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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 uL pPOT (25 ng/uL)
- 0.2 mM dNTPs
- 1 uM for primer
- 1 uM rev primer
- 1 uL PCR grade DMSO
- 5uL 10x buffer 2 (Roche)
- XX uL ddH20 for total volume of 49 uL
- Add 1 uL 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 1×10^6 -1×10^7 cells ml^{-1} for at least 72 hours prior to transfection to ensure they are in log growth phase.

3 Centrifuge 1×10^7 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

6 Add 50 mL of unpurified PCR to the cell suspension

7 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))

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

9 Add the appropriate selective drug to the final concentrations:

- Blasticidin(Melford)20mg/ml(<20mg.ml-1is notsufficientto kill off allnon transformedcells).
- Hygromycin b Gold (Invivogen)25mg/ml(>25mg.ml-1reduces transfection efficiency due to low readthrough transcription of the resistance cassette).

10 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.

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

12 FOLLOW UP

Transfection of bloodstream formT. bruceiusing theAmaxaNucleofector II
T. bruceican be efficiently transfected using theAmaxaNucleofector II using the human T-cell kit (VPA-1002 Lonza).

STEP CASE

Untitled case 12 steps

13 1. Completethe 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 onephenol chloroform extractionfollowed 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[31]between 1×105–1×106cells.ml-1for at least 72 hours prior to transfection to ensure they are in log growth phase.

17 Centrifuge 2×107log phase (<1.3×106cells.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 completeAmaxaT cell buffer per transfection, and transfer to anAmaxacuvette.

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.

22 Add the appropriate selective drug to the final concentrations:

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

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

24 Resistant clones emerge after 6 – 8 days.