Nucleofecting human pluripotent stem cells

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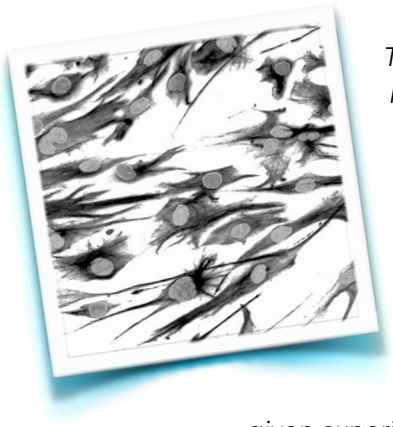

This protocol was developed in the old days before Amaxa had permission to use human pluripotent cells. Back then, there was no human PSC kit, so Dimitris Placantonakis developed his own. I tested the hPSC kit after it was developed but did not see a difference, so we have stayed with our original protocol.

One big variable in this protocol is the amount of DNA that you add. The Amaxa/Lonza protocol states 1-5 µg DNA, but our work with BACs has shown that you can add 100-150 µg of DNA before you begin to see cell death. For most applications, it's probably best to stick to their protocol, but varying the amount of DNA added is something that can be tried if you need a higher dose for a given experiment: just do not add more than 10% of the final reaction.

[DNA preparation]

You need a concentrated and clean DNA preparation. It is best to use an endotoxin-free DNA Maxi preparation but regular Maxi preps work too. You should also have plasmids that act as controls. Each reaction can only accommodate 10 µl of DNA so the dose nucleofected can be limited by the concentration of DNA. If needed, you can ethanol precipate a preparation to increase the concentration.

[hESC expansion]

- Expand cells on 15 cm dishes with MEFs.
- Passage onto Matrigel-coated dishes with conditioned media and 10 ng/ml FGF2.
- Expand on Matrigel 3-5 days with new conditioned media supplemented with 10 ng/ml FGF2 daily.

[Cell preparation]

- The day before, plate out 4 x 15 cm dishes of Neo resistant MEFs.
- The day of nucleofection, aspirate DMEM from MEF dishes and wash once with hESC media.
- After wash, place fresh hESC media on MEFs and add 10 µM Y-27632 to the media.
- Place in the incubator to warm and bring the media to the correct pH.
- Dissociate cells with 10 ml of Accutase (~20 minutes at 37˚C).
- Add cells/Accutase to 20 ml of hESC media to wash the cells and centrifuge (5 min., 200xg).
- Aspirate and resuspend cells in 10 ml hESC media with 10 µM Y-27632 and count cells on hemocytometer.

- Aliquot 5 million cells/15 ml conical tube for each nucleofection reaction. 2-3 nucleofections per 4 x 15 cm dishes are frequently performed per construct.

[Nucleofection]

- Gently resuspend cell pellet with 100 µl solution V Amaxa solution.
- Mix cell suspension with 5-75+ µg BAC DNA (volume of DNA should not exceed 10 µl).
- Nucleofect with protocol B-16. Immediately add 500 µl of prewarmed hESC media with Y-27632 (from MEF dishes). Distribute the cells to the 4 x 15 cm MEF dishes.

[Post Nucleofection]

- Feed the cells the next day with hESC media containing Y-27632. It is not needed on day 3 and beyond.
- Begin selection on day 4 with 25 µg/ml G418. Increase selection on day 14 with 40-50 µg/ml G418.
- Colonies can be picked after 14-21 days after nucleofection.

Some in COREdinates (coredinates.org) have reported problems with plating too many hESCs after nucleofection: this can be a problem with G418-based selection. They report paracrine survival resulting in clones without the selectable marker surviving. In our work,

Reagents

Kit V for Nucleofection
Lonza
VACA-1003

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 (or, to the entire bottle if you use it within two weeks). If you warm it, also warm just an aliquot - not the whole bottle. KSR does not like to be warmed.

DMEM:F12
Life Technologies
11330-032

Knockout Serum Replacement
Life Technologies
10828-028
Read the instructions on how to thaw and aliquot - aliquot into 50 ml tubes and freeze once (-20˚C). It is wise to lot test this reagent too, particularly for differentiation protocols.

MEM Non-essential amino acids
Life Technologies
11140-050

2-mercaptoethanol
Life Technologies
21985-023

L-Glutamine (200mM)
Life Technologies
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 (100µg/ml) in sterile PBS with 0.1% BSA, aliquot and store at -80˚C)

***StemBeads** (time-release FGF2 for the weekends)*
StemBeads FGF2
StemCulture, LLC
[Use 7.5 ul/ml of hES media or as directed by manufacturer. Media can be left on feeder-based, pluripotent stem cell cultures for up to 4 days.]

DMEM+10% FBS for plating MEFs

DMEM 900 ml
FBS 100 ml
Sterile filter. No need to aluminum foil.

DMEM
Life Technologies
11965-118

Fetal bovine serum (for plating MEFs)
Life Technologies
26140-095

Dispase (Neural Protease)
Stem Cell Technologies
07913
100 ml
Thaw, mix, and store in working aliquots at -20˚C. Warm to 37˚C for 10 minutes prior to use. Do not leave at 37˚C for too long or you will inactivate the enzyme.
[Many of us now simply thaw the bottle and store it at 4˚C for at least 2 weeks. It does not lose appreciable activity.]

2x Freezing media
60% Hyclone FBS 20% DMSO
20% hESC media
[Use freshly made freezing media, and sterile filter before use. Keep freezing media and pre-labeled cryotubes on ice before use.]