HDR TARGETING ESCs

ESC TRANSFECTION

1. Culture ESCs on feeders until ~70-80% confluent
2. Disassociate ESCs using Accutase or trypsin, depending on culture media and deplete feeders by passing through a 40um mesh filter. *Alternatively you can plate cells on a gelatin-coated plate for 30-45mins to deplete feeders. This may work better for trypsinized cells.*
3. Count ESCs (by hemacytometer) and spin down 200,000 cells for each transfection. If doing many (>4) transfections at once, include one additional sample to account for volume errors:
4. While cells are spinning, prepare Nucleofection mix from supplied components – this can be prepared as a master mix. For each 20ul transfection mix:
    A. 16.4ul of P3 Primary cell solution
    B. 3.6ul of Supplement 1
5. Also while cells are spinning, prepare DNA in individual eppendorf tubes for each transfection.
    A. 2ug Cas9/sgRNA vector (usually PX330 or PX458)
    B. 4ul ssODN HDR template (20μM)
6. Resuspend ESCs in Nucleofection mix and add 20ul of mixture to each DNA combination. *From this point on work as quickly as possible to minimize the amount of time the cells spend in the transfection mixture.*
7. Gently pipette up and down 3-4 times to mix the DNA and ESCs and transfer entire volume to one well of the 16-well cuvette strip (each cuvette is used only once). **Avoid bubbles**.
8. Pulse the transfection mixture using an Amaxa Nucleofector X-unit: Program “ES mouse”
9. After transfection, add 80ul of ESC culture media to each well then transfer entire 100ul to one well of a feeder-coated six-well plate, drop-wise. *Do not pipette up and down*.
10. Replace media every 24hrs

CRISPR QC

11. 4-5 days following transfection, collect the cell population (Accutase or trypsin) and plate 500 cells on a 6cm plate to enable low density clonal growth. Split the remaining culture (50:50) for cryopreservation (1 vial) and genomic DNA isolation (see gDNA isolation protocol).
12. Change media on the 6cm dish every day until colonies are visible without a microscope
13. While the clones grow, confirm on target CRISPR activity by PCR and T7 assay (see associated protocol). If your HDR template contains a new restriction site (or removes a restriction site), assess HDR targeting by digesting the PCR product from the bulk gDNA. After confirming CRISPR activity and HDR targeting, continue to clone picking.

CLONE ISOLATION

14. Pick individual colonies into 10ul of trypsin in a 96 well PCR plate, allow the clones to disassociate for 5-10mins.
15. Using a multi-channel pipette, gently mix the cells to complete trypsinization, and transfer 5ul of cell-trypsin mixture to a replica PCR plate containing 5ul 2x DNA lysis buffer (with Proteinase K).
16. Incubate the plate at 55C for 2hrs in a PCR machine, and inactivate the Proteinase K at 95C for 20mins.
17. To the remaining cells, add 100ul of media and transfer to one well of a 48 well plate containing 500uL of ESC media.

CLONE SCREENING

18. PCR amplify the region/s of interest directly from 1ul of the crude gDNA lysis in 16ul volume using Promega PCR master mix
19. Confirm amplification by gel electrophoresis or (preferably) Qiaxcel.
20. If your HDR template contains a new restriction site (or removes a restriction site), assess HDR targeting in each clone by digesting the PCR product: transfer half of the PCR product to a replica 96 well plate containing 8ul of restriction enzyme in 2x digest buffer.
21. Digest PCR product for 2hrs in a PCR machine. *We have used SfoI and NaeI enzymes – both are compatible with the Promega PCR master mix, although this may need to be optimized for each individual enzyme.*
22. Analyze digest by gel electrophoresis or (preferably) Qiaxcel.
23. Identify positive clones and, if necessary, perform additional validation (e.g. allele specific PCR, sequencing)
24. Expand positive clones into a 6 well-plate. Re-confirm correct targeting by digest and sequencing on independently isolated gDNA.

SOLUTIONS

2x (crude) DNA lysis buffer (5ml)

1ml	10x MGB
500ul	10% Triton X100
200ul	Proteinase K (20mg/ml)
100ul	β-ME
3.2ml	ddH_2O

10x MGB solution (10ml)

6.7ml	1M Tris, pH8.8
830ul	2M $(NH_4)_2SO_4$
650ul	1M $MgCl_2$
1.82ml	ddH_2O