Genetic Manipulation of Human Embryonic Stem Cells

A. Electroporation

Required Materials:

- confluent 6 well dish starting cell population
- ROCK inhibitor (use at 1:1000)
- Linearized plasmid DNA (20ug, reconstituted in water) For pCAG-NLS-Cre plasmid, linearization is not necessary since you are not selecting for stable integration.
- Electroporation buffer (EmbryoMax, Chemicon International)
- 4mm electroporation cuvette
- 10cm dish prepared with MEFs
- 0.05% Trypsin-EDTA
- PBS
- HESC media

1. Pretreat HESC with HESC media + ROCK inhibitor to prevent cell death associated with dissociation of cell colonies. Rinse cells once with PBS, replace with HESC media + ROCK inhibitor, and incubate 2 hours.
2. Trypsonize 3 confluent wells of HESC per electroporation. For electroporations, it is necessary to dissociate cells to single cells suspension, therefore increase trypsin incubation time to 4-5 min and titurate cells to dissociate.
3. Stop trypsin with an equal volume of MEFs media (DMEM + 10% FBS) and pellet cells by centrifugation (3 min, 1100 rpm).
4. Prepare electroporation cuvette by adding DNA (15-20 ug per electroporation)
5. Gently resuspend ES cells in 400uL pre-cooled electroporation buffer / electroporation.
6. Add ES cell suspension to electroporation cuvette.
7. Pulse cells at 170V, 1050 µF
8. Immediately transfer cells into 15 mL conical tube containing 9mLs HES media + ROCK inhibitor using glass Pasteur pipette (be extremely gentle with cells from this point on – avoid tituration)
9. Plate cell suspension on 10 cm dish of MEFs.
10. Following 24 hrs, change media to regular HESC media, and start drug selection 48hrs following electroporation.
11. Colonies of drug resistant cells should become apparent after approx. 1 week, and colonies should be large enough to pick following 10-12 days (at this point colonies should be visible when observing the 10cm plate from below).

B. Picking Colonies of HESC

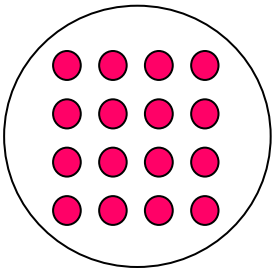
Required Materials:

- 0.05% Trypsin EDTA
- MEFs media (DMEM + 10% FBS)

- PBS
- HESC media
- 6 cm tissue culture dishes
- 4 well dishes prepared with MEFs (1 well for each colony to be picked)

The goal of picking colonies is to establish and expand distinct clonal populations containing your gene of interest. Care must be taken to avoid contamination between populations. Individual colonies will be picked and dissociated in drops of Trypsin-EDTA and then plated into separate wells of a 4 well dish of MEFs.

1. Prepare Trypsin by placing individual drops (approx 30uL) on the LID of a 6 cm dish. (make sure drops do not touch each other)



2. Prepare a 1.5mL epindorf tube of MEFs media (to stop trypsin)
3. Prepare the 4 well dishes of MEFs that you will be plating the individual colonies into by labeling wells appropriately, and changing to HESC media.
4. It often helps to mark colonies in 10cm dish by putting a colored dot on bottom of dish.
5. Remove media from 10cm dish of HESC colonies, rinse once with PBS, and replace with 9 mL PBS.
6. Work with either a dissecting microscope in a picking hood or an inverted microscope. Using a p200 pipette and new tip for each separate colony, pick colonies by first scraping around the circumference of the colony to detach cells from feeders, and then gently scrape colony off from bottom of the dish.
7. Pick up cells using pipette, and transfer into drop of trypsin, trying to minimize the volume of PBS being transferred along with the colony.
8. Change tip, and continue picking colonies.
9. The individual colonies should not remain in the Trypsin for longer than 2-4 minutes, so depending on your speed pick either 4 or 8 colonies at a time.
10. Once you have transferred 4-8 colonies, with a new tip and p200 set to 150 uL, aspirate approx 30uL MEFs media from epindorf tube and while looking under the microscope, titurate up and down in the first drop to dissociate the colony within. Transfer all liquid to first well of dish to plate. Continue until all colonies have been transferred to culture dishes.
11. Selection media can be added following 24 hrs and cultures are maintained until wells are ready to be passaged. From here, each confluent well can be passaged into 3 wells of a 12 well dish – 1 well for screening (Southern/qPCR) and 2 wells to be frozen down. If only 1 colony forms in 4 well dishes, cells can be trypsonized and plated into fresh well to let cells expand.