Human iPS Cell Boost Supplement II

Catalog No. SCM094

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Not for use in diagnostic procedures.

USA & Canada Phone: +1(800) 437-7500 Fax: +1 (951) 676-9209
Australia +61 3 9839 2000
www.millipore.com

Introduction

EMD Millipore has developed a single lentiviral vector that enables the expression of a "stem cell cassette" (STEMCCA) comprised of all four Yamanaka reprogramming transcription factors (OKSM) from a single polycistronic transcript. STEMCCA lentivirus reprogramming kits are available in both mouse and human gene expression formats. Both human and mouse STEMCCA lentivirus kits are available in constitutive and Cre/LoxP-regulated formats which enables efficient reprogramming of normal and diseased post-natal human somatic cells [2-5].

Despite these advances, reprogramming human somatic cells remains a highly inefficient and time-consuming process. Small molecules targeting specific signaling pathways have been shown to enhance the efficiency and/or replace transcription factors involved in somatic cell reprogramming. Chemical compounds were screened and selected based upon their positive effects in increasing colony number, quality and flat 2D morphology of human iPS cells.

Product Description

EMD Millipore's Human iPS Cell Boost Supplement II contains three proprietary small molecules (TGF-β RI Kinase Inhibitor IV, Sodium Butyrate and PS48) in amounts sufficient to supplement 350 mL of human ES cell maintenance medium. When used in conjunction with the Human STEMCCA lentivirus reprogramming kits (SCR544, SCR545, and SCR548), the Human iPS Cell Boost Supplement II enhances the efficiency of human iPS colony formation by 10 – 15 fold and shortens the timescale to establish fully reprogrammed colonies by 50%. The colonies formed possess the distinctive flat 2D morphology that are reminiscent of normal human ES cell colonies and can be easily passaged. Fully reprogrammed human iPS colonies that are SSEA-4-positive, TRA-1-60-positive and Hoechst-Dim are readily established in approximately 20 – 25 days.

The Human iPS Cell Boost Supplement II, in combination with the Human STEMCCA reprogramming kits, has been validated on p6 xeno-free human foreskin fibroblasts. Other cell types have not been tested and thus similar results can not be guaranteed.

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Kit Components

Contain amounts sufficient to supplement 350 mL of normal human ES cell maintenance medium.

1. TGF-β RI Kinase Inhibitor IV Supplement (1000X): (Part No. CS210445) One (1) vial containing 400 μL of the inhibitor in high quality DMSO. Store at -20°C. Aliquot into smaller working volumes. Avoid freeze thaw.
2. Sodium Butyrate Supplement (1000X): (Part No. CS210446) One (1) vial containing 400 μL of the inhibitor in water. Store at -20°C. Aliquot into smaller working volumes. Avoid freeze thaw.
3. PS48 Supplement (1000X): (Part No. CS210447) One (1) vial containing 400 μL of the inhibitor in high quality DMSO. Store at -20°C. Aliquot into smaller working volumes. Avoid freeze thaw.

Storage and Handling

Stable for 6 months at -20°C from date of receipt. Upon first thaw, centrifuge the vial and gently mix the solution. Aliquot into smaller working volumes and freeze at -20°C. Upon addition of the small molecule components to the media, filter the supplemented media with a 0.22 μM filtration unit. Supplemented media is good for up to 2 weeks when stored at 2-8°C.

Materials Required but Not Provided

1. Retro or lenti-virus based reprogramming systems. We recommend Human STEMCCA lentivurs reprogramming kits (SCR544, SCR545, and SCR548)
2. 6-well plates, culture flasks, dishes (TC grade)
3. Cell counter / hemocytometer
4. Optional: MEF expansion medium (see page 5)
5. Human ESC Media of choice (see page 6 for KOSR-based media, mTeSR or StemPRO)
6. Recombinant Human FGF-2 (Millipore Cat. No. GF003)
7. FibroGRO™ LS Complete Medium (Millipore Cat. No. SCMF002)
8. FibroGRO™ Xeno-Free Human Foreskin Fibroblasts (Millipore Cat. No. SCC058)
9. Accumax™ Cell Detachment Solution (Millipore Cat. No. SCR006)
10. PMEF cells, growth-arrested, mitomycin-C treated (Millipore Cat. No. PMEF-CF)
11. EmbryoMax® 0.1% Gelatin Solution (Millipore Cat. No. ES-006-B)

Reprogramming Human Somatic Cells

Important note 1: The Human iPS Cell Boost Supplement is expected to work in other retro and lenti-viral based reprogramming systems, but has only been validated using the Human STEMCCA lentivirus kits (SCR544, SCR545, and SCR548). Please follow the specific manufacturer's protocol for reprogramming.

Important note 2: The following protocol has been optimized using early passage Human Foreskin Fibroblasts (Cat. No. SCC058) and the Human STEMCCA lentivirus Kit (SCR548). The following protocol should only be used as a **reference** to begin optimizing conditions that will enable the generation of iPS cells from other human target cells.

EMD Millipore's FibroGRO Xeno-Free Human Foreskin Fibroblasts tend to proliferate significantly faster than human fibroblasts obtained from other sources and as such a lower cell seeding density (1×10^4) is called for, which reduces the amount of virus required. More virus may be required to achieve an equivalent MOI in slower growing cells that also require a higher cell seeding density (i.e. 1×10^5). **Depending upon the cell type and the reprogramming system, a higher MOI may be required.**

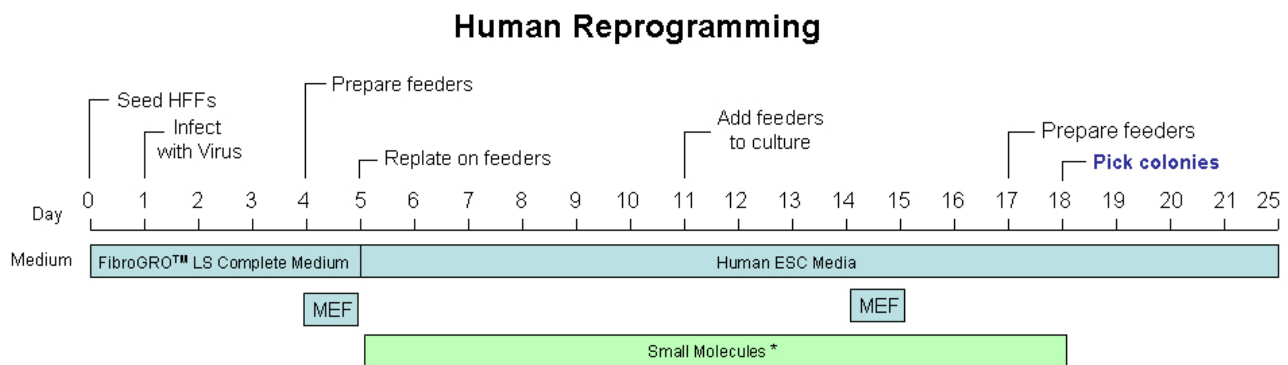

Figure 1. Time course schematic of reprogramming human somatic cells.

Day 0: Seed proliferating human somatic cells

1. Determine the plating density of target cells by plating out a range of cell numbers from 1×10^4 to 1×10^5 cells per well of a 6-well plate. Culture medium should be the same as that used to maintain the target cells in a proliferative state. Volume should be 3 mL per well of a 6-well plate. For each cell number range, a control well should be set aside for counting the number of cells on the day of transduction. **The optimal plating density is determined as the number of cells that should be plated at Day 0 in order to have the cells reach 90-95% confluency by Day 5.** The number of cells to be seeded at Day 0 will vary depending on the cell type as there are differences in cell size, morphology and rate of proliferation. For example, EMD Millipore's FibroGRO Xeno-Free Human Foreskin Fibroblasts plated at 1×10^5 cells per well on Day 0 had already reached 95% confluency by Day 3-4 instead of Day 5 and thus the initial plating density needed to be scaled back to 1×10^4 cells per well.

   If using FibroGRO Human Foreskin Fibroblasts (Cat. No. SCC058), seed 1×10^4 cells in 3 mL FibroGRO LS Complete Medium (Cat. No. SCMF002) into each well of a 6-well plate. Incubate overnight in a 37°C, 5% CO_2 incubator.

Day 1: Virus Infection

2. Before transduction, count the number of cells in one well of the 6-well plate. This cell count is used to calculate the volume of virus needed to achieve a target MOI.

3. Using the following equation, determine the volume of virus required to achieve an MOI of 20 – 50. **Please make note of the titer as it may vary slightly from lot to lot**. An MOI of 20 used to transduce 1 x 10^4 Human Foreskin Fibroblasts in the presence of Human iPS Cell Boost Supplement II will typically yield 20 – 50 human iPS cell colonies (~ 0.25% efficiency). ***Please note that an optimal MOI of 20-50 has been determined based on the Human STEMCCA lentivirus kits. If using retro or lentivirus-based reprogramming systems other than Human STEMCCA, an optimal MOI must be empirically determined.***

$$\text{Virus volume (µL) required} = \frac{\text{Number of cells seeded (from step 1)}}{\text{Virus Titer (IFU/mL)}} \times \frac{\text{Desired MOI}}{1\text{ mL}} \times 1000\text{ µL}$$

Example: If the number of cells in the well at the time of transduction is 1 x 10^4, the viral titer is 3 x 10^8 IFU/mL, and a desired MOI is 20, then the volume of virus required is:

$$\frac{1 \times 10^4 \text{ cells}}{3 \times 10^8 \text{ IFU/mL}} \times \frac{20}{1\text{ mL}} \times 1000\text{ µL} = 0.67\text{ µL virus required for 1 well of a 6-well plate}$$

4. Thaw the requisite amount of Lentiviruses at room temperature and quickly place the vial on ice after it is thawed. Quickly centrifuge the vial(s) to spin down the contents. Keep the virus on ice and proceed immediately to the next step.

5. Replace the medium from each well with 1 mL fresh FibroGRO LS Complete Medium or medium used to maintain target cells.

6. Dilute 1 µL of Polybrene transfection reagent into 9 µL of sterile distilled water to create a 1:10 dilution. Add 5 µL of the diluted Polybrene transfection reagent to each well to be transduced. Final polybrene concentration should be 5 µg/mL.

7. Add the required volume of thawed virus (from Step 4) directly to the wells containing the attached cells of interest. Gently rock the plate from side to side to thoroughly mix the virus onto the target cells. Incubate the plate overnight in a 37°C, 5% CO_2 incubator.

Day 2

8. Wash the cells 3 times with 3 mL 1 X PBS per well. Aspirate after each wash.

9. Replace with 3 mL fresh media (i.e. FibroGRO LS Complete Medium or medium used to maintain target cells) per well.

Day 3 – 4

10. Replace with 3 mL fresh media (i.e. FibroGRO LS Complete Medium or medium used to maintain target cells) per well. Monitor cell morphology daily.

Note: If using serum-free, feeder-free human expansion media system, skip steps 11 through 12b and go directly to step 12c. Please follow the manufacturer's protocol regarding ECM coating of 6-well plates (i.e. matrigel coating for mTeSR and Geltrex for StemPRO).

Day 4: Preparation of MEF feeder layer (if using feeder-based culture system)

11. Prepare inactivated Mouse Embryonic Fibroblast (MEF) feeder layers to support the cells being reprogrammed as follows.

a. Coat each well of a fresh sterile 6-well plate with 2 mL of 0.1% gelatin solution (Cat. No. ES-006-B). Incubate for 30 minutes at 37°C. Set aside until ready to receive inactivated MEFs.

b. Aspirate the 0.1% gelatin coating solution from each well before seeding the inactivated MEFs. Thaw inactivated MEFs (Cat. No. PMEF-CF). Count the number of thawed MEFs and seed 1.5×10^5 cells per well of a 6-well dish. Use normal MEF medium to culture the cells (see following Table). Total volume per well should be 3 mL. Incubate overnight in a 37°C, 5% CO_2 incubator.

Prepare 50 mL MEF Expansion Medium. Sterile filter using 0.22 µm filter.

Component	Quantity	Final Conc.	Millipore Cat. No.
DMEM High-Glucose Medium	44 mL		SLM-021-B
Fetal Bovine Serum	5.0 mL	10%	ES-009-B
L-Glutamine (200 mM)	0.5 mL	2 mM	TMS-002-C
Penicillin Streptomycin Solution (100X)	0.5 mL	1X	TMS-AB2-C

Day 5: Addition of Human iPS Cell Boost Supplement II (Cat. No. SCM094)

12. Replate virus-infected cells onto inactivated MEF feeder layer as follows.

a. Prepare 250 mL Human ESC Medium. Sterile filter using 0.22 µm filter. Set aside 100 mL to supplement with the Human iPS Cell Boost Supplement II. Store the rest at 2-8°C for up to 2 weeks.

Component	Quantity	Supplier	Cat. No.
DMEM/F12 Media	195 mL	EMD Millipore	DF-042-B
Knockout™ Serum Replacement	50 mL	Invitrogen	10828-028
Non-essential Amino Acids (100X)	2.5 mL	EMD Millipore	TMS-001-C
β-mercaptoethanol (100X)	2.5 mL	EMD Millipore	ES-007-E
bFGF	10 ng/mL final	EMD Millipore	GF003

b. To 100 mL Human ESC Media of choice (either KOSR-based (above), mTeSR or StemPRO), add 100 µL **each** of TGF-β RI Kinase Inhibitor IV Supplement (1000X) (Part No. CS210445), Sodium Butyrate Supplement (1000X) (Part No. CS210446) and PS48 Supplement (1000X) (Part No. CS210447). Sterile filter the supplemented media with a 0.22 µm filter. Store any unused medium at 2 – 8°C for up to 2 weeks.

Prepare the following supplemented media

100 mL Human ESC Media of choice (KOSR-based, mTeSR, or StemPRO)
100 µL TGF-β RI Kinase Inhibitor IV Supplement (1000X) (Part No. CS210445)
100 µL Sodium Butyrate Supplement (1000X) (Part No. CS210446)
100 µL PS48 Supplement (1000X) (Part No. CS210447)
~100.3 mL Total Volume

c. Remove the medium from the 6-well plate containing inactivated MEF feeder layer (from Step 11b). Wash once with 2-3 mL 1X PBS per well. Aspirate the PBS and replace with 3 mL of Human ESC Medium containing the Human iPS Cell Boost Supplement II per well (see above). Set plate aside until ready to receive virus-infected cells.

d. Aspirate the medium from the 6-well plate containing the virus-infected cells (from Step 10). Wash once with 3 mL of 1X PBS per well. Aspirate.

e. Add 1 mL Accumax solution to each well of the plate containing the virus-infected cells. Incubate for 8 – 10 minutes at 37°C to dissociate the cells. Inspect the plate and ensure the complete detachment of cells by gently tapping the side of the plate with the palm of your hand.

f. Add 2 mL of FibroGRO LS Complete Medium or medium used to maintain target cells.

g. Gently swirl the plate to mix the cell suspension. Using a 5 mL pipette, pipette up and down several times to dissociate into a single cell suspension. Transfer the dissociated cells to a 15 mL conical tube.

h. Centrifuge the tube at 800 rpm for 5 minutes to pellet the cells. Discard the supernatant.

i. Resuspend the cell pellet in 2 mL Human ESC Media of choice (KOSR-based, mTeSR or StemPRO) containing the Human iPS Cell Boost Supplement II (see Step 12b).

j. Count the number of cells using a hemocytometer.

k. If using feeder-based culture system, Seed virus-infected cells (from Step 12i) onto the 6-well plate containing inactivated MEFs (Step 12c). If using EMD Millipore's FibroGRO Human Foreskin Fibroblasts (Cat. No. SCC058), seed 2×10^4 virus-infected cells into each well of a 6-well plate. If using BJ human foreskin fibroblasts (ATCC; Cat. No. CRL-2522), seed 10^5 virus-infected cells into each well of the 6- well plate. Adjust the volume so that the total volume per well is 3 mL.

   If using serum-free, feeder-free culture system (mTeSR or StemPRO), remove ECM coating from the 6-well plate before seeding the virus-infected cells. If using EMD Millipore's FibroGRO Human Foreskin Fibroblasts (Cat. No. SCC058), seed 5×10^4 virus-infected cells into each well of a 6-well plate. Adjust the volume so that the total volume per well is 3 mL.

Day 6

13. Do not change the medium. Monitor cell morphology.

Day 7 – Day 11

14. Using a 5 mL pipette, carefully remove the media and replace with 3 mL fresh Human ESC Media of choice containing Human iPS Cell Boost Supplement II (see Step 12b). Use extreme care to prevent the feeder layer from peeling off if using feeder-based culture system.

15. Exchange with 3 mL per well of fresh Human ESC Media of choice containing Human iPS Cell Boost Supplement II every other day. Monitor cell growth and morphology daily. Small iPS cell colonies may start to appear around Day 9.

Day 11

16. If using feeder-based culture system: Thaw a new vial of inactivated MEFs (Cat. No. PMEF-CF). Count the number of viable cells and add 1.5×10^5 inactivated MEFs to each well of the 6-well plate containing virus-infected cells and inactivated MEFs. Add fresh inactivated MEFs every 7th day to replenish older MEFs during the reprogramming timecourse.

Day 15 – Day 20

17. Continue to monitor the growth of the human iPS cell colonies daily. Colonies should appear and are ready to be counted around Days 15-20. Do not wait too long as colonies may start to differentiate. Look for homogeneous colonies that are compact and have defined borders. When

iPS cell colonies reach approximately 200 cells or over in size, they are ready to be picked. The size of iPS cell colonies may vary, but colonies should possess a flat 2D morphology containing a monolayer of homogeneous cells. ***Note: Monitor the culture daily. Pick up those colonies that are large enough but have not undergone spontaneous differentiation or apoptosis yet.*** Some colonies may contain areas of differentiation; for these colonies, pick undifferentiated areas (i.e. characterized as monolayer of homogeneous cells) to manually passage. ***If using EMD Millipore's FibroGRO Human Foreskin Fibroblasts (Cat. No. SCC058), colonies may become large enough to be manually passaged anytime between Day 16 – Day 20.***

18. If using feeder-based culture system: One day prior to picking the iPS cell colonies, prepare a fresh 6-well plate with inactivated MEFs as described in Step 11 (Day 4).

    If using serum-free, feeder-free based culture system (mTeSR or StemPRO): One day prior to picking the iPS cell colonies, prepare a fresh ECM-coated 6- well plate following manufacturer's instructions.

19. On the day that iPS cell colonies are ready to be picked, aspirate the medium from the 6-well plate containing either inactivated MEFs (if using feeder-based culture system) or ECM coating mixture (if using mTeSR or StemPRO), plated from the day before (from Step 18). Wash the plate once with 2 mL 1X PBS. Aspirate and add in 3 mL fresh human ESC media of choice to each well. ***Note: Human iPS Cell Boost Supplement II is no longer required from this time onward.*** Set the plate in a 37°C, 5% CO_2 incubator until the manually passaged iPS are ready to be plated onto it.

20. On the day that colonies are to be picked, transfer the 6-well plate containing iPS cell colonies to a tissue culture hood containing a dissecting microscope. Using a 21 gauge needle attached to a 3 mL syringe, cut each iPS colony into 2-3 pieces depending upon the colony size. Using a p200 pipettor that has been set to 30 µL volume, transfer all the pieces from one well into a new well of a pre-equilibrated 6-well plate from step 21. Alternatively, if clonal expansion is desired, small pieces derived from a single colony can be replated onto a pre-equilibrated 4-well plate containing 2 x 10^4 inactivated MEFs (if using feeder-based culture system) or a pre-equilibrated ECM-coated 4-well plate (if using serum-free, feeder-free culture system; mTeSR or StemPRO). For a 4-well plate, use 0.5 mL final volume per well.

21. Agitate the plates **gently** from side to side and forward and backwards to ensure that iPS clumps are evenly distributed. Place the plate in 37°C, 5% CO_2 incubator for two days without any media exchanges.

22. DO NOT EXCHANGE MEDIA one day after passaging.

23. On the 2nd day after manual passaging, exchange with 3 mL fresh Human ESC Media of choice to each well of a 6-well plate. Alternatively, if using a 4-well plate, exchange with 0.5 mL fresh Human ESC Media of choice to each well.

24. Replace daily with 3 mL (for 6-well plates) or 0.5 mL (for 4-well plates) fresh Human ESC Media of choice for 5 – 7 days before the next passage. By the next passage, human iPS cells can be cultured similarly to human ES cells.

Representative Results

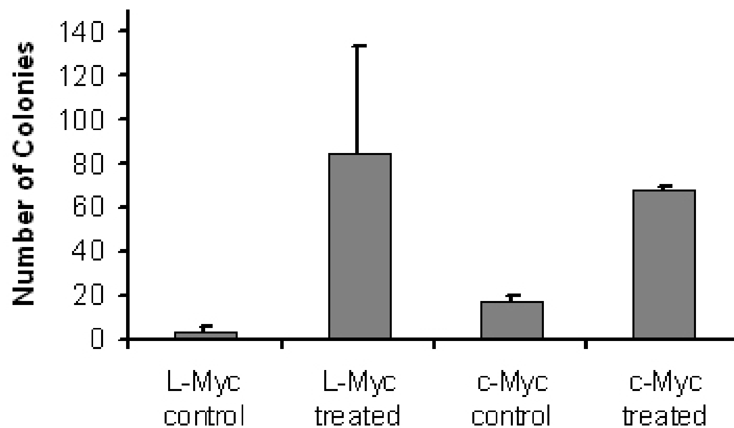

Figure 2. Human iPS Cell Boost Supplement II significantly enhanced colony formation by 28-fold and 4-fold when used in combination with EF1α-hSTEMCCA-LoxP (OKS/L-Myc) and EF1α-hSTEMCCA-LoxP (OKSM) lentivirus kits (Cat. No. SCR548 and SCR545), respectively. Results may vary between users and experimental constructs

Timecourse of human iPS colony formation

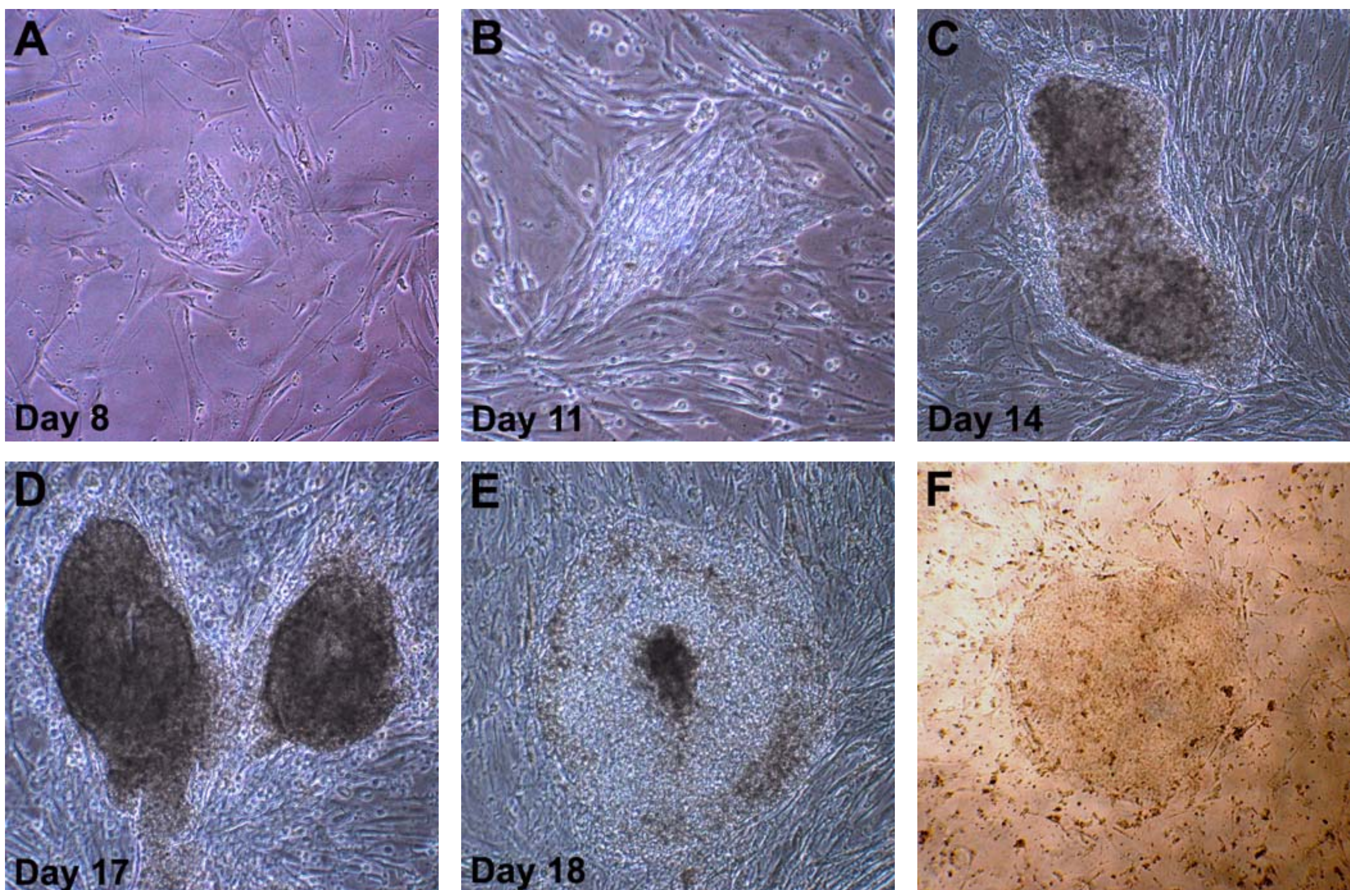

Figure 3. Timecourse of human iPS colony formation. At Day 5, virus-infected human foreskin fibroblasts were dissociated into a single cell suspension and replated at a density of 1×10^4 to 5×10^4 cells to each well of a 6-well plate containing irradiated MEFs. Morphology and approximate density of replated cells at Day 8 **(A)**. By Day 11, small iPS cell colonies are evident **(B)**. By Day 14, iPS cell colonies are more visible and different sized iPS cell colonies can be observed ranging in size from ~50 cells to several hundred cells **(C)**. Colonies that are compact, have ES-like morphology with defined borders can be selected and manually passaged around Day 15 – Day 20 **(D, E)**. In subsequent passages, human iPS colonies possessed characteristic ES-cell morphology **(F)**.

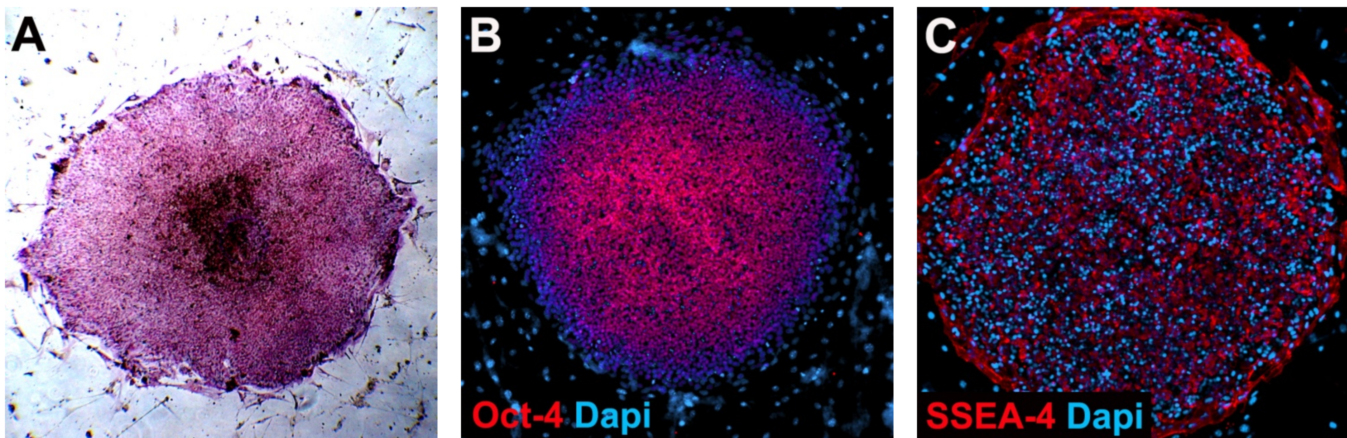

Figure 4. Passages 3 **(A)** and 4 **(B-F)** human iPS colonies generated using Human STEMCCA Cre-Excisable Constitutive Polycistronic (OKS/L-Myc) Lentivirus Reprogramming Kit (Cat. No. SCR548). Human iPS cells express human pluripotent markers including alkaline phosphatase **(A)**, Oct-4 **(B)**, SSEA-4 **(C)**, TRA-1-60 **(D)** and TRA-1-81 **(E)**. Human iPS cells do not express SSEA-1 **(F)**.

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