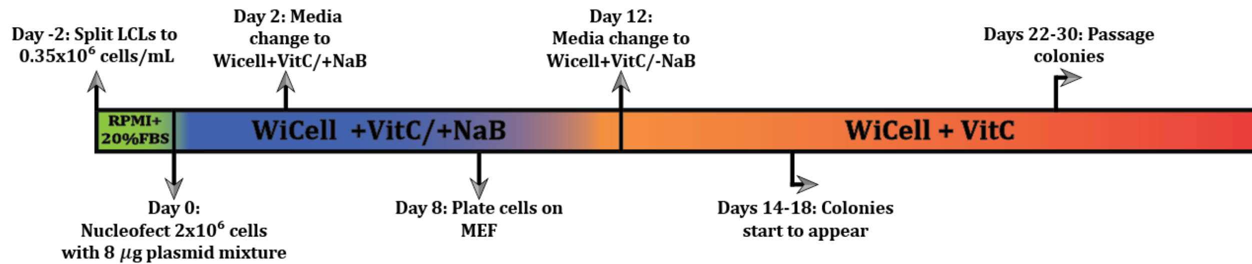
GILAD LAB LCL REPROGRAMMING PROTOCOL



Days -2: Splitting LCL Lines
Media used: RPMI+20% FBS

Two days before Nucleofection split LCL line into 2xT25 flasks containing 5-10 mL per flask at a density of $0.35x10^6$ cells/mL. If the line tends to grow slower, dilute to a cell concentration such that the cells will reach a density of $0.8\text{-}1x10^6$ cells by the day of Nucleofection.

Days 0: Nucleofection

Preprep:

- Let NF reagents warm to room temperature.
- NF supplement needs to be added to NF solution prior to use, each 100 µL transfection will contain 81.8 µL NF solution V and 18.2 µL supplement, **do not round these pipette measurements.** A master mix can be created for all transfections:

$$N\ x\ 81.8\ uL\ NF\ solution\ V = ______$$

$$N\ x\ 18.2\ uL\ NF\ supplement = ______$$

- Once supplemented the NF supplement is only good for 3 months and should be labeled with the expiration date (supplement date + 3 months) and stored at 4°C when not in use.
- Fill 1 well of a 12 well plate with 1 mL WiCell+VitC per line and allow to warm to 37 °C in an incubator for recovery post Nucleofection

Experimental Procedure:
Media used: WiCell +VitC

1. On the day of Nucleofection, take one of the two flasks and count the cells to ensure the cells are healthy and at the right density for Nucleofection, at least $0.8\text{-}1.2x10^6$ cells/mL and 65%+ live cells (cell lines that do not reach this density after two days can still be used, but should be half fed and Nucleofected once they reach a higher density).
2. Once the health of the cells has been verified, dissociate the cells in the second flask using a 10 mL pipette. Based on the count from above try to spin down ~$6\text{-}10x10^6$ cells in a 15 mL conical tube. Spin cells at 1000 RPM for 5 minutes.
3. Aspirate all the media being careful not to disturb the pellet. Resuspend in 6-10 mL PBS and count the cells.
4. Determine the volume needed for 2 million cells; transfer this volume to three different 15 mL conical (spin down 6 million cells in total in 3 different tubes) and spin at 1000 RPM for 5 minutes.
5. Aspirate the media from the three tubes; label two of the tubes with the LCL line number, the date, RNA pellet for one and DNA pellet for the other. Stick these two tubes in the -80°C for extraction later.

6. Flick the third tube to resuspend the pellet and add 100 µL supplemented Nucleofector solution V. Once the solution has been added to the cells, the following steps should be completed in less than 15 minutes.

7. Add 2 µL episomal vector stock (8 µg total, 2 µg per vector of the following: 27077, 27078, 27080 and 27082, for one transfection, numbers indicate Addgene ID for Yamanaka lab episomal vectors)[1] to the tube and mix gently using a pipette.

8. Transfer 100 µL of the cell/vector stock to a Nucleofector cuvette and Nucleofect using program X-005.

9. Using a transfer pipette remove 0.5 mL of media from 1 well of a 12 well plate. Gently dispense this into the cuvette, draw the cells back up into the pipette and carefully transfer them to the 12 well plate.

Days 2,4 and 6: Media Changes
Media used: WiCell + 50 μg/mL VitC and 0.5 mM Sodium Butyrate (NaB)

1. Using a 1 mL pipette, transfer cells to a 15 mL conical tube and spin down at 1000 RPM (~200xg) for 5 minutes.
2. Add 0.5 mL fresh WiCell media containing 0.5 mM NaB and 50 μg/mL VitC to the used wells of the 12 well plate. This will keep any cells that adhere to the plate from drying out. Add 0.5 mL to a new well (1 well per line, each line will be split into two wells at day 2 and maintained until day 8 in two separate wells).
3. Carefully aspirate the media from pelleted cells (from step 1), leave 100-200 μL to avoid removing cells.
4. Flick tube to resuspend the pelleted cells, add 1 mL media. On day 2, split each line into two separate wells by transferring 0.5 mL of these cells to the appropriate wells of the plate prepared in step 2.
5. By day 3-4 the level of GFP expression will reach its maximum.
6. Cells will begin to adhere to the plate and change morphology from day 4-8.

Day 7: Prepare MEF Plates
Media used: DMEM+10%FBS

1. Prepare ½ of a 6 well plate per line reprogramming, if reprogramming 4 different LCL lines you will need at least 2 MEF plates, but it may help to have one extra plate as a spare for different plating densities.
2. Coat each well of a 6 well plate with 2 mL of 0.1% gelatin and incubate plate for at least 15 minutes.
3. Aspirate gelatin and plate 20,000 irradiated MEF per cm^2 in DMEM+10% FBS (for TPP plates this is $1.08x10^6$ cells/plate or 2 mL per well at 0.0896 cells/mL).

Day 8: Plating Transfected LCLs on MEF
Media used: WiCell + 50 μg/mL VitC and 0.5 mM NaB

1. Inspect MEF plate to ensure even plating and accurate cell density.
2. Aspirate excess MEF media from plates and rinse with PBS, aspirate PBS and add 2 mL WiCell + 50 μg/mL VitC and 0.5 mM NaB to each well and return to incubator.
3. Spin down cells from reprogramming plate at 1000 RPM for 5 minutes (mix both wells from each line into one tube), aspirate old media and resuspend in 2 mL. Count cells and record the concentration.
4. Calculate the volume of cells needed for 2 mL of cells at 100,000 cells/mL ($0.2x10^6$ /cell count). Transfer this volume to a new tube and bring the volume up to 2 mL. Split the remaining undiluted cells into 2 tubes, spin down, remove all media and label with line number and: DAY8 RNA for one and DAY8 DNA for the other, place these two tubes in the -80°C for extraction.
5. Plate cells at 9000, 18,000, and 36,000 cells per well on the MEF plate (9 μL, 18 μL and 36 μL) and return plates to incubator.*

**The plating density will depend heavily upon the quality/age of the LCL line and the transfection efficiency (for a line that reaches 1 million cells/ mL after two days when split to $0.35x10^6$ with >70% live coupled with ≥60% transfection efficiency, as little as 6000-9000 cells is enough to get 5-10 colonies).*

Day 10: Media Change
Media used: WiCell + 50 μg/mL VitC and 0.5 mM NaB

- Look at reprogramming plates under the microscope using 4x objective and GFP channel, if there are a significant number of GFP+ cells attached to the plate, you do not need to spin the old media down, just aspirate the old media and replace with fresh WiCell media + 50 μg/mL VitC/+0.5 mM NaB.
- If there are few GFP+ cells attached AND lots of GFP+ cells still in solution (or you are not sure) transfer the media to a 15 mL conical tube, add 2 mL fresh media back to the plate. Spin down the 15 mL tube, aspirate and resuspend any pellet in media from the 6 well plate and return to well.

Day 12-30+: Media Change, No NaB
Media used: WiCell + 50 μg/mL VitC

- Change the media on the MEF plates every two days, from day 12 on use WiCell + 50 μg/mL VitC, No NaB!
- Colonies can generally appear starting at day 16-18 and can be picked between day 22-26.
- Look at the plates every day to ensure that you can prepare MEF plates for picking P#1s, if the plates are left too long they will overgrow and the colonies will differentiate. Generally day 24 is the most common day for picking colonies.
- Pick 2-4 colonies per line (only 1 colony per well for P#1) and passage to P#3 before picking the best "clones" to propagate for banking.

Media Formulations:

RPMI+20% FBS:

RPMI+ 20%FBS		
Component	Volume (mL)	
RPMI	500	1000
FBS	128.2	256.4
Pen/Strep/Lglut	6.4	12.8
Glutamax	6.4	12.8
Total Vol:	641 mL	1282 mL

DMEM+10%FBS:

DMEM BASE MEDIA		
Component	Volume (mL)	
DMEM	500	1000
FBS	56.8	113.6
NEAA	5.7	11.4
Glutamax	5.7	11.4
SUPPLEMENTS:		
VitC	32 mg	64 mg
Total Vol:	568 mL	1136 mL

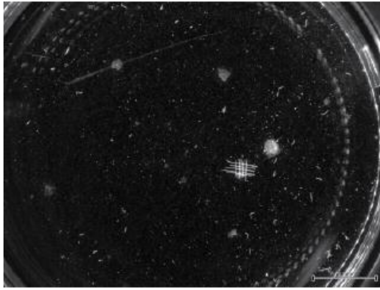
WiCell+ 50 µg/mL VitC* and 0.5 mM sodium butyrate (NaB):

Incomplete WiCell			
Ingredient	Volume (mL)	Final Conc	Stock Conc
DMEM/F12	500		
KOSR	128.2	20%	
NEAA (100x)	6.4	0.1 mM	10 mM
Glutamax (100x)	6.4	2 mM	200 mM
VitC	32 mg	50 µg/mL	
			Total Vol: 641 mL

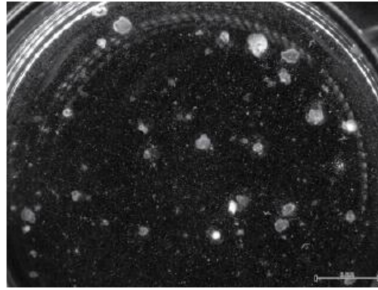
Add to 50 mL Incomplete WiCell to complete:		
Component:	Volume	Final Conc
bFGF (@ 25 µg/mL)	25 µL	12.5 ng/µL
BME (55 mM)	91 µL	0.1 mM
NaB (1000x,500 mM)	50 µL	0.5 mM

*We use L-Ascorbic acid 2-phosphate sesquimagnesium salt hydrate, Santa Cruz Biotec#sc-228390 or Sigma#A8960. It is important to use this derivative due to the instability of normal vitamin C in cell culture.

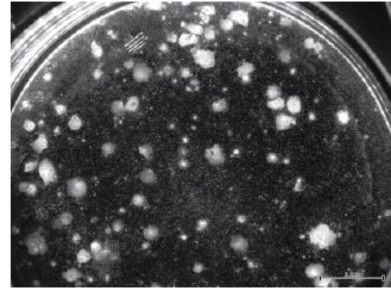
9,000 Cells



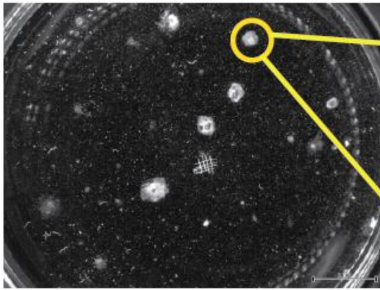
18,000 Cells



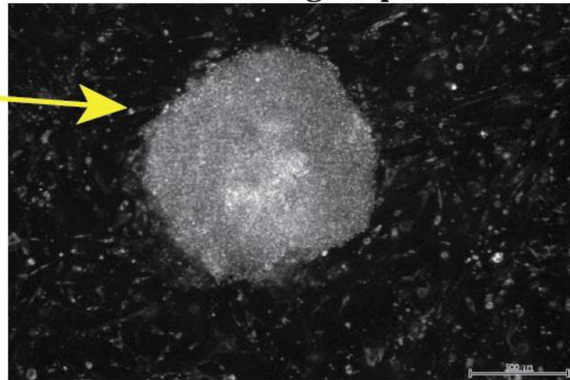
36,000 Cells



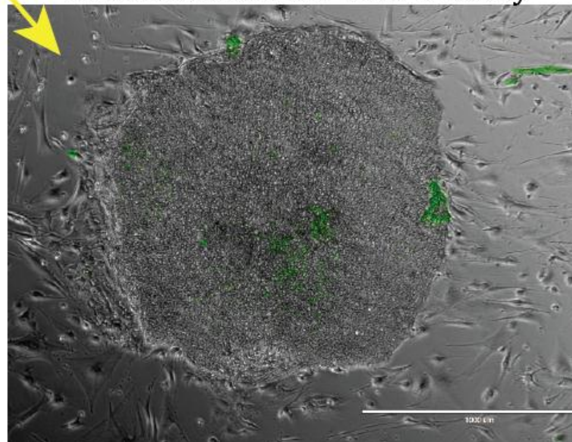
18,000 Cells



Dissecting Scope



4x Phase Contrast +GFP Overlay



YRI line 18909, day 22. Images show typical density and morphology of reprogramming colonies for a healthy LCL line with 60%+ transfection efficiency.

References:

1. Okita, K. *et al.* A more efficient method to generate integration-free human iPS cells. *Nature methods* **8**, 6-11 (2011).

2. Yu, J. *et al.* Human induced pluripotent stem cells free of vector and transgene sequences. *Science (New York, N.Y.)* **324**, 797-801 (2009).

3. Choi, S.M. *et al.* Reprogramming of EBV-immortalized B-lymphocyte cell lines into induced pluripotent stem cells. *Blood* (2011).doi:10.1182/blood-2011-03-340620

4. Rajesh, D. *et al.* Human lymphoblastoid B cell lines reprogrammed to EBV-free induced pluripotent stem cells. *Blood* (2011).doi:10.1182/blood-2011-01-332064