GLADSTONE INSTITUTES | STEM CELL CORE
GLADSTONE CORE SERVICES

iPSCs Blood Reprogramming	Revision Number: 08
	Effective Date: 12 April 2024
	Written/Revised By: Wendy Runyon

Table of Contents:

Note: Please acknowledge the Gladstone Stem Cell Core in your presentations and publications if you have used the core's infrastructure (lab space or equipment), reagents/media, expertise, and/or services. These acknowledgments help to justify the existence of the core facility, as well as allowing Gladstone to be more competitive in grants and pursue other resource opportunities in order to enhance the cores over time.

Version Number	Summary of Changes	Revised by/Date
01	Original Document	Daeun Jeong 10Sep16
02	Updated volumes and dilution after nucleofection.	Angela Liu 4Oct17
03	Restructured procedure. Added additional details and updated catalog numbers.	Wendy Runyon 15Mar18
04	Added mTeSR Plus as an option for feeding medium. Corrected StemFit+Cytokine Medium volumes. Updated colony picking to use Matrigel and 24-well plate.	Wendy Runyon 21Jan21
05	Added Addgene catalog numbers. Removed StemFit as a feeding medium option. Updated catalog number for CD34 Nucleofector kit and StemSpan.	Wendy Runyon 26Sep22
06	Updated StemSpan AOF to StemSpan SFEM II. Changed "SOP Number" to "Revision Number".	Wendy Runyon 27Sep23
07	Added optional feeding of PBMCs between thaw and nucleofection. Updated p2-p5 passaging directions.	Wendy Runyon 21Mar24
08	Added instructions to keep p0 backup plate. Noted p0 and p1 plates. Added example pictures of morphology.	Wendy Runyon 12Apr24

1. PURPOSE

To describe the procedure for generating iPSCs from PBMCs using episomal vectors.

2. SUPPLIES

iMatrix-511 (T304, Clontech)
Matrigel® Growth Factor Reduced Basement Membrane Matrix (356231, Corning)
DPBS (-/-) (14190250, Thermo Fisher)
StemSpan™ SFEM II (09605, StemCell Technologies)
IL-3 (203-IL, R&D Systems)

IL-6 (206-IL, R&D Systems)
SCF (255-SC, R&D Systems)
TPO (288-TP, R&D Systems)
Flt3 (308-FK, R&D Systems)
Human CD34 Cell Nucleofector Kit (VPA-1003, Lonza)
mTeSR Plus (05825, StemCell Technologies)
pCXLE-hOCT3/4-shp53-F (27077, Addgene)
pCXLE-hSK (27078, Addgene)
pCXLE-hUL (27080, Addgene)
pCXWB-EBNA1 (37624, Addgene)
ROCKi, Y-27632 (S1049, Selleck Chemicals)

3. SCOPE

This procedure applies to PBMCs to be reprogrammed into iPSCs using nucleofection of the episomal vectors OCT3/4, shRNA against p53, SOX2, KLF4, L-MYC, LIN28, and EBNA1.

4. PROCEDURE

4.1. Thawing PBMCs

4.1.1. Prepare one well of a 6-well plate for each vial of PBMCs to be thawed by adding 2mL DBPS (-/-) and 9.6µL iMatrix-511 (1µL iMatrix-511/cm^2). Incubate at 37°C for at least one hour.

4.1.2. Prepare 2mL PBMC Culture Medium for each vial of PBMCs to be thawed.

PBMC Culture Medium:

StemSpan AOF	1mL	For 2mL:	For 6mL:
IL-3 (100µg/mL)	10ng	0.2µL	0.6µL
IL-6 (100µg/mL)	100ng	2µL	6µL
SCF (100µg/mL)	300ng	6µL	18µL
TPO (100µg/mL)	300ng	6µL	18µL
Flt-3L (100µg/mL)	300ng	6µL	18µL

4.1.3. Thaw vial of PBMCs in a 37°C water bath until only a small ice pellet can be seen. Quickly transfer to a 15mL conical containing an equal volume StemSpan.

4.1.4. If desired, remove 10µL to perform a cell count.

4.1.5. Centrifuge at 400xg (RCF) for 4 minutes. Aspirate supernatant without disturbing pellet and resuspend in 2mL PBMC Culture Medium.

4.1.6. Plate cells to wells previously coated with iMatrix-511. If cell count was performed, ideal plating density is approximately 5x10^6 cells/well.

4.1.7. Place in a 37°C incubator, shaking plates left/right and up/down to ensure even seeding. Maintain without media change for 6 days.

Optional: Add 1mL PBMC Culture Medium to each well every other day. Do not remove spent media!

4.2. Nucleofection

4.2.1. Prepare three wells of a 6-well plate by adding 2mL DBPS (-/-) and 9.6µL iMatrix-511 to each well (1µL iMatrix-511/cm^2). Incubate at 37°C for at least one hour.

4.2.2. Prepare the pCXLE Plasmid/Transfection Reagent Mix.

pCXLE Plasmid/Transfection Reagent Mix:

Nucleofection Solution	82µL
Supplement Solution	18µL
pCXLE-hOCT3/4-shp53-F (1mg/mL)	2µL
pCLXE-hSK (1mg/mL)	2µL
pCLXE-hUL (1mg/mL)	2µL
pCLXE-EBNA1 (1mg/mL)	2µL

4.2.3. Prepare 6mL mTeSR Plus+Cytokine Medium for each 6-well plate.

Note: Fresh PBMC Culture Medium may be used instead of mTeSR Plus+Cytokine Medium.

mTeSR Plus+Cytokine Medium:

mTeSR Plus	1mL	For 6mL:
IL-3 (100µg/mL)	10ng	0.6µL
IL-6 (100µg/mL)	100ng	6µL
SCF (100µg/mL)	300ng	18µL
TPO (100µg/mL)	300ng	18µL
Flt-3L (100µg/mL)	300ng	18µL

4.2.4. Aspirate iMatrix-511 from the previously coated 6-well plate and add 4mL mTeSR Plus+Cytokine Medium to the first well. Add 1mL mTeSR Plus+Cytokine Medium to each of the remaining two wells.

4.2.5. Remove 6-well plate containing PBMCs and harvest cells in suspension by pipetting media across the well 2-3 times. Place in a 15mL conical.

4.2.6. If desired, remove 10µL to perform a cell count.

4.2.7. Centrifuge at 440xg (RCF) for 5 minutes.

4.2.8. Aspirate supernatant using a P1000, being careful not to disturb the pellet. Resuspend in 105µL pCXLE Plasmid/Transfection Reagent Mix and transfer to a cuvette.

4.2.9. Pulse by Nucleofector 2b, using program U-008.

4.2.10. Add approximately 800µL mTeSR Plus+Cytokine Medium from the first well containing 4mL medium to the cuvette. Using the plastic transfer pipette, transfer the entire volume from the cuvette to the first well of the 6-well plate and mix well.

4.2.11. Plate cells using a serial dilution by transferring 2mL from the first well into the second well. Mix well and transfer 1mL from the second well into the third well.

4.2.12. Place in a 37°C incubator, shaking flasks left/right and up/down to ensure even seeding. Label plate as passage 0.

4.2.13. Day 2: Add 1.5mL mTeSR Plus Medium to each well. (Do not remove spent medium!)

4.2.14. Day 4: Add 1.5mL mTeSR Plus Medium to each well. (Do not remove spent medium!)

4.2.15. Day 6: Add 1.5mL mTeSR Plus Medium to each well. (Do not remove spent medium!)

4.2.16. Day 8+: Aspirate spent medium and replace with 2mL mTeSR Plus Medium. Change media every other day until colony size is approximately 1mm in diameter.

4.3. Picking Colonies

4.3.1. Prepare a 24-well plate with 80µg/mL Matrigel. Incubate at 37°C for at least one hour.

4.3.2. Aspirate Matrigel and add 500µL mTeSR Plus Medium + 10µM ROCKi to each well.

4.3.3. Using a picking scope and a P200 set to about 50µL, remove colonies exhibiting iPSC morphology by scratching off the plate while aspirating.

Note: If colony size is very large, dissociate mechanically by pipetting in a 1.5mL microcentrifuge tube.

4.3.4. Transfer colony to the prepared 24-well plate. Seed one colony per well.

4.3.5. Repeat for all colonies exhibiting iPSC morphology. Label plate as passage 1.

Note: Maintain starting 6-well plate until picked clones are passaged to ensure backup in case of contamination or poor seeding efficiency.

4.3.6. Replace media after 24 hours, then every other day with 500µL mTeSR Plus Medium until colonies are almost building up in the center.

Note: Cells may be maintained in mTeSR Plus Medium + 10µM ROCKi for as long as deemed necessary after passaging, up to 48 hours.

4.3.7. Passage an appropriate number of clones, based on the desired final number of clones, to 6-well plate format following the iPSC Maintenance SOP. Maintain cells a minimum of five passages before freezing stocks.

Note: Manual picking of colonies with good morphology is usually required at passage 2 to reduce carryover differentiation.

Morphology examples:

Passage 0:

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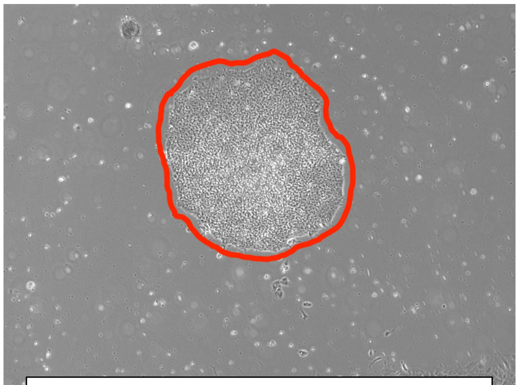
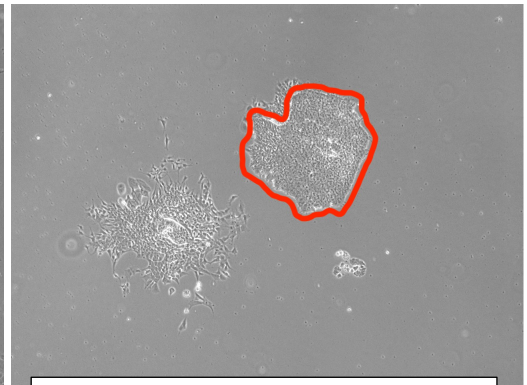
	Written/Revised By: Wendy Runyon

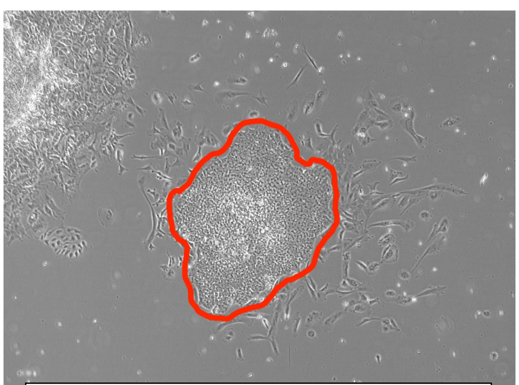


Excellent colony for picking
Good morphology, minimal surrounding differentiation

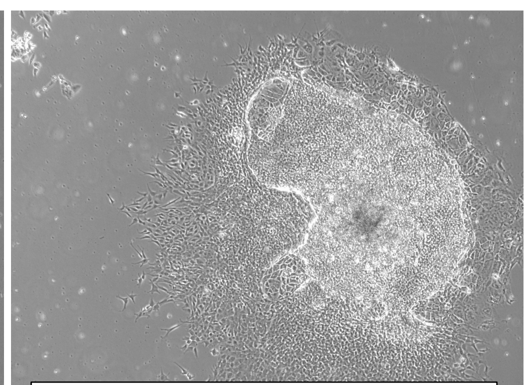


Good colony for picking
Good morphology, some differentiation nearby

Passage 0 (continued):

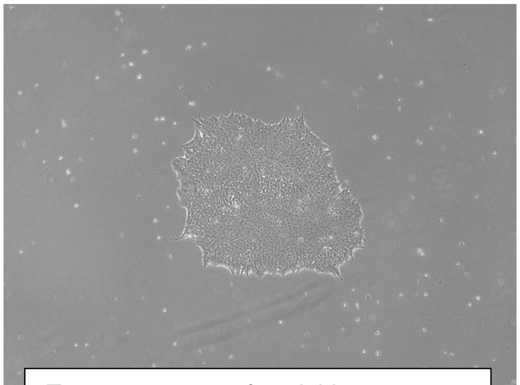


Good colony for picking
Good morphology, significant surrounding differentiation

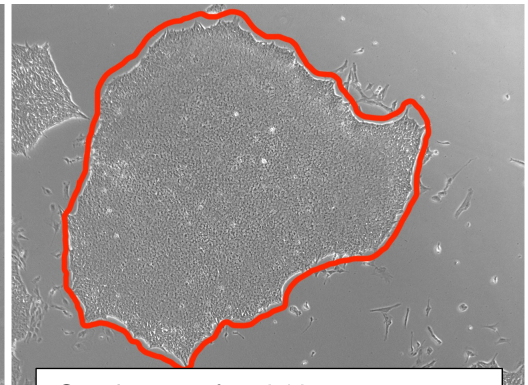


Very poor colony for picking
Poor morphology, significant surrounding differentiation

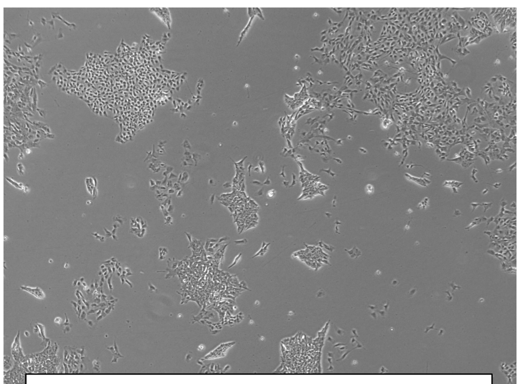
Passage 2:



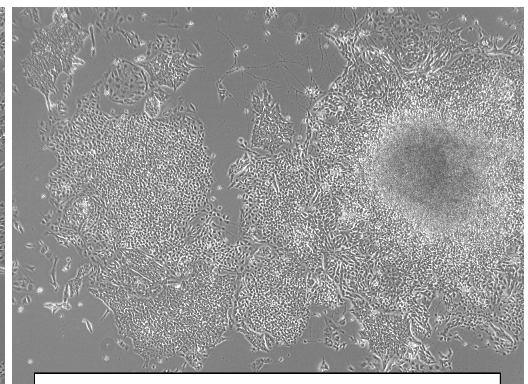
Excellent colony for picking
Good morphology, no surrounding differentiation nearby



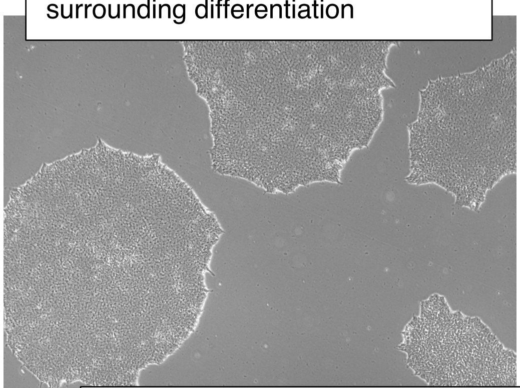
Good colony for picking
Good morphology, some differentiation nearby

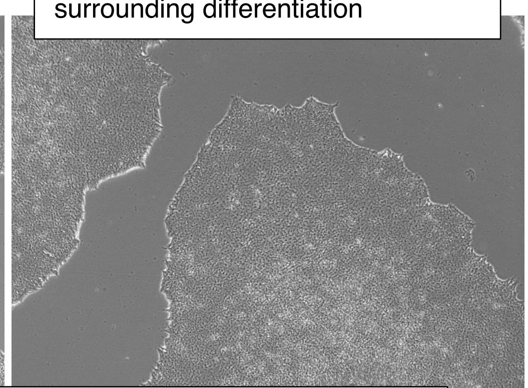


Very poor colonies for picking
iPSC colonies tiny, significant surrounding differentiation



Very poor colony for picking
Poor morphology, significant surrounding differentiation





Excellent colonies for freezing
Good morphology, no differentiation observed, cells 50-70% confluent