Lentiviral transduction of human PSCs

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Lentiviral transduction

1. Treat human pluripotent stem cell colonies with Accutase for 20-30 minutes at 37˚C.
2. The cells should simply float off the dish. Pipette them briefly to break them into single cells and wash with 5x the volume of hES media. Spin at 200xg for 5 min. and aspirate.
3. Resuspend and count on a hemocytometer. Replate at a density of between 30-50,000 cells/cm^2 in pluripotent media* supplemented with 10 µM Y-27632.
4. The day of transduction, prepare media. We typically use conditioned media, so that means collecting the media off feeders, adding FGF2 to 10 ng/ml, and maintaining it in the incubator to keep it warm and at the correct pH.
5. Remove an aliquot of the media before adding polybrene to control for polybrene-induced damage.
6. Add polybrene to the media to a final concentration 4 ug/ml. Use a fresh polybrene aliquot (thawed in the past week).
7. ***Transduction.*** Add minimal volume of media to your cells, but make sure that it stays wet in the center. Add appropriate amount of virus* to each well. *MAKE SURE YOU FOLLOW GOOD BSL2 PRACTICES WHILE WORKING WITH LENTIVIRUS! SEE APPENDIX.*
8. The next day, carefully remove inoculate and place it into a container with straight bleach. Carefully add new media and incubate overnight. Feed daily until desired density is reached.

Making clones

9. To make true clones, make a single-cell suspension and plate at low density. Since plating efficiency depends on the cell line and culture conditions, you might have to replate different densities to achieve well-isolated colonies (maybe 300, 1000 and 5000 cells/cm^2).
10. Feed cells daily until desired colony size is achieved.
11. Hand pick colony into a new dish and expand as a novel line.

(See notes on the next page)

NOTES:

Culture systems. As noted, we generally use MEF-conditioned media to maintain pluripotent stem cells in a feeder-free state. But other feeder-free media (such as mTeSR1 or E8) likely also work fine. A suitable substrate should always be used without feeders, such as Geltrex or Vitronectin.

Y-27632. This ROCK inhibitor is needed to maintain a single-cell suspension of hESCs. Make sure it is present for 24 hours after making the single-celled suspension.

Lentiviral safety. Lentiviruses are used in gene delivery because they are very efficient at entering cells and integrating into the genome. They cannot replicate but they can integrate into YOUR DNA or the DNA of your co-workers. Many people work with lenti vectors without the proper respect for themselves or others. Don't be "that guy/gal". Plan out the experiment in your head before you do it. Prepare the hood by having bleach ready and having enough empty tubes for waste. Wear a lab coat and safety glasses. Double glove. Double bag autoclave bags. Every tip that touches virus should be exposed to bleach afterwards. In other words, pull virus into the tip and expel into the well. Then, go directly to the bleach and pull it into the tip to inactivate it in place. Expel back into the bleach waste, then eject tip into a safe container. I like to remove the autoclave bags immediately after use, and make sure to carefully decontaminate the surface of the hood and everything that was touched during the procedure. It's best to use a 10% bleach solution for this. Check with your institute's biosafety department for the procedures that you should follow. Lentiviral vector experiments should also always be cleared by your local biosafety office - they should be in the lab's biosafety approval, and all proper BSL-2 procedures should be carefully followed.

Titration. Good titers are hard to come by and should not be taken literally, unless they were defined on the exact cell type (and density) that you are using. In general, it's a good idea to transduce over a range of multiplicities to make sure that you are within range. If you use too much virus, there can be non-specific cell death that occurs. And, of course, the higher the copy number of the vector, the greater the likelihood that insertional activation/inactivation of something important will happen. On the other hand, your GOI will be expressed at higher levels and will more likely withstand silencing.

Silencing. Small transgenes are prone to silencing. In my experience, there also seems to be an acute silencing that can occur (variegation) as well as one with more global but slower kinetics. This is just a matter

Our protocols were largely derived from the Thomson Lab's protocol (http://ink.primate.wisc.edu/~thomson/). Another wonderful resource is the WiCell Research Institute protocols (www.wicell.org).

I prefer to order **MEFs** from Applied Stem Cells, Inc. Carefully test your vendor's cells since some are very inconsistent.

Applied Stem Cell, Inc.
ASF-1114
1165 O'Brien Drive, Suite A
Menlo Park, California 94025, USA
info@appliedstemcell.com

Human ES media
780 ml DMEM:F12
200 ml Knockout serum replacement (KSR)
10 ml MEM non-essential amino acids
1 ml 2-mercaptoethanol
5 ml L-glutamine

Sterile filter and cover bottle with aluminum foil. Add FGF2 to 6 ng/ml to an aliquot of media just before feeding cells. If you warm it, also warm just an aliquot - not the whole bottle. KSR does not like to be warmed.

DMEM:F12
Invitrogen
11330-032

Knockout Serum Replacement
Invitrogen
10828-028
Read the instructions on how to thaw and aliquot - aliquot into 50 ml tubes and freeze once (-20˚C)

MEM Non-essential amino acids
Invitrogen
11140-050

2-mercaptoethanol
Invitrogen
21985-023

L-Glutamine (200mM)
Invitrogen
25030-081
L-glutamine does not go fully into solution. Keep it well suspended as you aliquot. Aliquot

into 5 ml tubes and freeze once (-20˚C).

FGF2 (bFGF)
R&D Systems
233-FB-025
reconstitute (10µg/ml) in sterile PBS with 0.1% BSA, aliquot and store at -80˚C

DMEM+10% FBS for plating MEFs
DMEM 900 ml
FBS 100 ml
Sterile filter. No need to aluminum foil.

DMEM
Invitrogen
11965-118

Fetal bovine serum (for plating MEFs)
Invitrogen
26140-095

Accutase
Innovative Cell Technologies
AT-104

Y-27632
Tocris Biosciences
1254
dissolve in sterile-filtered water to 10 mM and freeze aliquots at -80˚C.

Geltrex
Life Technologies
A141330l
We typically dilute Geltrex 1:30 and plate at room temperature for 1 hour before use. Simply aspirate and plate cells.

Making MEF-conditioned media (MEF-CM)

•Two days before you need it, plate MEFs in DMEM+10%FBS at high density (50,000 cells/cm^2). Let cells attach overnight.

• The next day, aspirate DMEM+10%FBS and wash once with PBS.

• Aspirate PBS, then add complete hPSC media and incubate overnight.

• After overnight incubation, remove conditioned media and place in a conical tube or other appropriate container, depending on the volume.

• Place fresh hPSC media back on the feeders and return to the incubator for the next day's conditioned media.

• Add FGF2 to 10 ng/ml final concentration to the conditioned media before use.

Notes: Conditioned media can be stored frozen, although likely with some loss of activity. We do not keep it for longer than one month after freezing at −20◦C although it is possible that it works longer.

Always add fresh FGF2 before use. Some suggest filtering it through a 0.22 µM filter. I do not do this and I think it might be a bad idea. While it can remove the few dead MEFs that float in these preparations, it likely also removes a lot of the protein from the CM, reducing its efficacy – at least in principle. Experience has demonstrated that the filtering is, at the very least, not necessary.

Additional hPSC media can be conditioned daily for up to two weeks using the same culture of high density MEF feeders.