Title	Reprogramming Fibroblasts with Sendai
Date Submitted	04/2012
Submitted by -	Sunita D'Souza
Adapted from -	Invitrogen protocol book: Reprogramming Fibroblasts with the CytoTune™-iPS Reprogramming Kit
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Affiliation(s) -	Life Technologies and Mount Sinai School of Medicine

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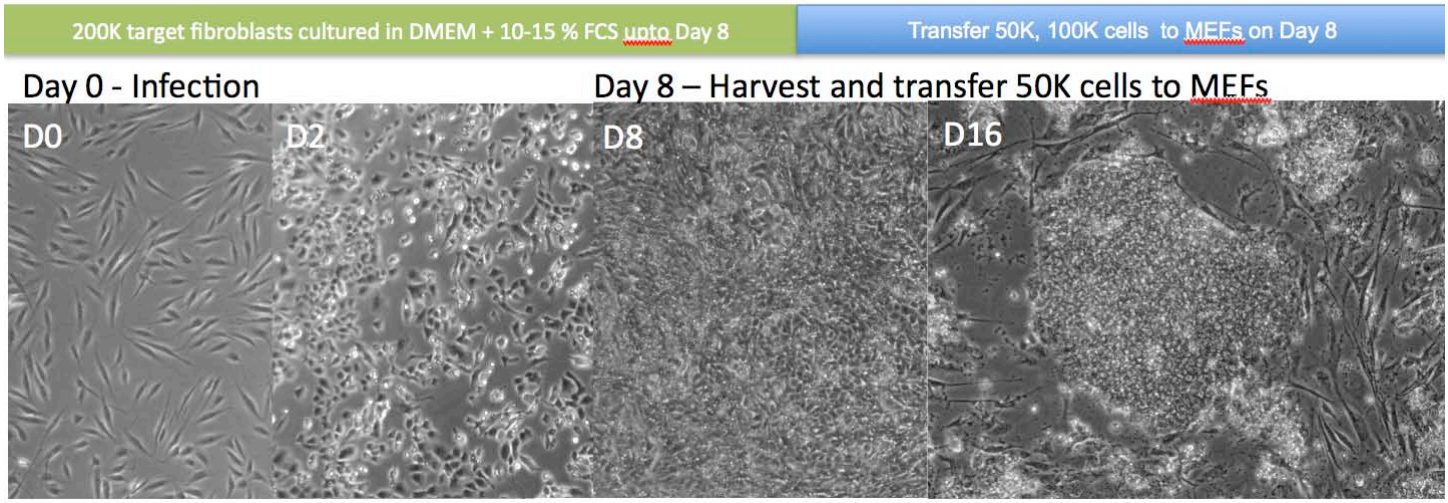
INTRODUCTION (adapted from Publication Part Number MAN0006711 – Life Technologies)

Induced pluripotent stem cells (iPSCs) are genetically reprogrammed adult cells that exhibit a pluripotent stem cell-like state similar to embryonic stem cells (Meissner *et al.*, 2007; Park *et al.*, 2008; Takahashi *et al.*, 2007; Takahashi & Yamanaka, 2006; Wernig *et al.*, 2007; Yu *et al.*, 2007). While these artificially generated cells are not known to exist in the human body, they show qualities remarkably similar to those of embryonic stem cells (ESCs); thus, they are an invaluable new source of pluripotent cells for drug discovery, cell therapy, and basic research. There are multiple methods to generate iPSCs, including retrovirus-mediated gene transduction and chemical induction. While retroviral vectors require integration into host chromosomes to express reprogramming genes, DNA based vectors, such as adenovirus, adeno-associated virus, and plasmid vectors exist episomally and do not require integration; however, they may still be integrated into host chromosomes at certain frequencies. Unlike these vectors, the CytoTune™ reprogramming vectors do not integrate into the host genome or alter the genetic information of the host cell (Fusaki *et al.*, 2009; Li *et al.*, 2000; Seki *et al.*, 2010).

CytoTune™-iPS Reprogramming System uses vectors based on replication in competent Sendai virus (SeV) to safely and effectively deliver and express key genetic factors necessary for reprogramming somatic cells into iPSCs. In contrastto many available protocols, which rely on viral vectors that integrate into the genome of the host cell, the CytoTune™ Reprogramming System uses vectors that are non-integrating and remain in the cytoplasm (i.e., they are zero-footprint). In addition, the host cell can be cleared of the vectors and reprogramming factor genes by exploiting the cytoplasmic nature of SeV and the functional temperature sensitivity mutations introduced into the key viral proteins. The CytoTune™-iPS Reprogramming Kit contains four SeV-based reprogramming vectors, each capable of expressing one of the four Yamanaka factors (i.e., Oct4, Sox2, Klf4, and c-

Myc) and are optimized for generating iPSCs from human somatic cells. The reprogramming vectors in this kit have been engineered to increase biological and environmental safety.

FLOWCHART



MATERIALS

	Reagent	Company	Cat #
1.	DMEM	Invitrogen	11965-084
2	Knockout Serum Replacement	Invitrogen	10828-028
3.	Cytotune Reprogramming Kit	Invitrogen	A1378001
4.	Penicillin/Streptomycin	Invitrogen	15070-063
5.	Fetal Calf Serum	Gemini Biologicals	100106
6.	Trypsin	Invitrogen	A-4544
7.	Collagenase B	Sigma	T-4799
8	Monothioglycerol (MTG	Sigma	M-6145
9	b-mercaptoethanol	Sigma	M3148
10	Trypsin	Invitrogen	25200-056
11	+/- Matrigel (1:1) *	BD	354230
12	DNAse I	Calbiochem	260913
13.	hbFGF	R&D Systems	233-FB
14.	anti-cardiac isoform of cTNT (clone 13-11	NeoMarkers; 1:400	MS-295-P1
15.	2-MERCAPTOETHANOL	Sigma	M3148-100
16.	MEM Non-Essential Amino Acids Solution	CellGro	11140-050
17.	Rabbit Anti-SeV Antibody	MBL International	PD029
18.	Human Nanog Goat polyclonal	R&D	AF1997
19.	Alexa 488 donkey anti goat	Invitrogen	A11055
20.	Sox-2 goat polyclonal	santa cruz	sc-17320

21.	Alexa 488 donkey anti goat	Invitrogen	A31573
22.	Purified Rabbit anti-mouse/human Oct4	Stemgent	09-0023
23.	Alexa 647 donkey anti rabbit	Invitrogen	A31573
24.	Chick anti-Sendai	Abcam	ab33988
25.	Goat anti-Chicken IgY H&L (DyLight® 488) secondary antibody	Abcam	ab96951
26	e-Myco PLUS Mycoplasma PCR Detection Kit, 48 tests	Boca Scientific	25237

MATERIAL PREPARATION

1. First confirm that the cells are mycoplasma negative using the e-Myco PLUS Mycoplasma PCR Detection Kit

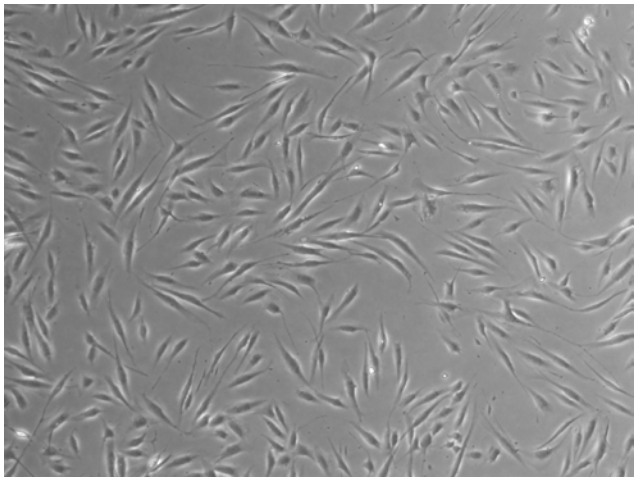
2. Prepare DMEM-15% FCS. Filter (at Day 0).

3. Prepare hESC media (at Day 8):
80% DMEM-F12 containing 2mM Glutamine and 50U/ml P/S,
20% KOSR,
1% NEAA
Beta mercaptoethanol (Use 700ul of a 1:100 dilution/ 1 liter of hESC media)
20ng of bFGF (to be added fresh)

4. Prepare 10cm MEF plate containing $1X10^6$ cells (Day 8)

PROTOCOL

D0 : Plate 200,000 target, mycoplasma-free, fibroblast cells onto one well of a 6-well plate. Labs have used three to five lines / Sendai virus vial.



D1: Take picstures of D0 fibroblasts. Remove one set of CytoTune™ Sendai tubes from the –80°C storage. Thaw each tube one at a time by first immersing the bottom of the tube in a 37°C water bath for 5–10 seconds, and then removing the tube from the water bath and allowing it to thaw at room temperature. Once thawed, briefly
centrifuge the tube and place it immediately on ice. Check certificate of analysis for # of particles.

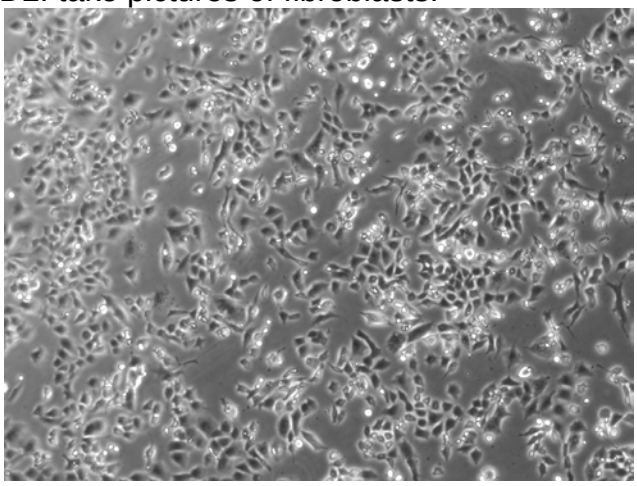
Warm up fibroblast media (DMEM, 15% FCS, 3-5ml depending on how many lines need to be infected) in a water bath. Aspirate media from fibroblast cells and add freshly warmed up media.
Add 20-30ul of EACH virus directly into fibroblast media. Swirl the 1ml around to make sure that the

virus is well distributed. Discard all virus tips into a bleach solution.

Incubate at 37OC for 24 hours .

Remove media for the well. Discard media after addition of bleach. Add 2ml of fresh DMEM-15% FCS /well.

D2: take pictures of fibroblasts.



D3: Add 2ml of fresh DMEM-15% FCS /well.

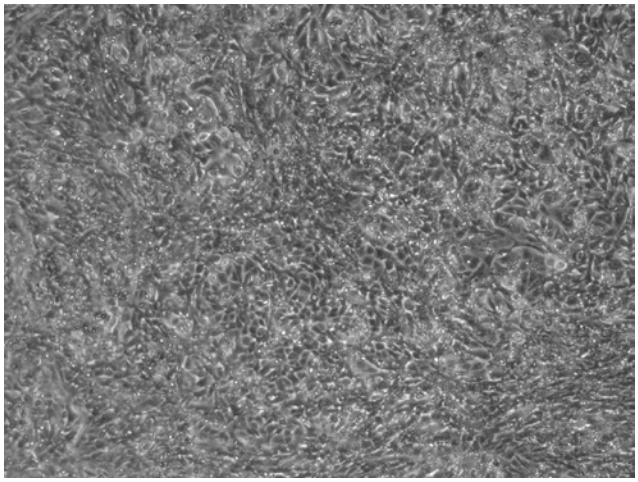
D4: Look at cells

D5: Add 2ml of fresh DMEM-15% FCS /well. Take pictures.

D6: Depending on confluency of cells, media may have to be changed every day from D5 onwards. Add 2ml of fresh DMEM-15% FCS /well. Take pictures.

D7: Add 2ml of fresh DMEM-15% FCS /well. Take pictures. Prepare atleast two ‘10cm plates of mefs (1million mefs/plate) / fibroblast line.

D8: take pictures of cells. Harvest cells. Count cells in the presence of trypan blue or eosin. Cells should have undergone a 7-10-fold expansion. Plate 50,000 cells on one 10cm mef dish in 8ml of fresh DMEM-FCS media. Plate 100,000 cells on the other 10cm mef dish in 8ml of fresh DMEM-FCS media. Invitrogen also suggests going upto 200,000 cells but in our case this resulted in “mess”. Incubate at 37 for 24 hours.



D9: Replace 50% of media with hESC media. Incubate at 37 for 24 hours.

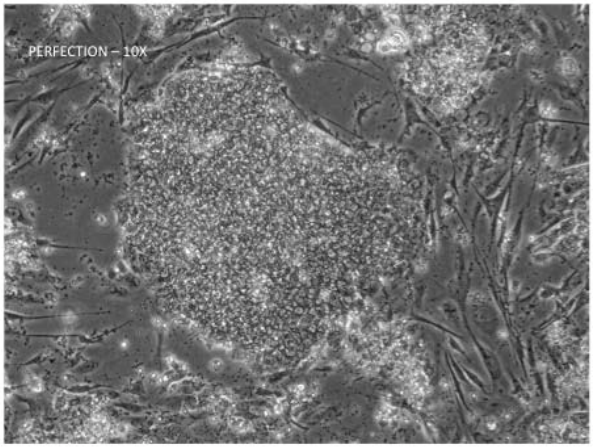
D10: Replace 100% of media with hESC media. Incubate at 37 for 24 hours.

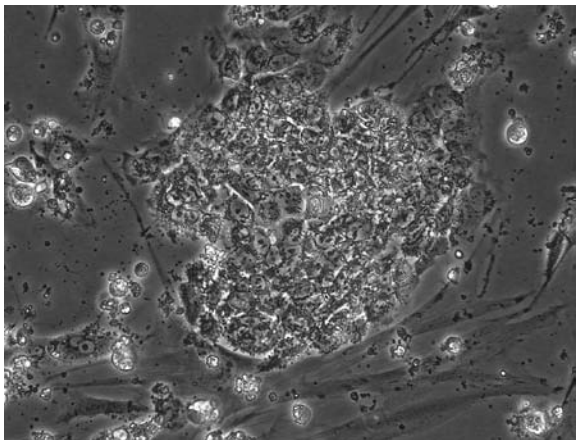
D11: Take pictures.

D12 : Replace 100% of media with hESC media. Incubate at 37 for 24 hours.

D13: -

D14: Replace 100% of media with hESC media. Incubate at 37 for 24 hours. Take pictures.





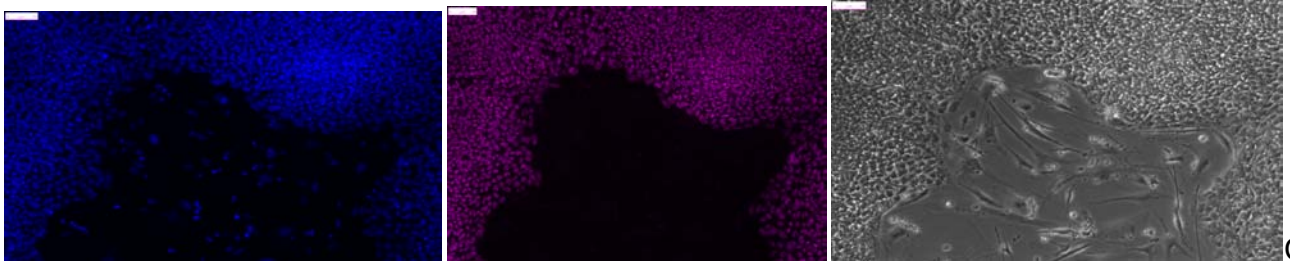
D15: Depending on confluency media may have to be changed everyday from now onwards.

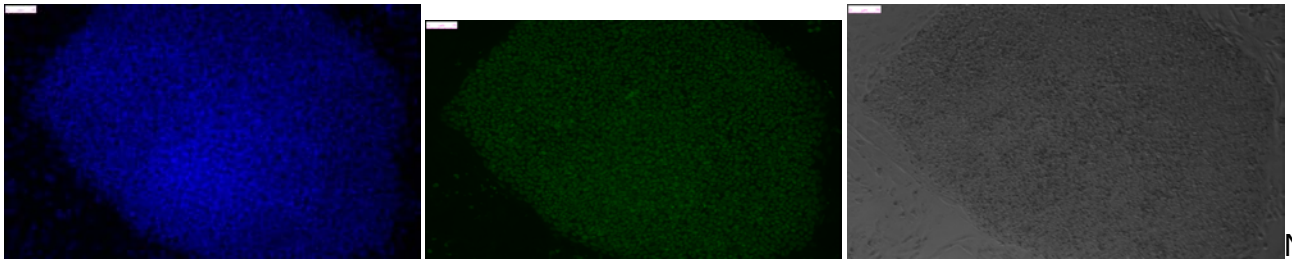
D18-D21: For the first few lines, we picked individual colonies and expanded and then after 5-6 passages, picked clones again and tested for lack of SV.

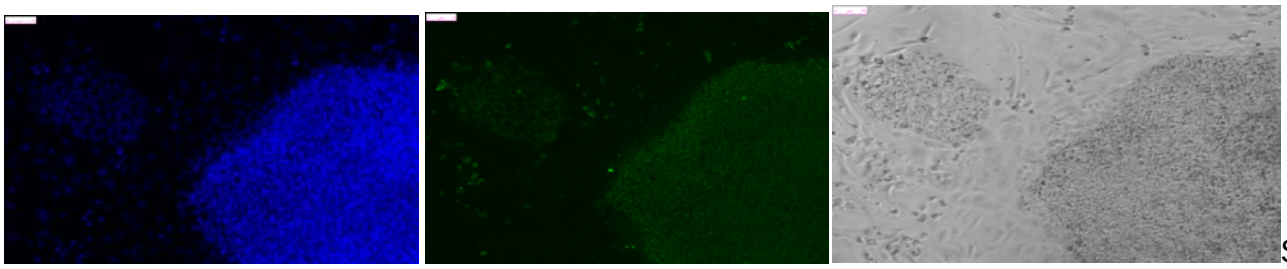
Picking method: Pick clones into 48-well plates. Wait for clones to grow and then expand gradually.

Immunohistochemistry on clones

DAPI Specific Genes Phase


OCT4


NANOG


SOX2

Antibodies used

Human Nanog Goat polyclonalAF1997 (R&D)/ Alexa 488 donkey anti goat A11055 (invitrogen)

Purified Rabbit anti-mouse/human Oct4 09-0023 (stemgent)/ Alexa 647 donkey anti rabbit A31573 (invitrogen)

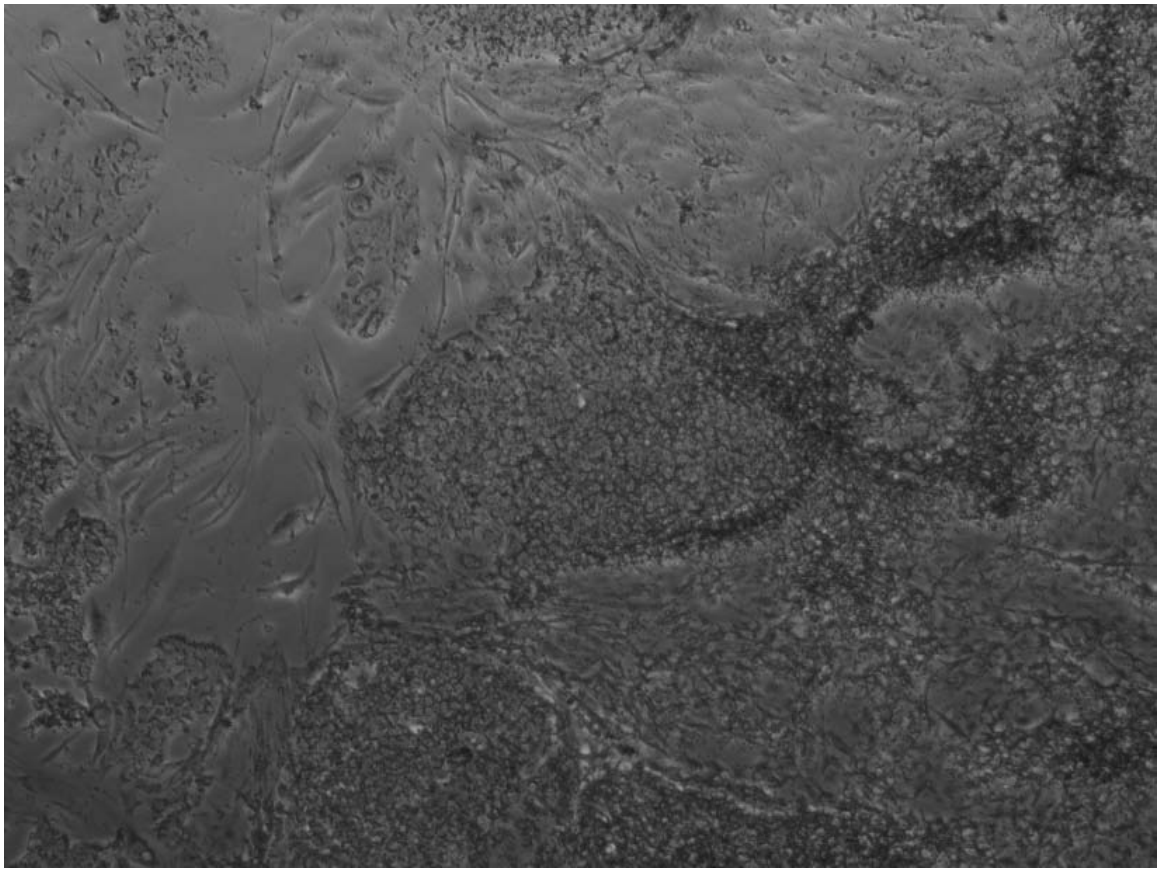
Sox-2 (Y-17) sc-17320 goat polyclonal (santa cruz),/Alexa 488 donkey anti goat A11055 (invitrogen)

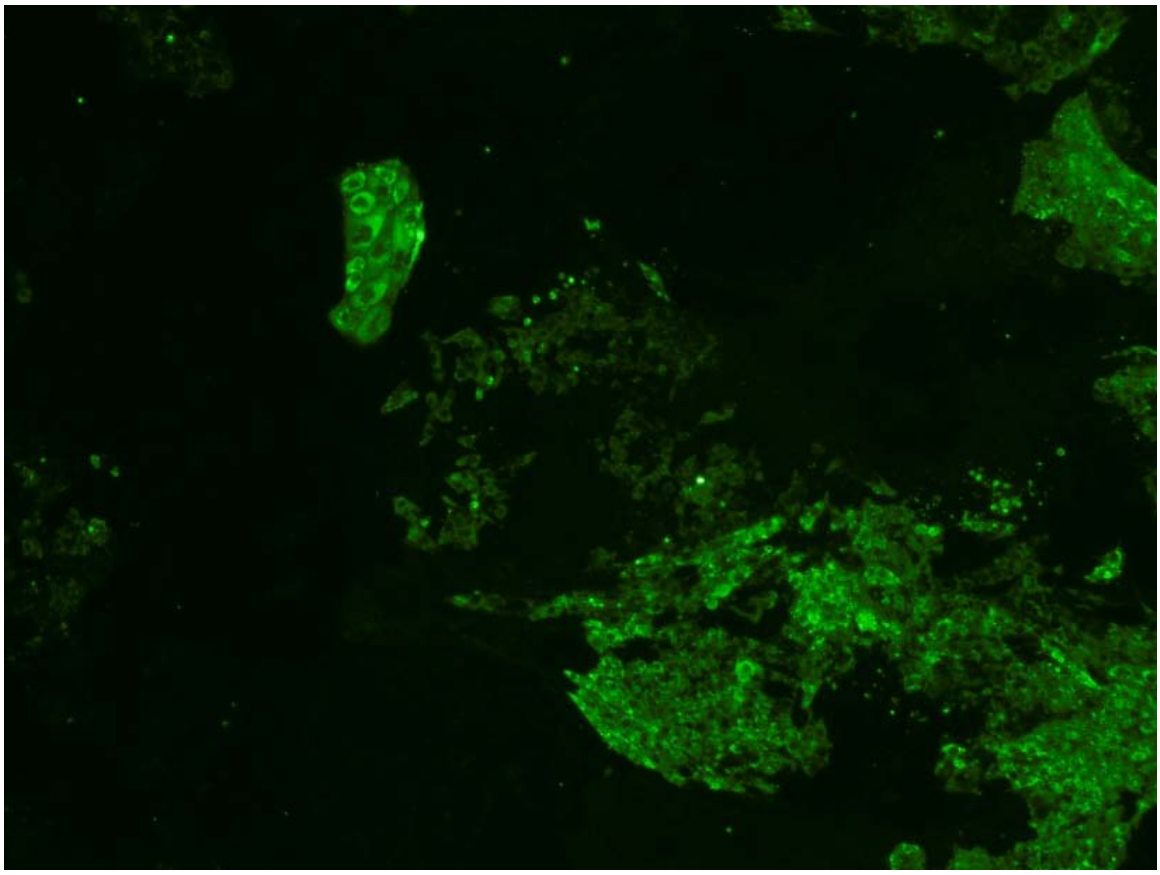
Rabbit polyclonal Ab to Lin28 ab46020 (abcam)/ Alexa 647 donkey anti rabbit A31573 (invitrogen)

Primary rabbit SV antiserum MBL PD029

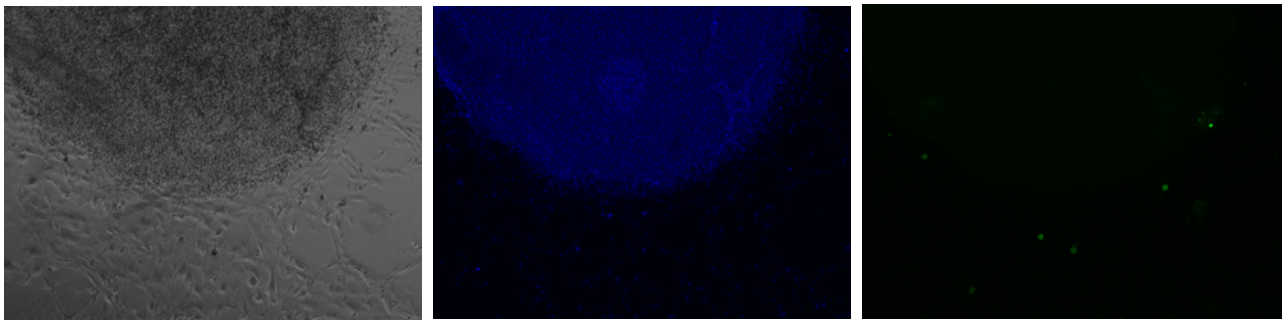
Secondary donkey anti rabbit AlexaFluor 647 (Cy5 spectral analog) A21208 (Invitrogen)

Sendai virus staining-p3 (need to overlay 2 phase and fluorescence images to see the SV-neg cells)





Sendai virus staining-p6 (need to overlay 2 phase and fluorescence images to see the SV-neg cells)



TROUBLE SHOOTING

The titer of each CytoTune™ Sendai reprogramming vector is lot-dependent. For the specific titer of your vectors, refer to the Certificate of Analysis (CoA) available on our website. Go to **www.lifetechnologies.com/cytotune** and search for the CoA by product lot number, which is printed on the vial. Viral titers can decrease dramatically with each freeze/thaw cycle. Avoid repeated freezing and thawing of your reprogramming vectors. Viral titer is not guaranteed for kits that have been refrozen or thawed.

For positive control, Invitrogen recommends performing a reprogramming experiment with human neonatal foreskin fibroblast cells (strain BJ; ATCC no. CRL2522). Note that experimental conditions may vary among target cells and need to be optimized for each cell type. The example given in the following protocol does not guarantee the generation of iPSCs for all cell types..

ACKNOWLEDGEMENTS

This protocol represents a modification of the protocol from the Publication Part Number MAN0006711 – Life Technologies. It was developed by the Core Facility at Mount Sinai School of Medicine.