RESOLUTE

Lab protocols to study Solute Carrier Transporters

Generation of Single Cell Knockout Clones

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Protocol description

Protocol for the generation of single cell knock out (KO) clones via lentiviral transduction.

Materials

Biological materials:	▪ Envelope plasmid pMD2.G (Addgene, 12259) ▪ Packaging plasmid psPax2 (Addgene, 12260) ▪ pLentiCRISPRv.2 plasmid (Addgene #52961) expressing target-specific gRNA) ▪ HEK293T cells ▪ Cell line to be transduced
Reagents:	▪ Lipofectamine 3000 (ThermoFisher, L3000001) ▪ Puromycin (Sigma-Aldrich, P8833-100MG) ▪ Opti-MEM (ThermoFisher, 31985070) ▪ Polybrene (Sigma-Aldrich, H9268) ▪ DirectPCR lysis buffer (Viagen Biotech, 301-C) ▪ Proteinase K (Bartelt, A4392,0100)
Equipment:	▪ Sterile tissue culture hood ▪ Optional: Cell Sorter (e.g., Sony SH800S)

Reagents setup

5 mg/ml stock solution of Polybrene

Dissolve 50 mg of Polybrene in 10 ml of ddH20 and sterilize through a 0.22-µm filter. Make 500 µl aliquots of the solution and store at -20°C.

10 mg/ml stock solution of Polybrene

Dissolve 100 mg of Puromycin in 10 ml of ddH20 and sterilize through a 0.22-µm filter. Make 500 µl aliquots of the solution and store at -20°C.

Procedure

Transfection

- **Day 1**

1. Seed HEK293T cells in 100 µl at a density of 15000 cells/well on a 96 well plate

- **Day 2**

2. Check HEK293T cells (should be 50-70% confluent)
3. Prepare transfection reagents:

4. Lipofectamine MM (scale up according to planned transfections, add 10% extra)

	µl per reaction
Optimem	7.15
Lipofectamine 3000	0.35
Total volume	7.5

5. Let the Lipofectamine MM incubate for 10 min at RT
6. DNA MasterMix (MM) (scale up according to planned transfections, add 10% extra)

	µl per reaction
Optimem	4.8
pMD2.G (100 ng/ul)	0.3
psPax2 (100 ng/ul)	0.5
P3000 Reagent	0.3
Total volume	6.0

7. Pipette 6 µl of DNA MM per reaction into clean PCR strips or plates
8. Add 1.5 µl of pLentiCRISPRv.2 plasmid (concentration: 50-100 ng/µl) to 6 µl of DNA MM
9. Mix 6 µl of Lipofectamine MM per reaction with each DNA MM
10. Incubate at RT for 5-15 minutes
11. Add 10 µl of mix to HEK293T cells (keep pipette tips in medium while dispensing but do not touch the bottom of the wells)

- **Day 3**

12. Add 120 µl fresh medium to transfected HEK293T cells (pipette very slowly to avoid detachment of HEK293T cells)

- **Day 4**

13. Transfer 200 µl of the virus-containing medium to a safe-lock tube and immediately freeze at -80°C

Transduction

- **Day 1**

1. Seed the cell line to be transduced at $1.5x10^4$ cells/well in a 96 well plate (Note: add one more well than needed as a negative control)

- **Day 2**

2. Thaw the safe-lock tube with the virus-containing medium at RT

3. Change the medium of the cell line to 100 µl of medium with 10 µg/ml polybrene
4. Add 100 µl of thawed lentivirus to the cells

- **Day 3**

5. If transduced cells are confluent: split transduced cells and transfer 200 µl to a 24 well plate
6. If not confluent: change medium on the cells

- **Day 4**

7. Change medium to selection medium (1 µg/ml Puromycin)
8. If cells are still on a 96 well plate, transfer to a 24 well plate and start Puromycin selection the next day

- **Day 5-12**

9. Monitor cells and split when reaching 80-100%. Keep under puromycin selection. Change medium every 3-4 days depending on confluency

- **Day 13-15**

10. Observe negative control (untransduced cells), remove puromycin from transduced cells if all cells in neg. control are dead
11. Monitor cells and freeze when reaching 80-100% confluency (after at least 10 days of selection)
12. If single cell cloning is planned for the next week, keep KO pools in culture, change medium every 3-4 days depending on confluency

Single Cell Clone Generation

- **Option 1: Limited Dilution**

1. Prepare 1.5 ml safe-lock tubes (3 per sample)
2. Prepare 50 ml falcon tubes (2 per sample)
3. Cells should be in logarithmic growth phase and 70-90% confluent
4. Transfer the cells to 50 ml falcon
5. Count the cells
6. Calculate volume of cells needed for $2.0x10^4$ cells in 1 ml, pipette this into the first 1.5 ml tube
7. Prepare tubes 2 and 3 for each sample with 900 µl
8. Add 4.5 ml of medium (conditioned medium if necessary) to a falcon

9. Transfer 100 µl from tube 1 to tube 2 and mix well, then transfer 100 µl from tube 2 to tube 3 and mix well
10. Transfer 500 µl from tube 3 to the falcon containing 4.5 ml medium, mix well
11. Use a multistep pipette to transfer 50 µl per well into a 96 well plate

- **Option 2: FACS sorting**

1. Add 200 µl of medium into 96-well dishes, place in incubator until used
2. Some cells require conditioned medium (CM) and/or a higher FBS concentration of the media to grow. CM can be collected either from WT stocks or the corresponding pools and have to be sterile filtered (0.2 µm filter).

Cell	Medium	Special conditions
1321N1	DMEM 10%FBS	Matrigel coated wells
SK-MEL-28	DMEM 10%FBS	Matrigel coated wells
HuH-7	DMEM 10%FBS	Conditioned medium
HCT116	RPMI 10%FBS	-
MDA-MB-468	RPMI 20% FBS	
LS180	RPMI 10%FBS	Conditioned medium

3. Start cell sorter before trypsinizing the cells
4. Once the cell sorter is set up and working well, start trypsinization of cell pools in batches of 6 pools.
5. Sort single cells into 96-well dish with medium

- **Expansion and Freezing**

6. Monitor cells after 1-2 weeks, add 50-100 µl fresh medium after 1 week. If necessary, change medium after 2 weeks.
7. As soon as the colonies reach a proper size (covering about 50% of the well), transfer the colonies to a new 96 well plate so that they are all in a row (depending on transduction efficiency, 12 – 24 clones per KO pool should be sufficient)

   Note: If possible, transfer clones from adjacent wells using a multichannel pipette to minimize pipetting steps. Transfer clones to get complete rows of 12 clones for each KO pool and assign subclone numbers 1-12 according to their position on the row of the plate.

8. Once the cells are near confluency, split half of them to a new 96 well plate for DNA lysis
9. When they are 80 – 100% confluent again, freeze the cells directly from one of the 96 well plates OR transfer the cells to a 24 well plate if it is known that they are sensitive to freezing conditions (e.g., HuH7 and LS180 cell lines)
10. Add 150 µl fresh medium to remaining 50 µl of cells and let grow until they reach confluency.
11. To freeze from a 96 well plate, prepare sterile PCR tubes and add 50 µl of medium with 40% DMSO

12. Wash the cells with PBS, add 30 µl of Trypsin, incubate for 5 min at 37°C, resuspend cells in 120 µl medium, then transfer 150 µl to the PCR tubes
13. Freeze the tubes in a Styrofoam box at -80°C for 24 h, then transfer them to a gas phase liquid nitrogen tank if available

- **Prep for genotyping**

1. After most clones reached confluency, completely remove medium and add 40 µl DirectPCR buffer with 1.25 µl Proteinase K per 1 ml lysis buffer (make fresh dilution every week) using a 12 channel pipette.
2. Incubate cells with lysis buffer at 37°C for at least 15 minutes
3. Carefully pipette up and down 3 times and transfer entire volume (40 µl) to PCR tubes (avoid generating air bubbles)
4. Run DirectPCR lysis program in a PCR machine (2 hours 55°C, 5 minutes 95°C)
5. Store lysed cells at -20°C until used for genotyping (lysate can be used directly for PCR)

Additional notes

Every cell line needs to go through an optimization process while trying to generate single cell clones. While some will grow quite easily from a single cell, others might need extra care, e.g. Matrigel coated plates, medium with 20% serum, conditioned medium.

Some cell lines do not survive the process of FACS sorting and must be diluted and pipetted by hand. Some cell lines do not grow from single cells at all.

Data/reagent availability

https://re-solute.eu/resources/reagents

References

Please write to contact@re-solute.eu in case of questions or errors.