. A running vial is maintained at 4°C. Selective drugs are added at a 1:1000 dilution to final concentrations of: HYG (100 µg/mL), BSD (10 µg/mL), SAT (100 µg/mL).

Steps:

1) Growth of parental lines in complete Schneider’s medium (C-SM) until stationary phase.
   
a. Use fresh isolated parasites with no more than 15 passages in culture.
   
b. Maintain parasites at 27°C without CO₂.
   
c. To have the parental lines ready for the mating setup, defrost the stock and grow it in C-SM for 24h or until the cells start to divide. Add respective selective drug to ensure the cells have the drug-resistant marker inserted into the genome. Let them grow for 4-5 days until late log phase.
   
d. Seed a new passage of the drug resistant line (1x10E5/mL) without drug pressure to get the parasites to grow in enough numbers. For this stage, grow parasites in 25 cm² culture flasks in an upright position. If a lot of parasites are needed, several flasks from each parental line should be established accordingly.
   
e. When the cultures are ready (stationary phase by day 5) (Mating protocol Fig. 1), spin down the cells and resuspend in 2 mL of PBS.
   
f. Proceed to metacyclic purification by adding 20 µL of PNA solution into the 2mL of parasite suspension. Mix (rapidly vortexing) and let it sit at room temperature for 15 min. Centrifuge at 60x g for 10 min and collect the supernatant without disrupting the pellet. Collect the supernatant into 10mL of PBS. Count to calculate the total number of cells. Pellet cells by centrifugation at 2500x g for 15 min and wash once with PBS. Discard the supernatant. Usually, the remaining amount of PBS in the tube (~200uL) is enough to have cells in a good concentration to dispense in wells. Make sure the metacyclic
promastigotes are enriched and are fit showing the right morphology (Mating protocol Fig. 2) and movement (Mating protocol Videos a and b).

2) Assembly of mating wells.

a. Use 24-well plates for this. Each well is seeded with 4x10E6 parasites/mL in 1.5 mL final volume in a final proportion of 1:1 of each parental line (2x10E6 parasites/mL/parental line). From this step forward supplement the C-SMA with adenosine (250 µM) to have enough nutrient support to encourage growth and establishment of hybrids resulting from rare hybridization events. As a first attempt it will be good to try around a total of 18 wells per experiment (6 wells per 3 independent medium/parasite preparations, set up over several days to account for an observed variation in parasite fitness from a single preparation).

i. Do not start a crossing trial if both parental lines are not in good condition (normal growth rates and good cell shape and movement), or if the recovered metacyclics are not well isolated after treatment with PNA (Mating protocol Fig. 2b, Mating protocol Video b).

ii. PNA works well for *Leishmania major* metacyclics enrichment; for other species it may not be the case. Then, alternative metacyclic enrichment methods (e.g. density gradient centrifugation), or stationary phase promastigotes may be used. Remember that optimization will likely be required.

b. Add the corresponding volume of a purified and preservative-free IgM from a concentrated stock to have it at the final concentration of 200 µg/mL. We arbitrary chose 50 µg/ml of IgM in this work as a reasonable physiological concentration, however, the yield of hybrids was low. By increasing to 200 µg/ml while ensuring clump dissociation (see step 3 below), the yield of hybrids increased considerably.

c. Mix by pipetting up and down with a P1000 micropipette set to the maximum volume (1000 µL) a couple of times.

d. Seal the plates well with parafilm to avoid evaporation of the media. Grow at 27°C.

i. The clumping formation will start immediately. By 24h clumps should be at their peak sphere formation and dense organization.

3) Transferring of clumps from mating wells to dissociation flasks.

a. At 24h after incubation with IgM, the spherical clumps will be visible at the bottom of the well. Collect the formed clumps and transfer to a new media and flask. To do that tilt the plate and allow the clumps to move to the edge of the well for 20 minutes (Figure 3; gently tapping can help if clumps are stuck). With a P1000 pipette, set up the volume to 100 µL and gently collect the clumps from the edge of
the well. One hundred µL will be more than enough to collect all the clumps. Do not transfer more volume as the media contains IgM. Gently dispense the clumps into a new flask with 15 mL of C-SMA (without IgM and without drug pressure) to allow the clumps to dissociate. Once a day, gently agitate the flasks from one side to another (~ 4 bounces as exemplified in the mating protocol video c). By 72-96 hours the clumps should all have dissociated. If not, further disrupt the clumps by pipetting up and down a couple of times with a P1000 set to 1 mL. Clumped parasites barely grow; multiplication will mostly start after they detach from the clump.

4) Transferring of parasites from dissociation flasks to selection flasks.

a. After 72-96 hours (or less if all clumps have dissociated) in the dissociation flask, transfer the volume of parasites into the selection flasks with new complete media (C-SM) + selection drugs (1 dissociation flask = 4 to 6 selection flasks). Altogether, each 15 mL dissociation flask will be redistributed in 60 to 90 mL of new complete media + drugs divided equally into 4 to 6 flasks. Procedure example: Divide the 15 mL from the dissociation flask into 4 new flasks (3.75 mL each). Wash the dissociation flask with 5 ml of new C-SMA and divide into 1.25 per selection flask. Add more 10 ml of new C-SMA per flask. Add selective drugs.

b. Follow the cultures weekly, up to 30 days, and look for the flasks with growing parasites.

c. Clone parasites growing under drug selection and proceed for PCR genotyping or NGS sequencing if needed.

   i. Cloning can be carried out in different ways. For instance, plate 0.5 cells/100 µL of C-SM under double drug selective pressure in a 96 well plate to collect clones. In most cases, 1-2 plates are enough to recover at least 3 clones. More plating can be done if needed – e.g. for slow growing hybrids.

Troubleshooting

Time Taken

Time taken per step is described on the step description.

One biological replicate experiment, that will contain several wells, will take at least 30 days to completion.
Figures

Figure 1

**Mating protocol Fig.1**: Representative growth curves of parentals used for the crossing experiments.
Figure 2

**Mating protocol Fig. 2:** Metacyclics enrichment by PNA. (a) Cultured promastigotes at day 5. (b) Following metacyclic enrichment by PNA negative selection. Also check respective mating protocol videos “a” and “b”.
Figure 3

Mating protocol Fig. 3: Collection of parasite clumps. (a) Plate tilting for clump collection. Test some different background colors under the plate to properly see the clumps. The steel background of traditional laminar flow chambers makes it difficult to see the clumps. (b-d) Clumps after a 24h incubation with IgM. (b) No IgM well with usual *Leishmania* culture turbidity and loose cells. (c) IgM 200 well. (d) IgM 200 well, after tilting the plate.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- MatingprotocolVideoa.mov
- MatingprotocolVideob.mov
• MatingprotocolVideo.mov