Catalog No. SCR544

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

Introduction

Induced pluripotent stem (iPS) cells can be generated from somatic cells by the ectopic expression of the four Yamanaka transcription factors, Oct4, Klf4, Sox2, and c-Myc (OKSM). Induction of reprogramming has been achieved mostly through the co-infection of these factors in four separate expression vectors[1-5] Successful reprogrammings have required that a sufficient number of each virus deliver the four factors simultaneously to the same cell. This has raised concerns over the high number of integration sites that must arise from the random incorporation of four viruses to the genome and the difficulty in removing these viral integrations from genomic DNA. Moreover, the inability to predict whether cells receive one, two, three or all four factors has created heterogeneous cell populations, further complicating detailed study into the mechanism and timing of reprogramming.

Recently, a single lentiviral vector was generated which enabled the expression of a "stem cell cassette" or STEMCCA comprised of all four transcription factors separated by the self-cleaving 2A peptide and IRES[6] sequences. This single polycistronic cassette enabled higher efficiency of reprogramming and reduced the number of viral integrations. In some cases, iPS clones were isolated which possessed only a single viral integrant[6].

STEMCCA lentivirus reprogramming kits are provided in two formats, dependent on whether you are working with a human or mouse system. The mouse STEMCCA kits include lentivirus that express the mouse OKSM factors from a single polycistronic transcript. These kits are ideal for reprogramming mouse and other rodent somatic cells. The Human STEMCCA kits contain lentivirus that express the human OKSM factors from a single polycistronic transript and can be used for efficient reprogramming of normal or diseased post-natal human somatic cells [8-10]. Both human and mouse STEMCCA lentivirus kits are available in constitutive and Cre/LoxP-regulated formats.

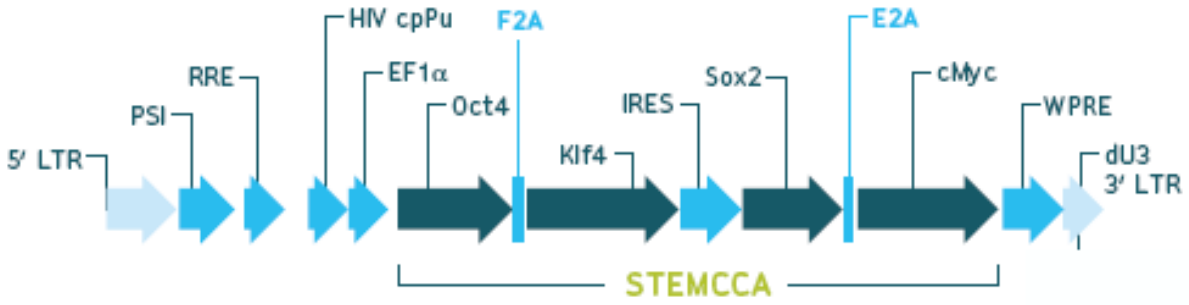

Figure 1. The Human STEMCCA vector is comprised of the humanized transcription factors Oct-4, Klf4, SOX-2, and c-Myc (OKSM), separated by the self-cleaving 2A peptide and IRES sequences driven by the EF-1alpha constitutive promoter [6-8].

Lentiviral particles were generated using the pPACKH1 Lentivector Packaging System at System Biosciences (SBI). www.systembio.com

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Product Description

EMD Millipore's Human STEMCCA Constitutive Polycistronic (OKSM) Lentivirus Kit contains high titer lentivirus that express the human Oct-4, Klf4, Sox2 and c-Myc transcription factors from a single polycistronic transcript. Also included in the kit is the Polybrene® transfection reagent. The human genes allow for more efficient reprogramming of human somatic cells with significantly less virus required (10 – 50 MOI instead of 200 MOI for mouse genes). Each virus lot is functionally validated to form human iPS cells from normal human foreskin fibroblasts (HFFs). Human iPS cells display characteristic ES cell-like morphology, express pluripotent markers and can be rapidly expanded in normal ES cell culture conditions. The use of a single lentiviral vector instead of four separate vectors for the derivation of iPS cells significantly reduces the risks of insertional mutagenesis and viral reactivation and is a step towards safer utilization of iPS technology for disease models and clinical therapies.

EMD Millipore's human STEMCCA lentivirus has been tested to confirm the generation of iPS cells from p6 human foreskin fibroblasts. Other cell types have not been tested and thus similar results can not be guaranteed.

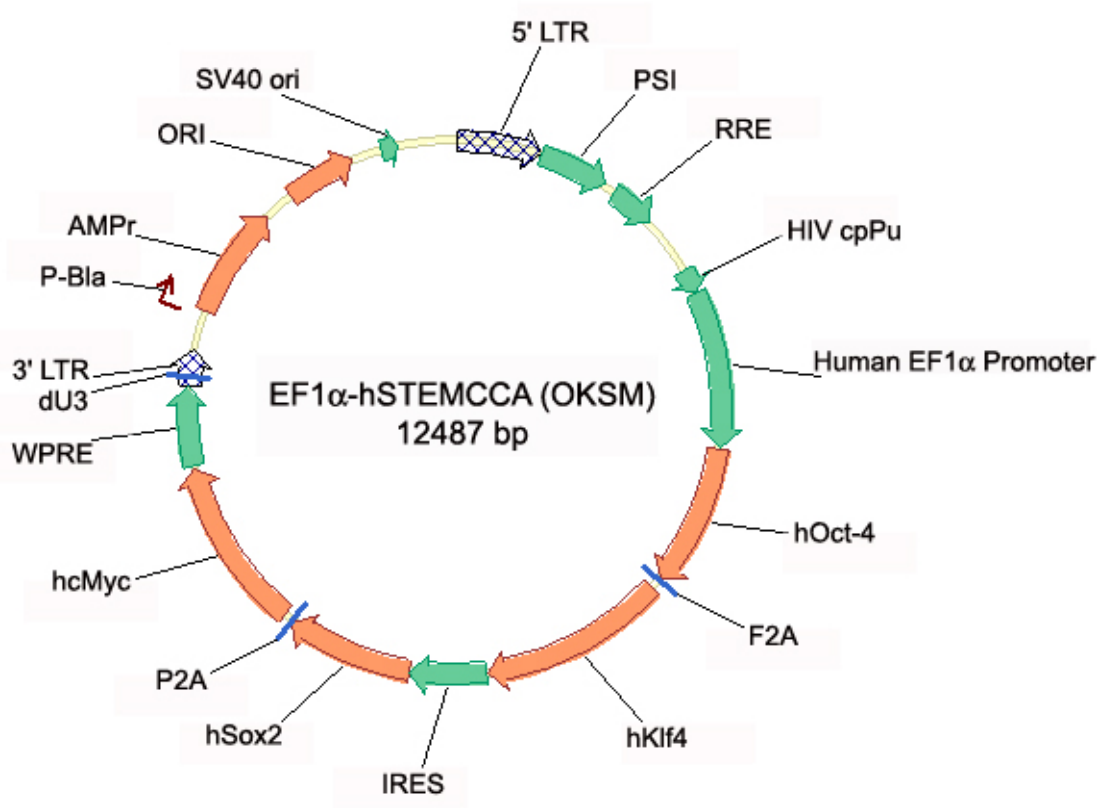

Figure 2. Schematic map of EF1α-hSTEMCCA (OKSM) lentiviral vector.

Kit Components

1. Human STEMCCA Constitutive (OKSM) Lentivirus: (Part number SCR544-1) Two (2) vials of EF1α-hSTEMCCA (OKSM) Lentivirus (Part number CS204502). Each vial contains 15 µL of high titer lentivirus. For exact titer refer to the label on the front of manual.
2. Polybrene 10 mg/mL: (Part number TR-1003-50UL) One (1) vial containing 50 µL of 10 mg/mL stock of Polybrene transfection reagent.

Storage and Handling

Lentivirus is stable for at least 6 months when stored at -80°C. After first thaw, place immediately on ice and store in working aliquots to avoid further freeze thaws. Avoid freeze thaws as this will result in a decrease in the virus titer. Polybrene transfection reagent is stable for at least 1 year when stored at -20°C.

Important Safety Note: Replication-defective lentiviral vectors are not known to cause any diseases in humans or animals. However, lentiviruses can integrate into the host cell genome and thus pose some risk of insertional mutagenesis. Material is a risk group 2 and should be handled under BSL 2 controls.

Materials Required but Not Provided

1. 6-well plates, culture flasks, dishes (TC grade)
2. Cell counter / hemocytometer
3. MEF expansion medium (see page 6)
4. Human ESC medium (see page 6)
5. Recombinant Human FGF-2 (Millipore Cat. No. GF003)
6. FibroGRO™ LS Complete Medium (Millipore Cat. No. SCMF002)
7. FibroGRO™ Xeno-Free Human Foreskin Fibroblasts (Millipore Cat. No. SCC058)
8. Accumax™ Cell Detachment Solution (Millipore Cat. No. SCR006)
9. PMEF cells, growth-arrested, mitomycin-C treated (Millipore Cat. No. PMEF-CF)
10. EmbryoMax® 0.1% Gelatin Solution (Millipore Cat. No. ES-006-B)

Reprogramming Human Somatic Cells

Important note: The following protocol has been optimized using early passage Human Foreskin Fibroblasts (Cat. No. SCC058). 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, a higher MOI may be required.**

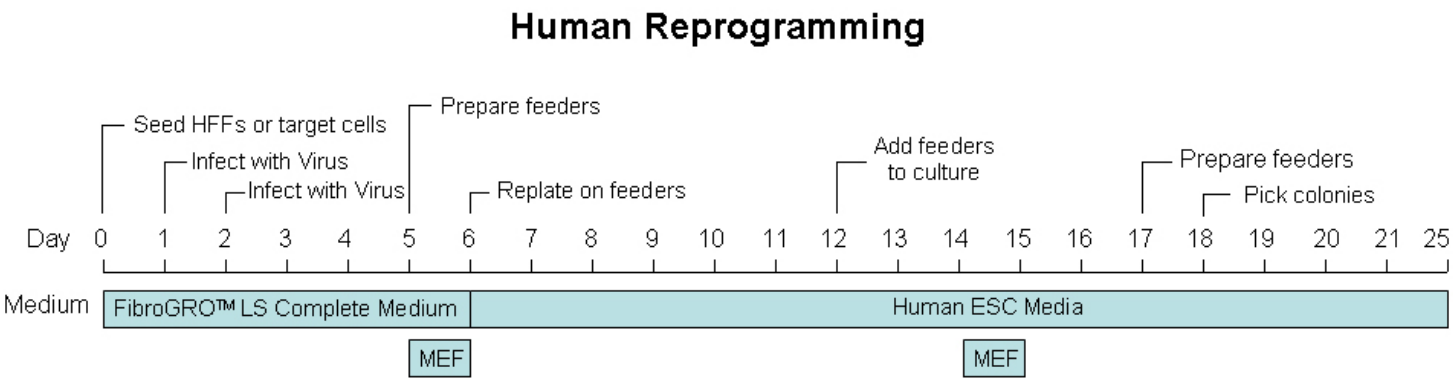

Figure 3. Time course schematic of reprogramming human somatic cells.

Day 0

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 1×10^4 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

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 50 used to transduce 1 x 10^4 Human Foreskin Fibroblasts will typically yield 5-15 iPS cell colonies (~ 0.15% efficiency).

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

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) 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°C, 5% CO_2 incubator.

Day 2

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

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

Day 3

10. Wash cells 3 times with 3 mL 1 X 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.

Day 5

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°C. Set aside until ready to receive inactivated MEFs (see next page).

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°C, 5% CO_2 incubator.

Make up 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

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

a. 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 10 ng/mL FGF-2 (Cat. No. GF003) per well. Set plate aside until ready to receive virus-infected cells.

b. Make up 250 mL Human ESC Medium. Sterile filter using 0.22 µm filter. Set aside 10 mL. Store the rest at 2-8°C for up to 2 weeks.

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

c. 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.

d. 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.

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

f. 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.

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

h. Resuspend the cell pellet in 2 mL Human ESC Medium containing 10 ng/mL FGF-2 (Cat. No. GF003).

i. Count the number of cells using a hemocytometer.

j. Seed approximately 1 x 10^4 to 5 x 10^4 of the virus-infected cells (from Step 14h) onto the 6-well plate containing inactivated MEFs (from Step 14a). Total volume per well should be 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 Medium containing 10 ng/mL FGF-2 to each well. Use extreme care to prevent the feeder layer from peeling off.
17. Exchange with 3 mL per well of fresh Human ESC Medium containing 10 ng/mL FGF-2 every other day. Monitor cell growth and morphology daily. Small iPS cell colonies may start to appear around Day 10 - 15.

Day 12

18. 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. ***Note: Monitor the culture daily. Colonies may become large enough to be manually passaged anytime between Day 18 – Day 25.***
20. 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).
21. On the day that iPS cell colonies are ready to be picked, aspirate the medium from the 6-well plate containing inactivated MEFs plated from the day before (from Step 20). Wash the plate once with 2 mL 1X PBS. Aspirate and add in 3 mL fresh Human ESC Medium containing 10 ng/mL FGF-2 to each well of inactivated MEFs. Set the plate in a 37°C, 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 a pre-equilibrated 6-well plate containing inactivated MEFs (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. 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 over the inactivated MEF feeder layer. Place the plate in 37°C, 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 Medium containing 10 ng/mL FGF-2 to each well of a 6-well plate. Alternatively, if using a 4-well plate, exchange with 0.5 mL fresh Human ESC Medium containing 10 ng/mL FGF-2 to each well.

26. Replace daily with 3 mL (for 6-well plates) or 0.5 mL (for 4-well plates) fresh Human ESC Medium containing 10 ng/mL FGF-2 for up to 10 -12 days. For the first 3 - 5 passages, colonies may require a longer length of time to grow to sufficient size to be ready for passaging. Monitor iPS colony formation every day to determine optimal time for next passage. By the 3rd to 5th passage, iPS cells can be cultured similarly to human ES cells.

Representative Results

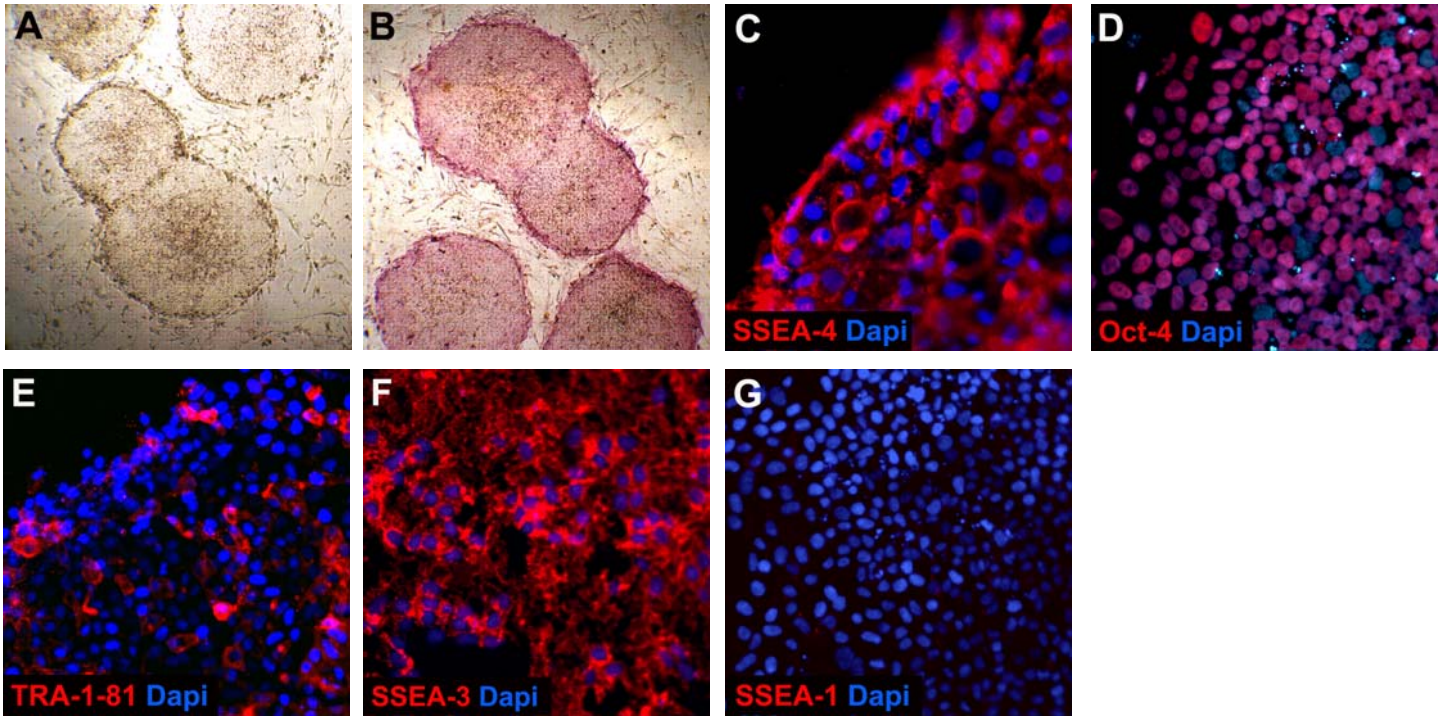

Figure 4. Passage 1 human iPS colonies generated using Human STEMCCA Constitutive Polycistronic (OKSM) Lentivirus Reprogramming Kit (Cat. No. SCR544) **(A)**. Human iPS cells express human pluripotent markers including alkaline phosphatase **(B)**, SSEA-4 **(C)**, Oct-4 **(D)**, TRA-1-81**(E)**, and SSEA-3 (**F**) Human iPS cells do not express SSEA-1 (**G**).

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