STEMCCA Constitutive Polycistronic (OKSM) Lentivirus Reprogramming Kit

Catalog No. SCR510
Catalog No. SCR530

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Not for use in diagnostic procedures.

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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[6,10] 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]. The polycistronic cassette technology has also been applied toward generating single-gene transgenic mouse strains[8-9].

Product Description

Millipore's STEMCCA Constitutive Polycistronic (OKSM) Lentivirus Kit contains high titer polycistronic (OKSM) lentivirus and Polybrene® transfection reagent that have been validated for the generation of mouse and human induced pluripotent stem cells from mouse embryonic fibroblasts (MEFs) and human foreskin fibroblasts (HFFs), respectively. Both mouse and human iPS cells display characteristic ES cell-like morphology, express pluripotent markers and can be rapidly expanded in normal ES cell culture conditions. Mouse iPS cells can be directly adapted to serum-free and feeder-free conditions and differentiated into neurons. 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.

Millipore's STEMCCA lentivirus has been tested to confirm the generation of iPS cells from p3 mouse embryonic fibroblasts and p6 human foreskin fibroblasts. Other cell types have not been tested and thus similar results can not be guaranteed.

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

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Kit Components

Catalog number SCR510:

1. EF1α-STEMCCA (OKSM) Lentivirus: (Part number CS204279) One (1) vial containing 15 µL of high titer lentivirus. For exact titer refer to the label on the front of manual.
2. Polybrene 10mg/mL: (Part number TR-1003-50UL) One (1) vial containing 50 µL of 10 mg/mL stock of Polybrene transfection reagent.

Catalog number SCR530:

1. EF1α-STEMCCA (OKSM) Lentivirus: (Part number CS204279) Three (3) vials containing 15 µL each of high titer lentivirus. For exact titer refer to the label on the front of manual.
2. Polybrene 10mg/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℃. 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℃.

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 3)
4. PMEF cells, not mitomycin-C treated (Millipore Cat. No. PMEF-CFL)
5. EmbryoMax® 0.1% Gelatin Solution (Millipore Cat. No. ES-006-B)
6. EmbryoMax Complete ES Cell Media w/15% FBS and mLIF (Millipore Cat. No. ES-101-B)
7. ESGRO Complete™ Plus Clonal Grade Medium (Millipore Cat. No. SF001-500P)
8. Accutase™ Cell Dissociation Solution (Millipore Cat. No. SCR005)
9. Human ESC medium (see page 7)
10. Recombinant Human FGF-2 (Millipore Cat. No. GF003)
11. FibroGRO™ LS Complete Medium (Millipore Cat. No. SCMF002)
12. FibroGRO™ Xeno-Free Human Foreskin Fibroblasts (Millipore Cat. No. SCC058)
13. Accumax™ Cell Detachment Solution (Millipore Cat. No. SCR006)
14. PMEF cells, growth-arrested, mitomycin-C treated (Millipore Cat. No. PMEF-CF)

Reprogramming Mouse Somatic Cells

Important note: The following protocol has been optimzed using early passage primary mouse embryo fibroblasts (MEFs) and can be used as a guide to further optimize reprogramming of other somatic cells derived from rodents.

Day 0

1. Coat a sterile 6-well plate with 0.1% gelatin solution (Cat. No. ES-006-B). Use 2 mL volume per well. Incubate for at least 30 minutes before using. Aspirate the gelatin solution just before seeding the MEFs or target rodent cells.

2. Make up 50 mL MEF Expansion Medium. Sterile filter with 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

3. Seed 1 x 10^5 actively proliferating p3 mouse embryonic fibroblasts (Cat. No. PMEF-CFL) in 3 mL MEF Expansion media into each well of a 0.1% gelatin coated 6-well plate. Incubate overnight in a 37℃, 5% CO_2 incubator. It is recommended to use early passage MEFs.

Day 1

4. Replace the media with 3 mL fresh MEF Expansion Media per well. Add 1.5 µL Polybrene transfection reagent (Part No. TR-1003-50UL) to each well that is to be infected with the virus. Final polybrene concentration should be 5 µg/mL. Set the plate aside in 37℃, 5% CO_2 incubator until ready to add the virus.

5. Using the equation provided below, determine the volume of virus required to achieve a multiplicity of infection (MOI) of at least 20. **Please make note of the titer as the viral titer may vary slightly from lot to lot.** An MOI of 20 will typically yield 5-10 mouse iPS cell colonies from p3 MEFs. Using an MOI < 20 is **not** recommended as the results are variable and may yield very few to no colonies.

$$\text{Virus volume (µL) required} = \frac{\text{Number MEFs seeded (from step 3)}}{\text{Virus Titer (IFU/mL)}} \times \frac{\text{Desired MOI}}{1\text{ mL}} \times 1000\ \mu\text{L}$$

Example: If the number of cells in the well at the time of transduction is 1 x 10^5, the viral titer is 3 x 10^8 IFU/mL, and a desired MOI is 20, then the volume of virus required is:

$$\frac{1 \times 10^5 \text{ cells}}{3 \times 10^8 \text{ IFU/mL}} \times \frac{20}{1\text{ mL}} \times 1000\ \mu\text{L} = *6.6\ \mu\text{L virus required for 1 well of a 6-well plate}$$

Note: *Use the actual viral titer located on the label on the front of the manual in the equation above to determine the actual volume of virus to add.*

6. Thaw 1 vial of EF1α-STEMCCA (OKSM) Lentivirus at room temperature and quickly place the vial on ice after it is thawed. Quickly centrifuge the vial to spin down the contents. Keep the virus on ice and proceed immediately to the next step.

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

Day 2

8. Exchange MEF Expansion Media with 3.0 mL Complete ES Cell Media with 15% FBS and LIF (Cat. No. ES-101-B).

Day 4 – Day 13

9. Exchange with 3 mL fresh Complete ES Cell Media with 15% FBS and LIF every other day for a total of 10 – 13 days. Mouse iPS cell colonies start to emerge around day 7 – 10.

10. Mouse iPS cell colonies can be selected and clonally expanded (typically around Day 12 – 14) when they reach an approximate size where the colony fits into the frame of a 10X Magnification view (please refer to Figure 1C for an example of an iPS colony that can be picked).

Expansion of mouse iPS cell colonies in serum-free, feeder-free conditions:

11. After the first passage (around day 14), mouse iPS cell colonies can be directly adapted to serum-free, feeder-free expansion medium by dissociating cells with Accutase and passaging them into a 0.1% gelatin coated plate or flask containing appropriate volume of ESGRO Complete Plus Media (Cat. No. SF-001-500P). Use 5 mL volume for T25 flasks and 6-cm plates and 10 mL volume for T75 flasks and 10-cm plates.

12. Replace with fresh ESGRO Complete Plus Media every other day and culture as normal. Please see instructions for Cat. No. SF-001-500P for detailed protocols.

Mouse iPS Cells Derived from MEF cells

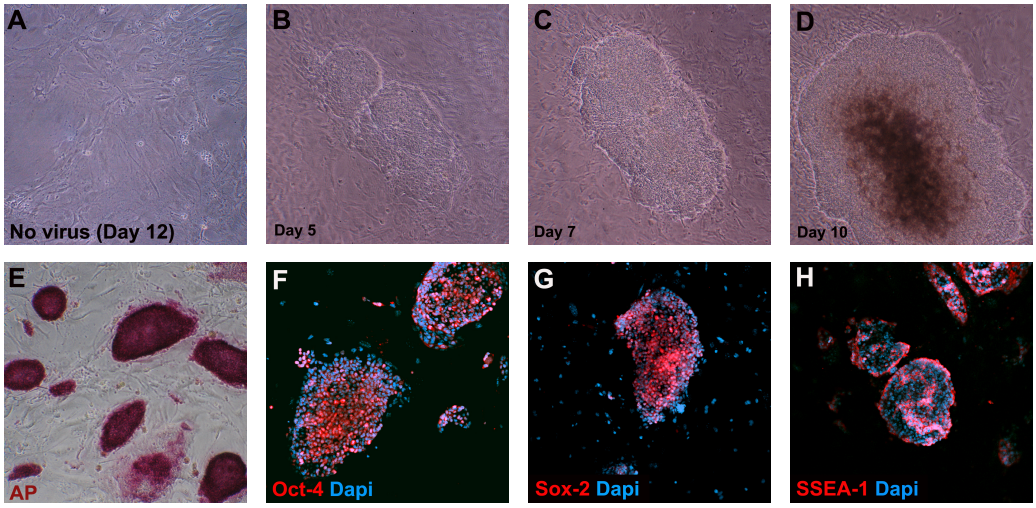

Figure 1. Mouse iPS cells derived from MEFs (p3) infected with the STEMCCA Constitutive Polycistronic (OKSM) Lentivirus have cell morphology and staining characteristics of mouse ES cells. Lentiviral infection was performed with an MOI of 20 and 5 µg/mL Polybrene reagent. Control well of MEFs that had not been infected with the STEMCCA Constitutive Polycistronic (OKSM) Lentivirus, day 12 (**A**). Non-infected MEFs remained in a monolayer culture with no ES cell-like colonies observed (**A**) while infected MEFs formed multilayered, tightly packed cells with well-defined borders (**B-D**). Phase contrast images of a single mouse iPS cell colony monitored over the course of five (**B**), seven (**C**), and ten (**D**) days after infection. Figure 1C is an example of an iPS colony that can be picked. Figure 1D is an example of a colony that has overgrown and has necrotic cells at the center. Passage 3 mouse iPS cells exhibit high alkaline phosphatase activity (**E**, Cat. No. SCR004) and expressed high levels of Oct-4 (**F**, Cat. No. MAB4419), Sox-2 (**G**, Cat. No. AB5603), and SSEA-1 (**H**, Cat. No. MAB4301). Cell nuclei were counterstained with DAPI (blue).

Reprogramming Human Somatic Cells

Important note: The following protocol has been optimized using Millipore's 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.

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.**

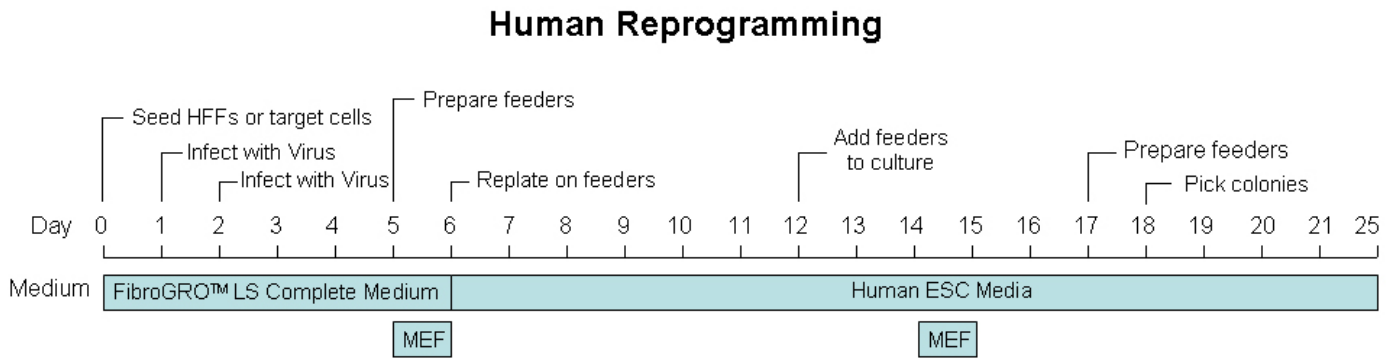

Figure 2. Time course schematic of reprogramming human somatic cells.

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 target cells in proliferative state. Volume should be 3 mL per well of 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, 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 Millipore's 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℃, 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 equation provided below, determine the volume of virus required to achieve an MOI of 75 - 200. **Please make note of the titer as it may vary slightly from lot to lot**. An MOI of 75 used to transduce 1 x 10^4 Human Foreskin Fibroblasts will typically yield 5-10 iPS cell colonies (~ 0.1% efficiency).

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

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 75, then the volume of virus required is:

$$\frac{1 \times 10^4 \text{ cells}}{3 \times 10^8 \text{ IFU/mL}} \times \frac{75}{1\text{ mL}} \times 1000\ \mu\text{L} = 2.5\ \mu\text{L virus required for 1 well of a 6-well plate}$$

4. Thaw the requisite amount of vial(s) (1 vial = 15 µL) of EF1α – STEMCCA (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℃, 5% CO_2 incubator.

Day 2

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

9. Perform a 2nd virus infection by repeating Steps 4 through 7. If the volume of virus remaining is less than that used in the initial infection, use the total amount remaining for this step.

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℃. Set aside un til 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 page 3). Total volume per well should be 3 mL. Incubate overnight in a 37°C, 5% CO_2 incubator.

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 with 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 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 (refer to Figure 3). When iPS cell colonies reach approximately 200 cells or over in size, they are ready to be picked. Please refer to Figures 4E–4H for representative images of human iPS cell colonies. Colonies such as shown in Figures 4E and 4F are not yet of sufficient size to be passaged. These colonies should be allowed to grow until they reach the approximate size of colonies exemplified in Figures 4G and 4H. ***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℃, 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. 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 3.1 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℃, 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.

Timecourse of human iPS colony formation

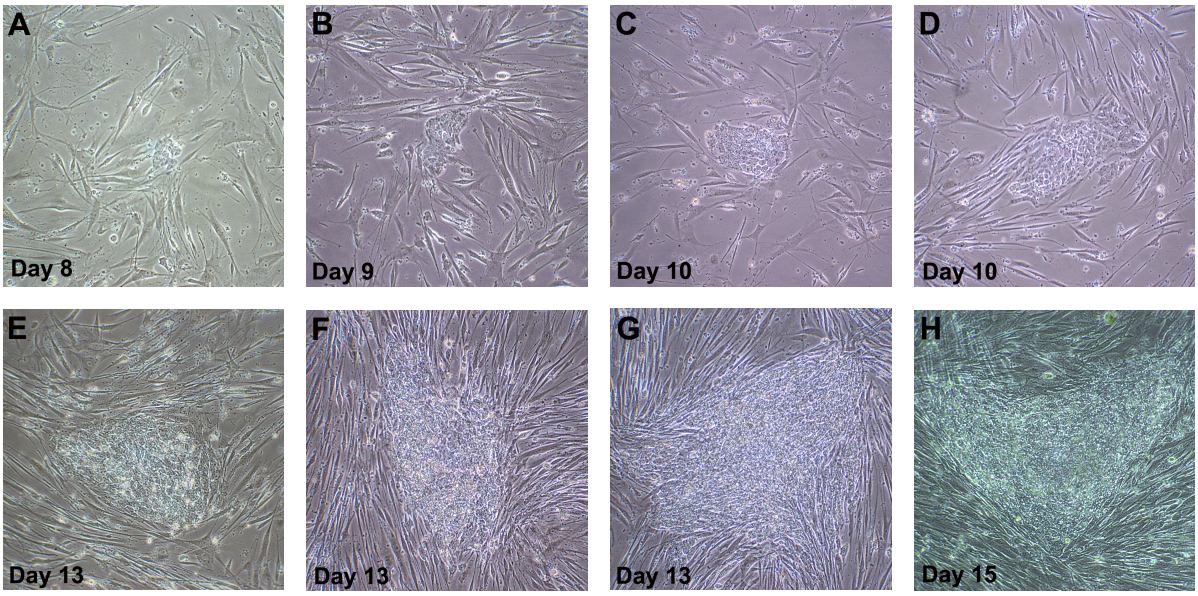

Figure 3. Timecourse of human iPS colony formation. At Day 6, virus-infected human foreskin fibroblasts were dissociated into a single cell suspension and replated at a density of 2 x 10^4 to 5 x 10^4 cells to each well of a 6-well plate containing inactivated MEFs. Morphology and approximate density of replated cells at Day 8 (A) and Day 9 (B). By Day 10, small iPS cell colonies will be evident (**C**, **D**). By Day 13, iPS cell colonies are more visible and different sized iPS cell colonies can be observed ranging in size from ~50 cells (**E)** to several hundred cells (**F**, **G**). Colonies that are compact with defined borders can be selected and manually passaged at around Day 18 – Day 25 (**H**).

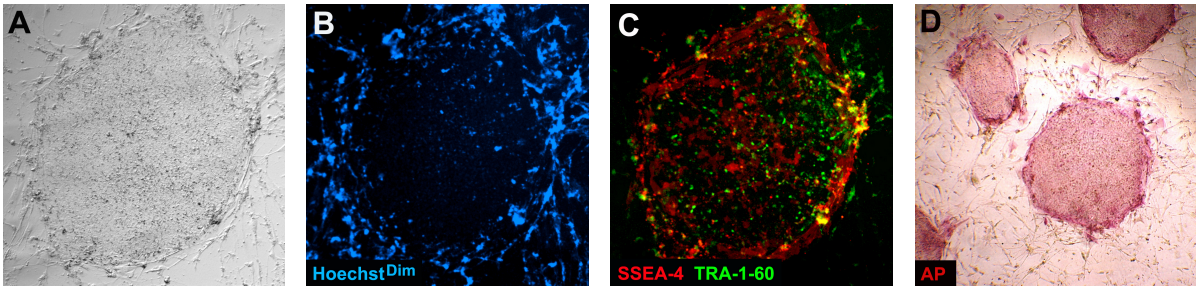

Figure 4. Live staining of p5 human iPS colonies using the Human iPS Selection Kit (Cat. No. SCR502). Fully reprogrammed human iPS cells express human pluripotent markers, TRA-1-60 FITC (**C**, green) and SSEA-4 PE (**C,** red) and exhibit Hoechst dim phenotype (see colony center in **B**) while non-iPS and differentiated cells exhibit a Hoechst bright phenotype (see the periphery of the colony in **B** which is surrounded by fibroblast cells and is Hoechst bright). Human iPS cells also express alkaline phosphatase (**D**).

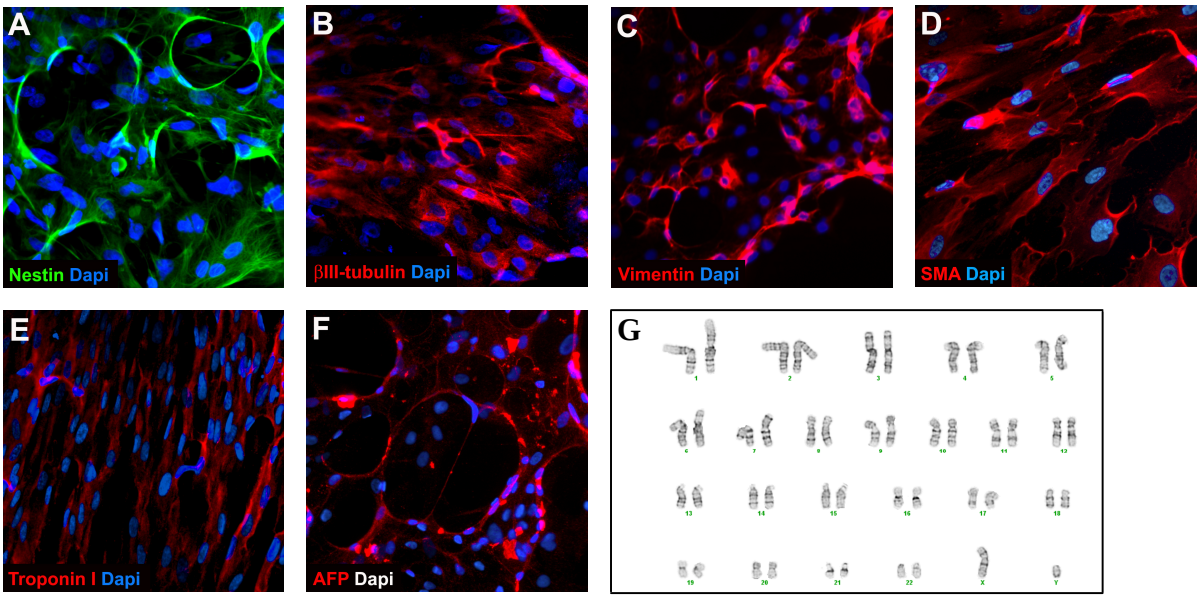

Figure 5. Human iPS cells generated using the STEMCCA Constitutive Polycistronic (OKSM) Lentivirus Reprogramming Kit possess an apparently normal karyotype (**G**) and are pluripotent. Embryoid bodies spontaneously differentiated for 13 days can give rise to cells of the 3 germ layers; neuroectoderm, Nestin (**A**, Anti-Nestin, Cat. No. AB5922), and βII-tubulin (**B**, Anti-βIII tubulin, Cat. No. MAB1637); mesoderm, Vimentin (**C**, Anti-Vimentin, Cat. No. CBL202), smooth muscle actin (**D**, Anti-SMA, Cat. No. CBL171) and Troponin I (**E**, Anti-Troponin I, Cat. No. MAB1691), and endoderm, AFP (**F**, Anti-AFP, Cat. No. 2004189). Cytogenetic analysis was performed on twenty G-banded metaphase cells from p6 human iPS cells. Seventeen cells demonstrated an apparently normal male karyotype, while three cells demonstrated non-clonal chromosome aberrations which are most likely technical artifacts (**G**, Cell Line Genetics).

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