Human iPS Cell Boost Supplement

Catalog No. SCM088

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Not for use in diagnostic procedures.

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Introduction

The ability to “reprogram” differentiated adult cells to a state that resembles embryonic stem cells has created wide-ranging opportunities for development of relevant in vitro disease models and patient-specific cell replenishment therapies. EMD Millipore offers a single lentiviral vector that enables the expression of a “stem cell cassette” (STEMCCA) comprised of the four Yamanaka transcription factors Oct-4, Klf4, Sox-2, and c-myc (OKSM) from a single polycistronic transcript [1]. STEMCCA reprogramming kits are available in mouse or human formats and include lentivirus that expresses either mouse or human OKSM factors, respectively. Both human and mouse STEMCCA kits are available in constitutive and Cre/LoxP-regulated formats [1-3].

Even with use of a single vector, however, 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 the transcription factors required for reprogramming. EMD Millipore has screened multiple chemical compounds and selected the compounds in this kit based on their effects on enhancement of reprogramming efficiency as assessed by the number and overall quality and flat 2D morphology of human iPS colonies generated.

Product Description

EMD Millipore’s Human iPS Cell Boost Supplement contains two proprietary small molecules in amounts sufficient to supplement over 250 mL human ES/iPS cell maintenance medium. When used in conjunction with the Human STEMCCA lentivirus reprogramming kits (SCR544, SCR545), the Human iPS Cell Boost Supplement enhanced the efficiency of human iPS colony formation over ten-fold and reduced the time to establish full reprogrammed colonies by nearly 50%. The colonies formed possessed the distinctive flat 2D morphology that are reminiscent of normal human ES cell colonies and could be easily passaged. Full 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 in combination with the Human STEMCCA reprogramming kits has been validated on multiple human fibroblast cell lines including EMD Millipore’s xeno-free human foreskin fibroblasts and BJ human foreskin fibroblasts (ATCC) using either feeder-based culture system (KOSR) or serum-free, feeder-free culture systems (mTeSR and StemPRO media).

For Research Use Only; Not for use in diagnostic procedures

Kit Components

Contain sufficient amounts to supplement over 250 mL of normal human ES/iPS cell maintenance medium.

1. TGF-β RI Kinase Inhibitor VI Supplement (1000X): (Part No. CS204420) One (1) vial containing 300 µL of the inhibitor in high quality DMSO. Store at -20℃. Aliquot into smaller working volumes. Avoid freeze thaw.
2. HDAC Inhibitor-A Supplement (1000X): (Part No. CS204423) One (1) vial containing 300 µL of the inhibitor in water. Store at -20℃. Aliquo t into smaller working volumes. Avoid freeze thaw.

Storage and Handling

Components of the Human iPS Cell Boost Supplement are stable for at least 6 months from date of receipt when stored at -20℃. Upon first thaw, cen trifuge the vial and gently mix the solution. Aliquot into smaller working volumes and freeze at -20℃ or -80℃. 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.

Important Safety Note: Wear personal protective equipment when using this product. Avoid skin contact or ingestion of all chemicals used in this protocol. TGF-β RI Kinase Inhibitor VI Supplement (1000X) contains DMSO; avoid contact with eyes and skin. HDAC Inhibitor-A Supplement (1000X) is suspected of damaging fertility or the unborn child.

Materials Required but Not Provided

1. Retro- or lentivirus based reprogramming systems. We recommend Human STEMCCA lentivirus reprogramming kits (SCR544 and SCR545)
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 medium formulation)
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 lentiviral based reprogramming systems, but has only been validated using the Mouse (SCR530, SCR531) and Human (SCR544, SCR545) STEMCCA lentivirus kits. 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 Kits (SCR544 and SCR545). 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).

Mouse STEMCCA lentivirus kits, SCR530 and SCR531 contain mouse transcription factors and thus will require a higher MOI (typically MOI = 200) to reprogram human somatic cells. Human STEMCCA lentivirus kits, SCR544 and SCR545 contain human transcription factors and thus require a lower MOI (typically MOI = 20 – 50). **Depending upon the cell type and the reprogramming system, a higher MOI may be required.**

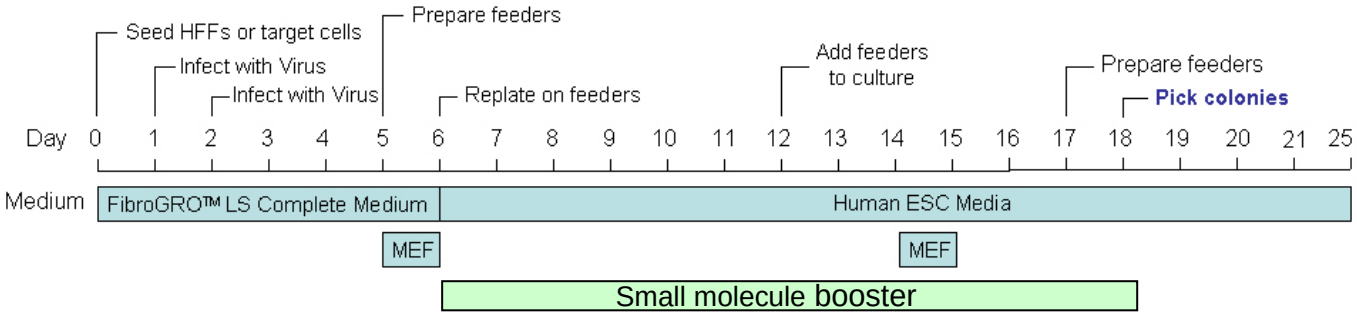

Figure 1. Time course schematic of reprogramming human somatic cells.

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 6.** 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 6 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 x 10^4 or 5 x 10^4 cells in 3 mL FibroGRO LS Complete Medium (Cat. No. SCMF002) into each well of a 6-well plate. For BJ Human Foreskin Fibroblasts (ATCC, Cat. No. CRL-2522), seed 5 x 10^4 cells in 3 mL BJ culture media according to the manufacturer's instructions into each well of a 6-well plate. Incubate overnight in a 37℃, 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 will vary slightly from lot to lot**. An MOI of 50 used to transduce 1 x 10^4 Human Foreskin Fibroblasts will typically yield 10-30 iPS cell colonies (~ 0.05-0.15% efficiency) when used in the absence of the Human iPS Cell Boost Supplement.

   Note: *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.*

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

   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 50, then the volume of virus required is:

   $$\frac{1 \times 10^4 \text{ cells}}{3 \times 10^8 \text{ IFU/mL}} \times \frac{50}{1\text{ mL}} \times 1000\ \mu L = 1.67\ \mu L \text{ virus required for 1 well of a 6-well plate}$$

4. Thaw the requisite amount of vial(s) (1 vial = 15 µL) of EF1α-hSTEMCCA (OKSM) (SCR544) or EF1α-hSTEMCCA-LoxP (OKSM) (SCR545) lentivirus 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℃, 5% CO_2 incubator.

Day 2: 2nd Virus Infection

8. Remove the medium from the infected wells.

9. Perform a 2nd virus infection by repeating Steps 4 through 7.

Day 3

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

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

Day 4 – 5

12. 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 a serum-free, feeder-free human expansion media system, skip steps 13 through 14b and go directly to step 14c. *mTeSR®1 Medium (STEMCELL Technologies) and StemPro® hESC SFM (Life Technologies) are two serum-free, feeder-free culture media that have been validated for use with the Human iPS Cell Boost Supplement. If using either of these two media, please follow the manufacturer's protocol regarding ECM coating of 6-well plates (i.e. matrigel coating for mTeSR and Geltrex for StemPro).*

Day 5: Preparation of MEF feeder layer (if using feeder-based culture system)

13. 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℃. 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 x 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℃, 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 6: Addition of Human iPS Cell Boost Supplement (Cat. No. SCM088)

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

a. Prepare 250 mL Human ESC Medium (see following table). Sterile filter using 0.22 µm filter and add FGF-2 (Cat. No. GF003) for final concentration of 10 ng/mL. Set aside 100 mL to supplement with the Human iPS Cell Boost Supplement. Store the remainder at 2-8℃ 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. Remove the medium from the 6-well plate containing inactivated MEF feeder layer (from Step 13b). Wash once with 2-3 mL 1X PBS per well. Aspirate the PBS and replace with 3 mL of Human ESC Medium containing final concentration of 10 ng/mL FGF-2 per well. Set plate aside until ready to receive virus-infected cells.

c. To 100 mL Human ESC Media of choice (KOSR-based (above), mTeSR or StemPRO), add 100 µL **each** of TGF-β RI Kinase Inhibitor VI Supplement (1000X) (Part No. CS204420) and HDAC Inhibitor-A Supplement (1000X) (Part No. CS204423). 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 VI Supplement (1000X) (Part No. CS204420)

100 µL HDAC Inhibitor-A Supplement (1000X) (Part No. CS204423)

~100.2 mL Total Volume

d. Aspirate the medium from the 6-well plate containing the virus-infected cells (from Step 12). 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℃ 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 media) containing the Human iPS Cell Boost Supplement (see Step 14c)

j. Count the number of cells using a hemocytometer.

k. If using a feeder-based culture system, remove media from the 6-well plate containing inactivated MEFs (from Step 14b) before seeding the virus-infected cells. Seed virus-infected cells (from Step 14i) onto the 6-well plate containing inactivated MEFs. If using EMD Millipore's FibroGRO Human Foreskin Fibroblasts (Cat. No. SCC058), seed 2 x 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 using media prepared in step 14c so that the total volume per well is 3 mL.

If using a serum-free, feeder-free culture system (mTeSR or StemPRO media), 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 x 10^4 virus-infected cells into each well of a 6-well plate. Adjust the volume using media prepared in step 14c so that the total volume per well is 3 mL.

Day 7

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

Day 8 – Day 12

16. 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 (see Step 14c). Use extreme care to prevent the feeder layer from peeling off if using feeder-based culture system.

17. Exchange with 3 mL per well of fresh Human ESC Media of choice containing Human iPS Cell Boost Supplement every other day. Monitor cell growth and morphology daily. Small iPS cell colonies may start to appear around Day 10-15.

Day 12

18. If using a 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 x 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 18 – Day 25

19. Continue to monitor the growth of the human iPS cell colonies daily. 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.*** 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 FibroGRO Human Foreskin Fibroblasts (Cat. No. SCC058), colonies may become large enough to be manually passaged anytime between Day 17 – Day 20.***

20. If using a 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 13 (Day 5).

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

21. 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 media), plated the day before (step 20). 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 is no longer required from this time onward.*** Set the plate in a 37℃, 5% CO_2 incubator until the manually passaged iPS are ready to be plated onto it.

22. 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 the 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). For a 4-well plate, use 0.5 mL final volume per well.

23. 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℃, 5 % CO_2 incubator for two days without any media exchanges.

24. DO NOT EXCHANGE MEDIA one day after passaging.

25. 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.

26. 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

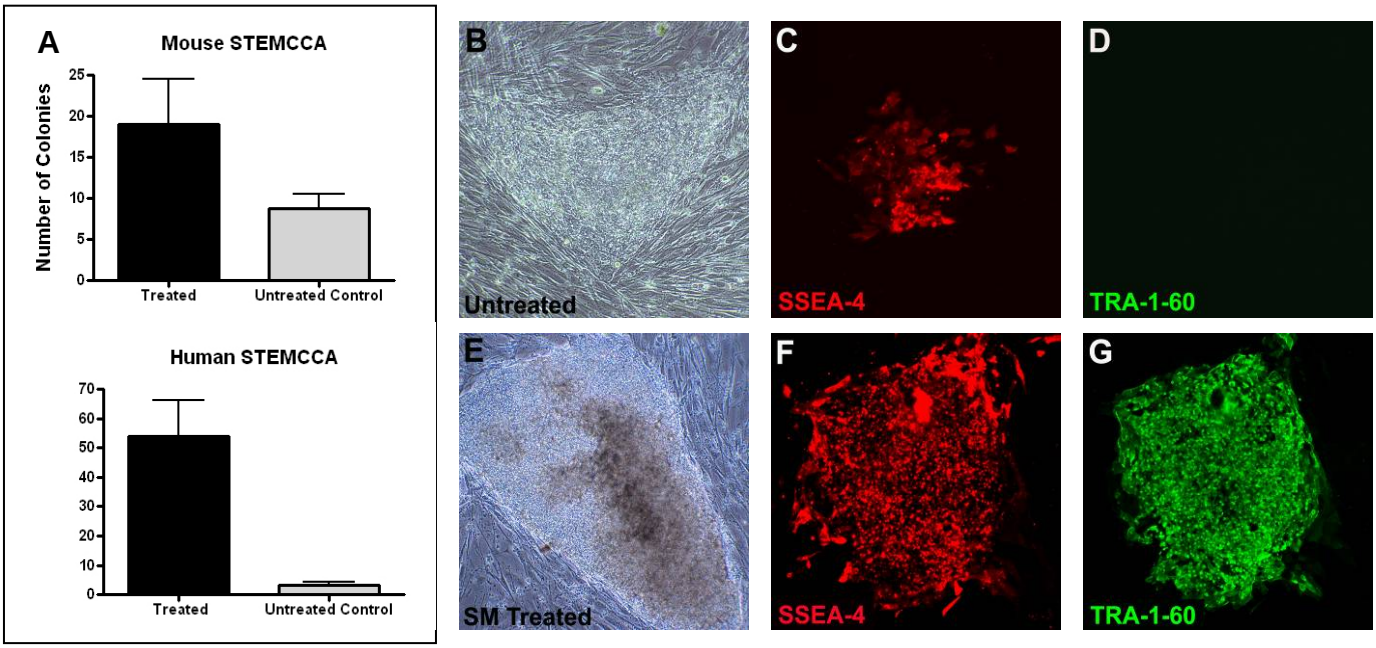

Figure 1. Addition of Human iPS Cell Boost Supplement to a polycistronic lentivirus-based reprogramming regime (STEMCCA) dramatically increased the efficiency of colony formation (**A**) and shortened the time to establishment of full reprogrammed human iPS clones (**E, F, G**). Human iPS Cell Boost Supplement enhanced colony formation by 2-3 fold when used in combination with the mouse STEMCCA lentivirus kit (SCR530, SCR531) and 15-fold when used in combination with the human STEMCCA lentivirus kits (SCR544, SCR545) (**A**). p0 human iPS colonies generated from FibroGRO Xeno-Free Human Foreskin Fibroblasts reprogrammed with mouse STEMCCA lentivirus (SCR530) in the presence of Human iPS Cell Boost Supplement exhibited larger colony sizes, a flat 2D morphology (**E**), and are SSEA-4-positive (**F**), and TRA-1-60-positive (**G**). This is in contrast to untreated control where the colonies are smaller, 3D in morphology (**B**) and are SSEA-4 positive (**C**) but TRA-1-60 negative (**D**) at p0.

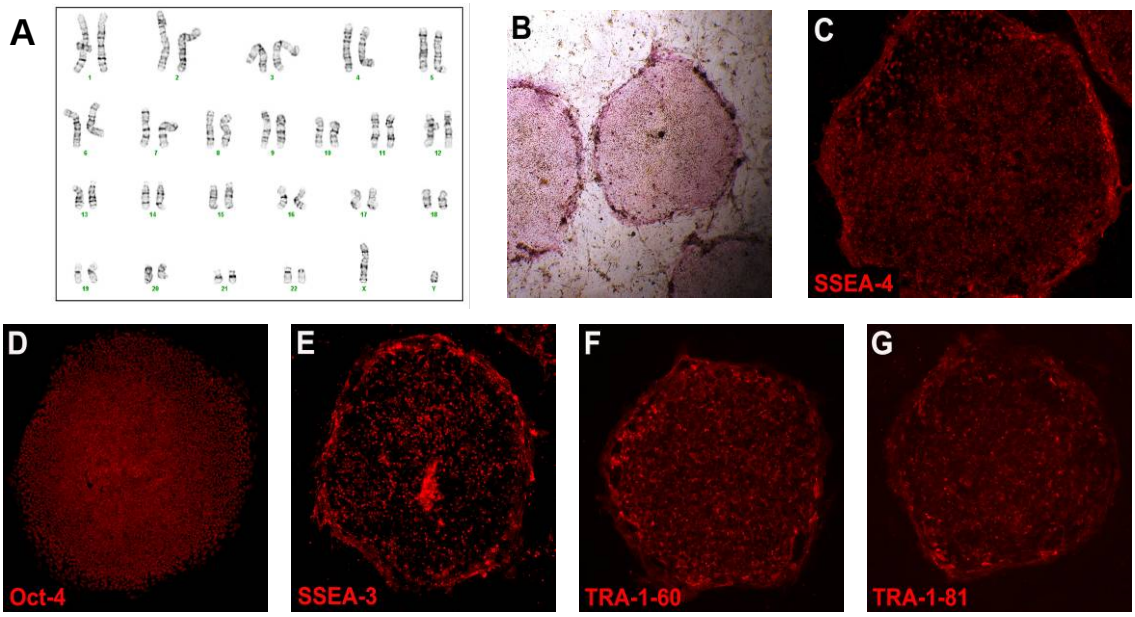

Figure 2. Human iPS cells generated using the Human STEMCCA Cre-Excisable Constitutive Polycistronic (OKSM) Lentivirus Reprogramming Kit (SCR545) in combination with Human iPS Cell Boost Supplement possessed an apparently normal karyotype (**A**) and expressed the appropriate human pluripotent markers, alkaline phosphatase (**B**), SSEA-4 (**C**), Oct-4 (**D**), SSEA-3 (**E**), TRA-1-60 (**F**), and TRA-1-81 (**G**). Cytogenic analysis was performed on twenty G-banded metaphase cells from p9 human iPS cells. All twenty cells demonstrated an apparently normal male karyotype. No abnormal cells were detected. (**A**, Cell Line Genetics)

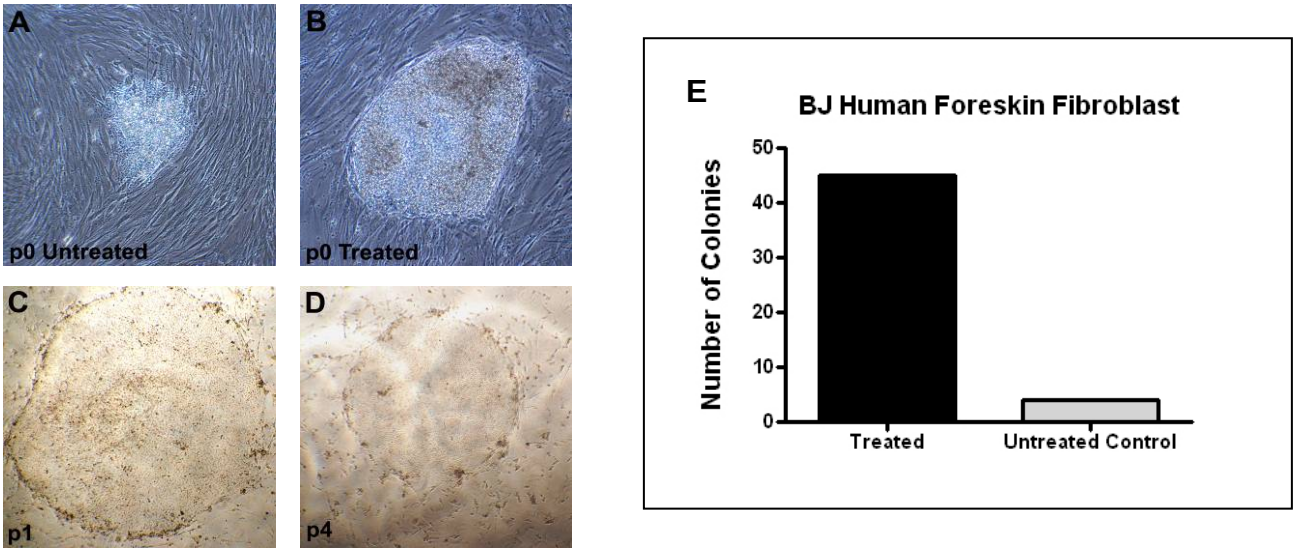

Figure 3. Human iPS colonies generated from BJ human foreskin fibroblasts (ATCC) using Mouse STEMCCA Constitutive Polycistronic (OKSM) Lentivirus Reprogramming Kit in combination with Human iPS Cell Boost Supplement exhibited a 10-fold increase in colony numbers (**E**) and displayed increased colony size (**B**) relative to untreated control (**A**). After the first passage, human iPS cell colonies exhibited similar flat 2D morphology (**C**, **D**) and similar proliferation kinetics as human ES cells (data not shown).

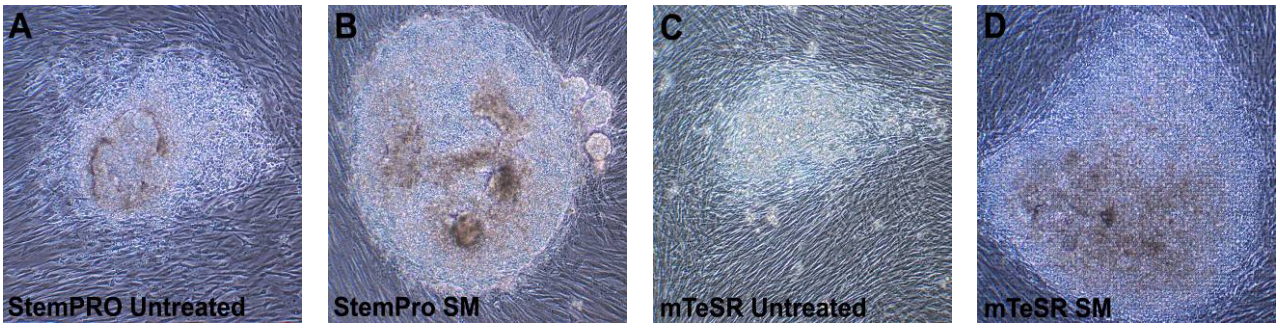

Figure 4. Human iPS Cell Boost Supplement improves reprogramming efficiency in serum-free, feeder-free based culture systems (data not shown) and increased colony size relative to untreated controls (Compare **B**, **D** to **A**, **C**). Human iPS colonies were generated using Mouse STEMCCA Constitutive Polycistronic (OKSM) Lentivirus Reprogramming Kit in the absence or presence of Human iPS Cell Boost Supplement. Cells were cultured in either Geltrex-coated plates in StemPRO medium (**A**, **B**) or Matrigel-coated plates in mTeSR medium (**C**, **D**).

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