HEK293s

(Thawing, Suspending Cells, Maintaining attached cells, freezing attached cells, maintaining suspension cells)

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Thawing HEK293s cells

Overall Scheme:

P2	→	P3	→	P4	→	P5
Frozen		attach cells		expand		suspend

Thawing HEK cells:

1) Add 30mL new media to 50mL Falcon tube
2) Remove cells from small liquid nitrogen dewar
   [Chen Lab section, 1st box on top, Hek 293S p#2]
3) Thaw in H_2O bath at 37°C for 30sec to 1 min.
   [Hold tube; do not put orange top under water. Leave cells a little bit frozen, flick the bottom of the tube once in a while when keeping it upside down to see if cell clump is loose]
4) Pour whole tube in 50mL Falcon tube (from Step 1).
5) Spin down at 130 x G at room temperature for 5min. Press start 2x. [This media is only used to wash DMSO off cells]
6) Make 2 new flasks→ 12mL new media in each for now
7) Remove tube from centrifuge
8) Aspirate media
9) 25mL to resuspend pellet. Pipette up and down. Make sure cells disperse well.
10) 12mL into each new flask
11) Label flask w/Cell Type, Name, Passage, Date
12) Put in 37°C 5% CO_2 incubator

Subculturing of HEK293s cells in monolayer

Media: DMEM F-12, 10% FBS, 1% anti anti

Medium renewal: every 2 to 3 days

1) Wipe down hood with 70% EtOH
   Have DPBS, trypsin, supplemented DMEM F-12, and CellBind flask ready
2) Aspirate old media on top of cell monolayer
3) Add 5mL Ca^{2+}/Mg^{2+}-free Dulbecco's phosphate buffered saline (DPBS) to wash/rinse cells. DO NOT add directly on the cells, add it to the side of the flask and let the DPBS roll on the cell layer
4) Aspirate DPBS

5) Add 2mL Trypsin-EDTA solution. Leave in incubator 2-5min until cell layer is dispersed.
   Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach.
6) Prepare new flask: Cell Type, Name, Passage#, Date, Dilution
7) Add 20mL supplemented DMEM F-12 to the flask from step 6
8) Remove plate from incubator. Add 8mL fresh media to stop rxn.
   Gently pipette up and down 8-10 times to get single cells.
9) Make a 1:10 or 1:5 dilution
10) Put in 37°C, 5% CO_2 incubator

*Notes:

- These cells adhere poorly to standard tissue culture flasks. Attachment can be enhanced by using Corning CellBIND® flasks.
- Volumes used in this protocol are for 75 cm^2 flasks; proportionally reduce or increase amount of dissociation medium for culture vessels of other sizes.

Freezing HEK293s cells

Notes:

- Freeze cells at a density of at least 3 million viable cells/mL
- Use a freezing medium composed of 90% DMEM (with 10% FBS and no anti) and 10% DMSO. Prepare this immediately before use. Filter-sterilize the freezing medium and store at 4°C until use. Discard any excess freezing medium after use.
- You may check the viability and recovery of frozen cells 24 hours after storing cryovials in liquid nitrogen (see thawing cells protocol)

Before starting, label cryovials and prepare freezing medium. Keep the freezing medium on ice.

1) Culture the desired quantity of HEK293 cells to 70-90% confluency
2) Remove the cells from the tissue culture flasks by washing with DPBS, adding 2mL trypsin, adding 8mL media to stop the reaction, then transferring to a 15mL tube.
3) Determine viable and total cell counts and calculate volume of freezing medium required to yield a final cell density of > 3 Million viable cells/mL
4) Prepare the required volume of freezing medium
5) Centrifuge the cell suspension (from step 2) at 150 x g for 5 min at room temp. Aspirate the medium and re-suspend pellet in the pre-determined volume of chilled freezing medium.
6) Dispense aliquots of this suspension (frequently mixing to maintain a homogenous cell suspension) into cryovials
7) Freeze cells in an automated, control-rate freezing apparatus or using a manual method following standard procedures. For ideal cryopreservation, the freezing rate should be a decrease of 1°C per minute. Do this overnight in the -80°C freezer.
8) Transfer vials to liquid nitrogen storage the next morning.

Put HEK293s into suspension (make them into RIC cells)

After thawing cells and attaching them to plate: (This would be Passage #4=expansion)

1) Same protocol as monolayer HEK293s but expand to 5 flasks
   Label all with Cell Type, Name, Passage#, Date, Dilution
2) After 3 days, prepare a baffled flask, DPBS, and supplemented Freestyle 293 (with 2% FBS, 1% anti) media

Label baffled flask: RIC, name, passage #, date, cell volume, and count

3) Aspirate media from each flask. Wash with 10mL DPBS
4) Add 20mL of supplemented Freestyle 293 media to each flask
   (**No trypsin** - trypsin induces clumping of cells)
5) Use 10mL pipette to suspend cells. Make adherent cells come off by pipetting media up and down on them directly until all are off or use a cell scraper.
6) Put all of the media from all 5 flasks into a baffled flask
7) Put the 100mL of cells in shaker for 3 or 4 days.

Growth and maintenance of Ric15(Gntl(-)) suspension cells

Hek 293 S/RIC/human kidney= suspension cells, mutation in glycosylation gene, for large scale tests, not kept past 25th passage, kept in baffled flasks

Media: Freestyle 293, 2% FBS, 1% anti anti
(write date media opened and your name on bottle)
If kept at 30°C then wait more than 4 days before splitting
If kept at 37°C, split every 4 days

1) Take out 1mL sample in Eppendorf tube
   Can dilute sample 1:1 with Trypan Blue before loading hemocytometer
2) Load 10ul in hemocytometer. Count cells.
3) Split to 0.3-0.4 million cells/mL
4) If using ≥ 400mL of media → use big flask
   < 400mL→small flask
5) Add prewarmed media to new flask, then add appropriate amount of cells.
6) Label flask with: Cell Type, Passage#, Name, date, volume/conc
7) Place in 37^{o}C, shaking, 8% CO_2 incubator

SF-9 Cells
Thawing, Culturing, Freezing

By: Ana-Maria Tanasescu, November 2017

*Insect cells/SF-9= grow baculovirus in them, infect human cells, keep at 0.4 - 4 to 6 million, split 2x a week to keep stock going

Media: SF900II SFM (1x), 5% FBS, 1% anti anti
(write date media opened and your name on bottle)

Info:

- Doubling time is ~27 hours
- No need to filter media
- Don't keep past passage #30
- Expect to split about every 3 days

Overall Scheme:

Monday	→	Tuesday	→	Wednesday	→	Thursday
Thaw 2 vials		grow to 1.5-2million/mL		increase volume to 30-40mL		check growth

Thawing:

1) Prepare an ice bucket and 50mL conical tube with 30mL media in advance
2) Take the cell vials out (usually 2 vials with $1x10^7$ cells/mL density) from the liquid nitrogen tank. [Sf-9 cells are kept in the small dewar, 2nd box in the Chen Lab rack]
3) Quickly thaw cells in a 37°C water bath for ~1.5min. DO NOT let the cells thaw completely, since cell viability will be impacted. Invert tube to check if cells drop to the bottom.
4) Pour the cells into the conical tube with 30mL media (from Step 1). Spin down at 1,000 rpm/r.t. for 3 min.
5) Aspirate the supernatant. Resuspend cells with 10mL media.
6) Perform a cell count to determine actual cell density. Count the top left 4x4 square. Dilute the cells to $1x10^6$ cells/mL.
7) Place the flask in the incubator: shaking 90rpm, 28C
8) The cells should reach a density of $>2x10^6$ cells/mL after 2-3 days in culture.
9) When the cell density reaches $2x10^6$ to $3x10^6$ cells/mL passage cells at a seeding density of 0.8 to 1 million cells/mL.

*Check every day for growth
*At the beginning, do not split lower than 0.5 million/mL

Maintenance:

1) Spray down hood, inner panel, flasks, and media bottles with 70% EtOH

2) Count cells: Use 1mL stripette, swirl as you take sample out of flask, put sample in Eppendorf tubes, load 10ul in hemocytometer.
   [Can also do 1:1 dilution with trypan blue]
3) Calculate concentration of cells you desire. Use (M1)(V1)=(M2)(V2)
   Want roughly 3.5-5 million cells prior to split and 0.5 million cells after split
   Sample dilution:
   a. Counted 5 million cells
   b. Want ~.5 million cells
   c. 5 million * (1/10)= 0.5
      i. Want 1:5 dilution. To do so, add 20 mL of cells to 80 mL of new media
      ii. Want to maintain around 100 mL
4) On new flask write: Cell Type, Lab stock, Passage #, concentration made(volume and cell number), date, your name

Freezing:

- Once cell lines are established and are doubling regularly, they can be frozen.
- Freeze the cells between 1-2 million so passage the cells to 0.6 million/mL the day before you freeze.
- Passage number not important; health of the cells matters more (co clumps, no dead cells)

1) Have cryogenic storage vials ready, DMSO, and put isopropanol in freezing container.
2) Count cell density in log phase. Transfer the cells evenly to two 50mL conical tubes. Calculate how many vials you will need (round the number down)
3) Prepare cryogenic storage vials, label (cell name, storage media, date, your initials),
4) Prepare freezing medium: 50% fresh media+40% old media (added after spinning down cells) +10% DMSO
5) Spin down cells at 10,000rpm/r.t. for 4 min
6) Aspirate supernatant after taking some for the freezing medium
7) Resuspend pellet with freezing media to achieve a cell density of $1.0x10^7$ cells/mL
8) Dispense aliquots of the cell suspension into labeled cryovials. As cells are dispensed into cryovials, frequently and gently mix the cell suspension to maintain a homogenous solution. [Add 1mL in each vial using 10mL pipette]
9) Freeze the cells in the -80°C freezer in a controlled rate freezing container with isopropanol by decreasing the temp approximately 1°C per min. Leave the cap loose.
10) Next day, transfer the cells from -80°C to liquid nitrogen storage tank.