Long Primer PCR (for Trypanosoma brucei)

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Protocol status: Working
We use this protocol in our group and it is working!

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1 PRC Mix

- 1 μL pPOT (25 ng/μL)
- 0.2 mM dNTPs
- 1 μM for primer
- 1 μM rev primer
- 1 μL PCR grade DMSO
- 5μL 10x buffer 2 (Roche)
- XX μL ddH2O for total volume of 49 μL
- Add 1 μL Expand High Fidelity polymerase (Roche) once mixture has reached 94 C.

1.1 PCR conditions

- 94 C 5 mins
- 94 C 15 sec
- 65 C 30 sec (30 cycles)
- 72 C 2 min
- 72 C 7 min

2 Maintain procyclic form SMOX P9 cells [31] between 1x10^6 - 1x10^7 cells ml^-1 for at least 72 hours prior to transfection to ensure they are in log growth phase.

3 Centrifuge 1x10^7 log phase procyclic cell per transfection at 800 g for 10 min at room temp

4 remove all supernatant

5 Resuspend cells in 500 mL of room temperature cytomix per transfection, and add to a 4 mm gap electroporation cuvette
Add 50 mL of unpurified PCR to the cell suspension

Electroporate the cell once with 1.7 kV, 25 mF (gene pulser (Bio-Rad) or three times 1.7 kV for 100 Ms, 200 ms interval (BTX ECM830 (hardvard Apparatus))

Recover the cells for 8-16 hours in 10 ml SDM-79 at 28 C

Add the appropriate selective drug to the final concentrations:

- Blasticidin (Melford) 20 mg/ml (<20 mg/ml is not sufficient to kill off all non-transformed cells).
- Hygromycin b Gold (Invivogen) 25 mg/ml (>25 mg/ml reduces transfection efficiency due to low readthrough transcription of the resistance cassette).

If clones are required, dilute 5 ml of recovered cells into 50 ml of selective medium and distribute 1 ml aliquots into a 48 well plate.

Resistant populations of cells emerge after 7 – 10 days, and clones emerge after 10 – 14 days.

FOLLOW UP

Transfection of bloodstream form T. brucei using the Amaxa Nucleofector II

T. brucei can be efficiently transfected using the Amaxa Nucleofector II using the human T-cell kit (VPA-1002 Lonza).

Step 12 includes a Step case.
13. Complete the human T-cell Nucleofector solution by addition of supplement 1. The combined solution and supplement can be stored and is stable for 3 months at 4°C.

14. Purify 100ml of long primer PCR (~8mg) with one phenol chloroform extraction followed by ethanol precipitation at -80°C for 1 hour with two 70% ethanol washes (the pellet should be easily visible). Note that using a silica membrane column instead of phenol chloroform to purify the DNA will reduce transfection efficiency by 10–50 fold.

15. Resuspend the dried pellet in 10ml 5mM Tris pH8.

16. Maintain bloodstream form SMOX B4 cells between 1x10⁵–1x10⁶ cells ml⁻¹ for at least 72 hours prior to transfection to ensure they are in log growth phase.

17. Centrifuge 2x10⁷ log phase (<1.3x10⁶ cells ml⁻¹) bloodstream form cells per transfection at 800 g for 10 minutes at room temperature.

18. Carefully remove all supernatant.

19. Resuspend the cell pellet in 100ml of complete AmaxaT cell buffer per transfection, and transfer to an Amaxacuvette.

20. Add the purified DNA to the cell suspension, and electroporate once using Program X-001.

21. Recover cells for 8 - 16 hours in 50 ml HMI-9 at 37°C with 5% CO₂.
Add the appropriate selective drug to the final concentrations:

Blasticidin (Melford) 5mg/ml, Hygromycin B Gold (Invivogen) 1.5mg/ml.

Distribute 1ml aliquots of cells into two 48 well plates.

Resistant clones emerge after 6 – 8 days.