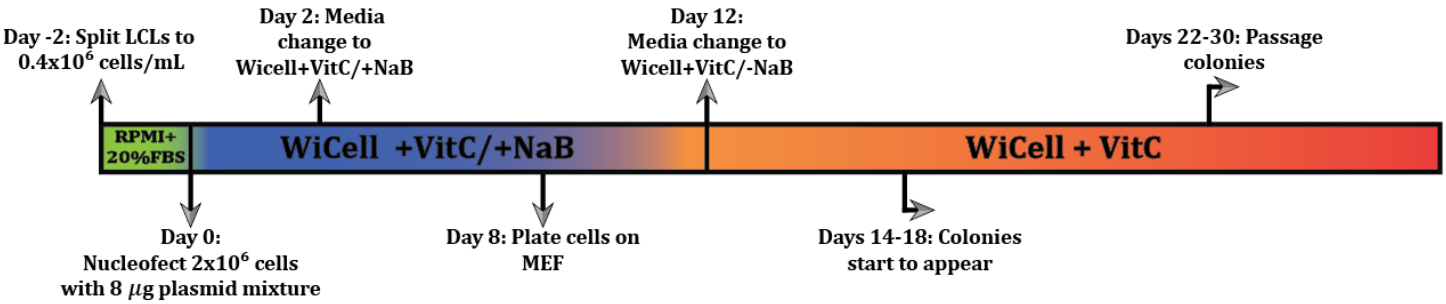
GILAD LAB LCL REPROGRAMMING PROTOCOL



Days -2: Splitting LCL Lines

Media used: RPMI+20% FBS (10-20% is commonly used, use whichever makes your LCL lines grow the best).

Two days before Nucleofection split LCL line into 1xT25 flasks containing 15 mL per flask at a density of 0.4x10^6 cells/mL. If the line tends to grow slower, dilute to a cell concentration such that the cells will reach a density of 0.8-1x10^6 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=# transfections, always make up 1 TF excess to account for liquid loss during pipetting):

$$(N+1)\ x\ 81.8\ uL\ NF\ solution\ V = \_\_\_\_\_$$

$$(N+1)\ x\ 18.2\ uL\ NF\ supplement = \_\_\_\_\_$$

- 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 complete WiCell per line and place in 37 °C incubator until needed.

Experimental Procedure:

Media used: RPMI+20% FBS and Complete WiCell

1. On the day of Nucleofection, dissociate the LCLs using a serological pipette (as you would normally do for passaging) and count the cells (using trypan blue) to ensure the cells are healthy and at the right density for Nucleofection. The optimal density (indicative of a healthy cell line) is 0.8-1.2x10^6 cells/mL and >65% live cells[1].
2. Calculate the volume of cells needed for 6 million cells (use a gate from 7-14 µM when counting and take the average of two counts), transfer this volume to a 15 mL conical and spin down at 50 x g for 5 minutes[2].
3. Aspirate all the media being careful not to disturb the pellet. Resuspend in 6 mL *RPMI+20% FBS* and count the cells again (don't need to use trypan blue, just use gate from 7-14 µM and take the average of 2 counts).
4. Determine the volume needed for 2 million cells; transfer this volume to a 15 mL conical (if you would like to collect pellets for RNA/DNA now, spin down 2 million cells for each per 15 mL conical).
    a. Spin down cells at 100 x g for 5 minutes.
5. While cells are centrifuging, take prepared 12 well plate out of incubator and place it in the hood.

---

[1] *Cell Lines that have a lower viability (if the line normally has a lower viability) may still be used, however they are more difficult to reprogram and some adjustments will need to be made when plating them on MEF.*

[2] *This step is to remove debris and dead/dying cells.*

6. Aspirate the media from the 15 mL conicals.
    a. If you have tubes for RNA/DNA, aspirate media from them and place those tubes in the -80°C.
7. Flick 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.
8. Add 5 µL premixed episomal vector stock[3] to the tube and mix gently using a 200 µL pipette.
9. Transfer the entire contents (between 105-115 µL) containing the cell/vector mix to a Nucleofector cuvette and Nucleofect using program X-005.
10. Using a transfer pipette remove 0.5 mL of Wicell from the destination well of the 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.
11. Once all transfections are complete, place 12 well plate in the incubator and leave undisturbed until media change at day 2.

[3] *5 µL of episomal vector stock contains a total of 8 µg vector, which is made up of 2 µg per vector of the following: 27077, 27078, 27080 and 27082. Numbers indicate Addgene ID for Yamanaka lab episomal vectors*

Days 2: Media Changes
Media used: Complete WiCell + 0.5 mM Sodium Butyrate (NaB)

1. Using a 1 mL pipette tip, transfer cells to a 15 mL conical tube and spin down at 100 x g for 5 minutes.
2. Add 1.5 mL fresh complete WiCell media containing 0.5 mM NaB to the used wells of the 12 well plate. This will keep any cells that adhere to the plate from drying out while the cells are spinning down.
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, using 0.5 mL of complete Wicell from the destination well (prepared in step 2), transfer the cells back to the plate.
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.

Days 4 and 6: Media Changes
Media used: Complete WiCell + 0.5 mM Sodium Butyrate (NaB)

1. Take cells out of incubator being careful not to swirl the plate (you want all the cells to stay in the bottom of the well). Let the cells settle in the plate for 5 minutes before proceeding.
2. Using a 1 mL pipette tip, tilt plate towards you to pool cells in the bottom and carefully remove 1 mL of old media[4], being careful not to remove any cells. There should still be 0.5 mL left in each well.
3. Add 1 mL of fresh complete WiCell media containing 0.5 mM NaB to each well and mix up and down one time to gently dissociate clumps (make sure to use a new pipette tip with each well so you don't cross contaminate).
4. Add 1.5 mL fresh complete WiCell media containing 0.5 mM to the used wells of the 12 well plate. This will keep any cells that adhere to the plate from drying out while the cells are spinning down.

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: Complete WiCell + 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 complete WiCell + 0.5 mM NaB to each well and return to incubator.
3. Transfer cells from 12 well plate to 15 mL conicals, rinse out the well with 1 mL fresh complete Wicell and pool into the 15 mL conical. Centrifuge at 100 x g for 5 minutes, aspirate old media and resuspend in 2 mL.

[4] You have to be very careful to pipette up slow otherwise you will remove cells during feeding, I do not advise using an aspirator as they generate too much suction and will remove a substantial number of cells at each feeding. If you are worried about removing cells, you can do media changes by spinning cells down as performed at day 2.

Count cells (using gate from 6-17 μM, this should enclose main peaks, anything to the left of 6 μM is debris, and generally anything to the right of 17 μM are clumps) 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[5], 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 (90 μL, 180 μL and 360 μL when cells have been standardized to 100k cells/mL as instructed in step 4) and return plates to incubator.
    a. The optimal 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). A density that is too high generally results in overgrowth of partially transformed junk and over crowding of iPSCs resulting in increased differentiation before good colonies can be picked. If the density is too low no, or few colonies will result.

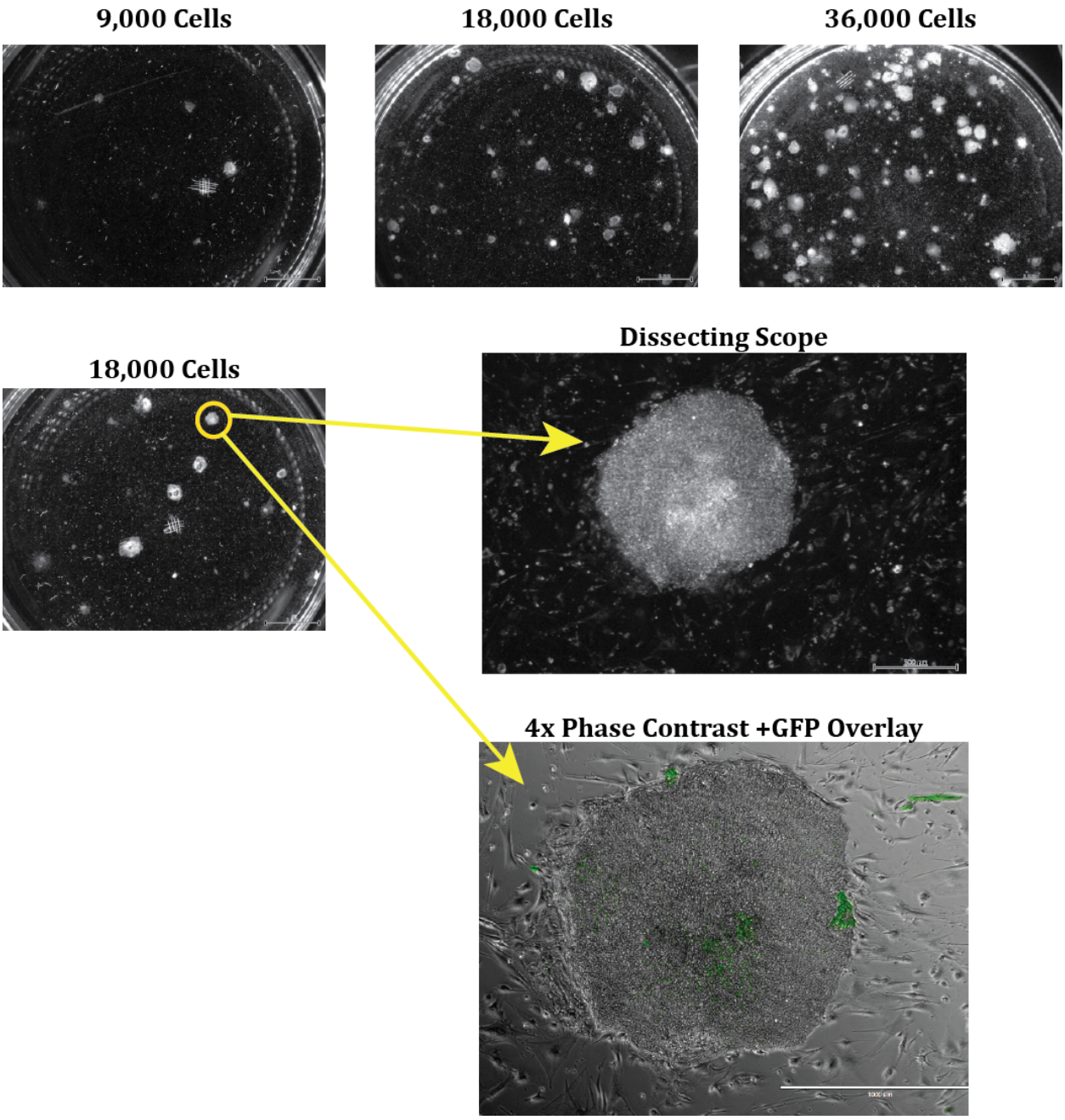
Day 10: Media Change
Media used: Complete WiCell + 0.5 mM NaB.

1. Aspirate old media and replace with 2 mL per well of fresh complete WiCell+*0.5 mM NaB*.

Day 12-30+: Media Change, No NaB
Media used: Complete WiCell

- Change the media on the MEF plates every two days, from day 12 on use WiCell without NaB!
- Colonies 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.
- If cultures accumulate excess partially transformed cells (they look like clumps of LCLs that stick in globs to the MEF), they should be removed by aspirating well and spraying over cells with 1 mL fresh complete WiCell to dislodge the partially transformed cells. Aspirate to remove them and feed with fresh media.
- 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.

[5] *This is used later as a positive plasmid control during integration analysis and as a reference for qPCR, it is not necessary to collect Day 8 pellets from every line.*



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

Media and stock formulations:

0.1% gelatin (900 mL)

- Add 0.9 g type A gelatin to 900 mL Millipore water and autoclave.
- Sterile filter using 0.22 µM filter before use and store at room temp for up to one year.

VitC stock (25 mL@32 mg/mL):

- Weigh out 800 mg of L-Ascorbic acid 2-phosphate sesquimagnesium salt and transfer to a 50 mL conical.
- Add 25 mL PBS and pipette mix. Transfer to 37°C water bath for 10 minutes to dissolve.
- Filter sterilize using 0.22 µM syringe filter. Store at 4°C for up to 1 month for longer storage, aliquot and store at -20°C.

NaB Stock (100 mM) (200x)

- Weigh out 200 mg Sodium Butyrate, transfer to a 50 mL conical.
- Add 18 mL molecular grade H2O, Transfer to 37°C water bath for 10 minutes to dissolve.
- Filter sterilize using 0.22 µM syringe filter. Aliquot and store at -20°C until thawed for use.

RPMI LCL media:

	RPMI+ 10%FBS		RPMI+ 15%FBS		RPMI+ 20%FBS	
	VOLUME (mL)		VOLUME (mL)		VOLUME (mL)	
RPMI	500	1000	500	1000	500	1000
FBS	57	114	90	181	128	256
Pen/Strep	5.7	11.4	6.0	12.0	6.4	12.8
GLUTAMAX	5.7	11.4	6.0	12.0	6.4	12.8
TOTAL	568 mL	1136 mL	602 mL	1205 mL	641 mL	1282 mL

DMEM+10%FBS:

	DMEM MEDIA	
	VOLUME (mL)	
DMEM	500	1000
FBS	57	115
NEAA	5.7	11.5
GLUTAMAX	5.7	11.5
PEN/STREP	5.7	11.5
TOTAL	575 mL	1149 mL

Incomplete WiCell:

	iWiCELL	
	VOLUME (mL)	
DMEM/F12	500	1000
KOSR	130	260
NEAA	6.5	13.0
GLUTAMAX	6.5	13.0
PEN/STREP	6.5	13.0
VitC	1 mL	2 mL
TOTAL	649 mL	1299 mL

Complete WiCell:

- To complete WiCell, you need to add BME and bFGF, we make up smaller stock solutions (50-250 mL) rather than completing the whole batch of media. This is because bFGF is relatively unstable and BME oxidizes over time.

	WiCELL ADDITIVES:				
	50 mL	100 mL	200 mL	250 mL	FINAL CONC
bFGF (100 µg/mL stock)	12.5 µL	25 µL	50 µL	100 µL	25 ng/mL
BME (55 mM)	91 µL	182 µL	91 µL	455 µL	0.1 mM
For Reprogramming Days 2-12, Add NaB:					
NaB (100 mM)	0.25 mL	0.5 mL	1 mL	1.25 mL	0.5 mM

Reprogramming Vector Prep:

It is essential to use high purity (A260/A280 and A260/A230 ≥2.0) endotoxin free episomal DNA. I usually do the following:

- Mega prep each episomal vector using endotoxin free specific kits. For each vector you will want to grow 500-750 mL of bacterial culture (the yield for these plasmids can often be lower than a standard smaller vector). Generally I will elute in ~5 mL H2O or elution buffer for a single mega prep column.
- Concentrate down to ~2 µg/µL (this is very concentrated and the solution will often get thick) using a speed vac.
- Centrifuge the concentrated DNA at ~20,000 x g, there will probably be a sizable pellet, this is usually just the fiber from the columns that always elutes with them, but it may also be residual debris/precipitated salts from the extraction. Pull off the DNA containing supernatant and leave the pellet.

To prepare concentrated vector stock:

- Calculate the volumes of each vector required for 2 µg (I usually make up a mastermix for 50-100 transfections, so just multiply each volume by the number of transfections, don't forget to do the same with the H2O at the end) mix these together and bring final volume to 5 µL with H2O. An example is shown below for 50 transfections with the current concentrations of my vectors.

Vector	27077	27078	27080	27082
Stock Conc(µg/µL)	1.544	1.931	2.155	1.735
# of TFs	50			

µg total per vector:	100					
Vector:	27077	27078	27080	27082	H2O	Final Vol (µL)
Volume (µL):	64.8	51.8	46.4	57.6	29.4	250.0

Part Numbers:

Item:	Manufacturer	Part#
DMEM/F12 With HEPES and L-glutamine - 1L	Fisher	10-092-CM
DMEM-1L	Fisher	10-017-CM
RPMI 1640 without L-glutamine 1L	Fisher	15-040-CM
Penicillin-Streptomycin Solution, 100X	Fisher	30-002-CI
GlutaMAX	LifeTechnologies	35050-061
MEM Non-Essential Amino Acids Solution 10 mM (100X), Liquid	LifeTechnologies	11140-050
2-Mercaptoethanol (1,000X), Liquid	LifeTechnologies	21985-023
Knockout Serum Replacement (KOSR)	LifeTechnologies	10828-028
L-Ascorbic acid 2-phosphate sesquimagnesium salt	Santa Cruz Biotech	sc-228390
bFGF	Gold Bio	1140-02-1000
Sodium Butyrate	Stemgent	04-0005
Cell Line Nucleofector Kit V	Lonza	VCA-1003
Gelatin from porcine skin - Type A, powder	Sigma	G1890-500G
TPP 6-well TC Plates-4/bag	Midsci	TP92406
TPP Tissue Culture Plate 6-well, 1/bag, 126/case	Midsci	TP92006
TPP Tissue Culture Plate 12-well, 1/bag 126/cs	Midsci	TP92012
TPP 12-well TC Plates 72/case	Midsci	TP92412
E.Z.N.A. Endo-Free Plasmid DNA Mega Kit	Omega Bio-tek	D6228-01
pCXLE-hOCT3/4-shp53-F (Vector 27077)	Addgene.org	27077
pCXLE-hSK (Vector 27078)	Addgene.org	27078
pCXLE-hUL (Vector 27080)	Addgene.org	27080
pCXLE-EGFP (Vector 27082)	Addgene.org	27082

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