Human STEMCCA Cre-Excisable Constitutive Polycistronic (OKS/L-Myc) Lentivirus Reprogramming Kit

Catalog No. SCR548

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Not for use in diagnostic procedures.

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Introduction

Induced pluripotent stem (iPS) cells were first generated from somatic cells by the ectopic expression of the four Yamanaka transcription factors, Oct4, Klf4, Sox2, and c-Myc (OKSM)[1]. Four factor reprogrammed mouse iPSCs closely resemble mouse embryonic stem cells (ESC) in their morphology, proliferation, and global gene expression profiles. In addition, fully reprogrammed mouse iPSCs have been shown to give rise to chimeric mice that are competent for germline transmission [2-4]. However, both the chimeras and progenies derived from mouse iPSC have an increased incidence of tumor formation, mainly due to the expression of the oncogene c-Myc [2]. Reprogramming is possible using three factors (OKS) without c-Myc; however the efficiency is extremely low and the kinetics of reprogramming is significantly delayed compared to reprogramming with four factors [5,6]. Recent studies have shown that of the three members of the Myc proto-oncogene family, L-Myc possessed significantly lower transformation activity in cultured cells than c-Myc while promoting the efficient generation of human iPS cells[7]. In mice, L-Myc promoted germline transmission, but not tumor formation[7].

EMD Millipore has developed a Cre-excisable polycistronic lentiviral vector[8-10] that allows for expression of a modified "stem cell cassette" or STEMCCA comprised of the Human OKS and L-Myc (OKS/L-Myc) transcription factors separated by the self-cleaving 2A peptide and IRES sequences. This single polycistronic cassette enabled higher efficiency of reprogramming, reduced transformation activity and enabled subsequent Cre-mediated excision of the viral reprogramming transgenes. The results are iPS cells that are virtually free of the exogenous viral transgenes. A proprietary reprogramming boost supplement (Human iPS Cell Boost Supplement II, Cat. No. SCM094) is also included. The Human iPS Cell Boost Supplement II contains three small molecules that modulate specific signaling pathways and epigenetic status to increase reprogramming efficiency. When used in combination with the Cre-Excisable Constitutive Polycistronic (OKS/L-Myc) Lentivirus, the number of iPS colonies is increased 30-fold.

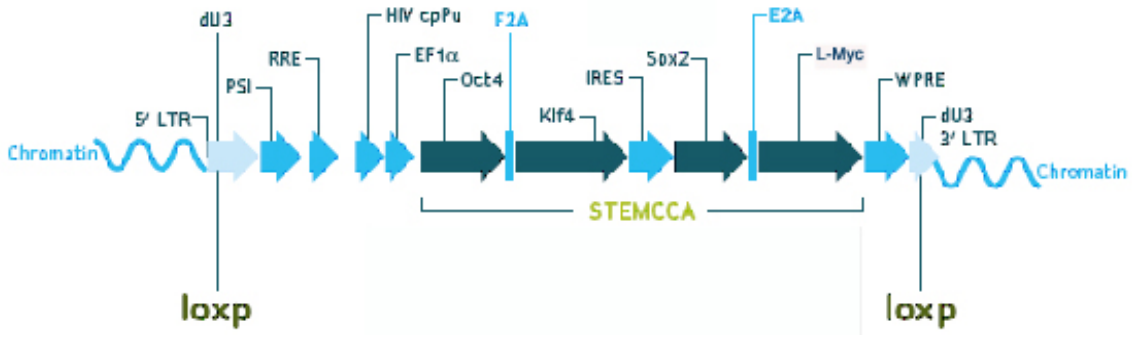

Figure 1. The Human STEMCCA vector is comprised of the humanized transcription factors Oct-4, Klf4, SOX-2, and L-Myc (OKS/L-Myc), separated by the self-cleaving 2A peptide and IRES sequences driven by the EF-1α constitutive promoter. The Cre/LoxP-regulated version has flanking LoxP sites incorporated into the vector.

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

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Product Description

EMD Millipore's Human STEMCCA Cre-Excisable Constitutive Polycistronic (OKS/L-Myc) Lentivirus Kit contains high titer Cre-excisable polycistronic (OKS/L-Myc) lentivirus, three small molecule rerogramming supplements, and Polybrene® transfection reagent. Efficient reprogramming of human somatic cells is achieved with significantly less virus required (20 MOI) and each lot of virus is functionally validated to form human iPS cells from EMD Millipore's FibroGRO Xeno-Free Human Foreskin Fibroblasts (HFFs). Human iPS cells displayed characteristic ES cell-like morphology, stained positive for alkaline phosphatase, expressed the correct human pluripotent markers (Oct4, SSEA-4, TRA-1-60 and TRA-1-81) and can be expanded in normal human ES cell culture conditions. The use of L-Myc in the context of an excisable single lentiviral vector instead of four separate vectors for the derivation of iPS cells significantly reduces the risks of transformation, 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.

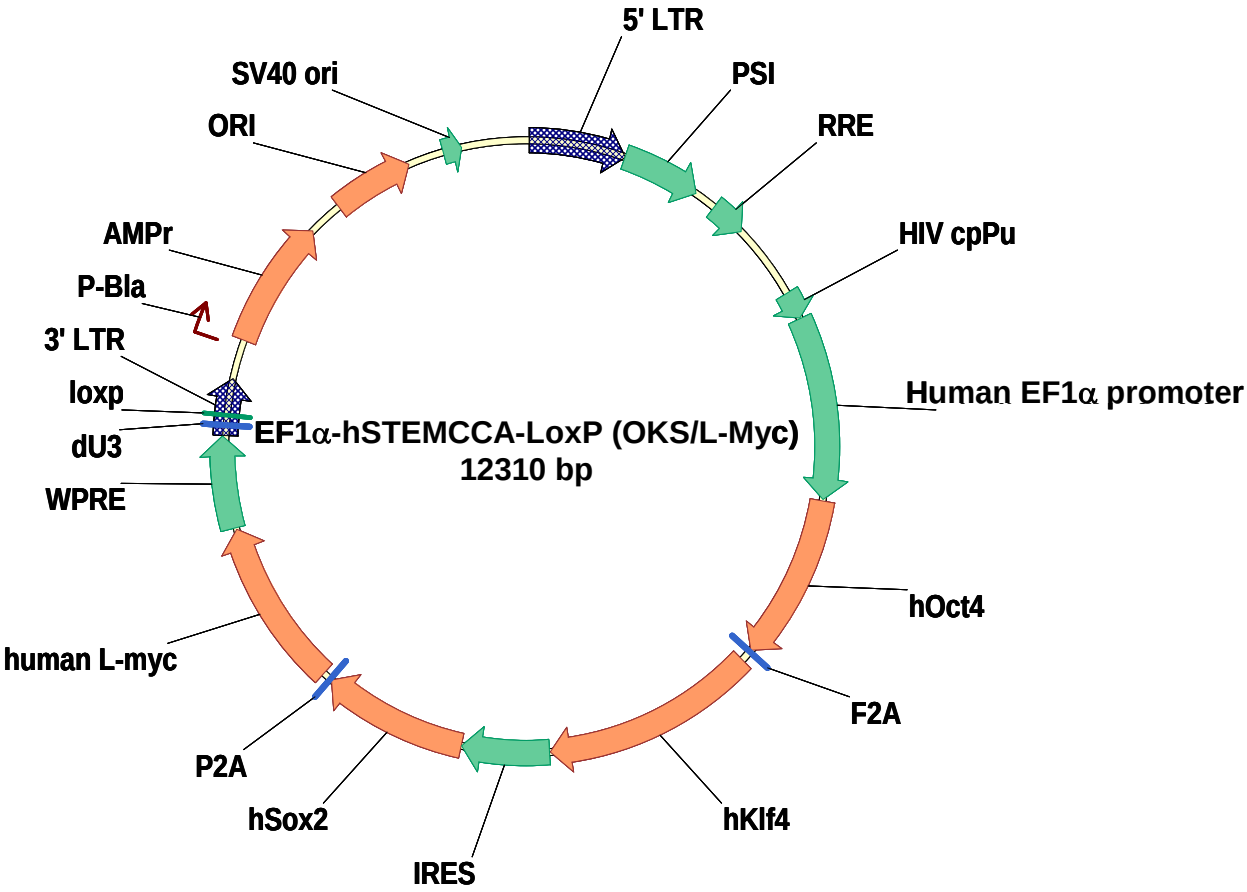

Figure 2. Schematic map of EF1α-hSTEMCCA-LoxP (OKS/L-Myc) lentiviral vector.

Kit Components

Human STEMCCA Constitutive LoxP (OKS/L-Myc) Lentivirus Kit (-20˚C):

1. Human iPS Cell Boost Supplement II (Part No. SCM094): Contains the following:

   a. 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.

   b. 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.

   c. 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.

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.

Human STEMCCA Constitutive LoxP (OKS/L-Myc) Lentivirus Kit (-80˚C):

3. EF1a-hSTEMCCA-LoxP (OKS/L-Myc) Lentivirus: (Part No. CS207826): One (1) vial containing 15 μL of high titer lentivirus. For exact titer, refer to the label on the front of the manual.

Storage and Handling

- Components of the Human iPS Cell Boost Supplement II are stable for at least 6 months when stored at -20°C. Upon first thaw, centrifuge the vial and gently mix the solution. Aliquot into smaller working volumes and freeze at -20°C or -80°C. Upon addition to cell culture media, filter the supplemented media with a 0.22 μM flitration unit. Supplemented media is good for up to two 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 IV Supplement (1000X) and PS48 Supplement (1000X) contain DMSO; avoid contact with eyes and skin.**

- Polybrene reagent is stable for at least 1 year when stored at -20°C.

- 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. **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.**

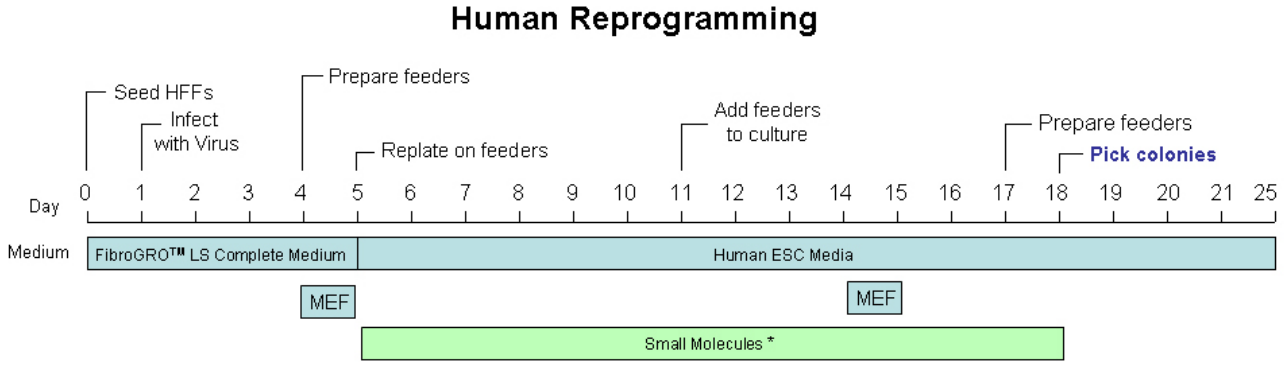

Figure 3. Time course schematic of reprogramming human somatic cells using Human STEMCCA Cre-Excisable Constitutive Polycistronic (OKS/L-Myc) Lentivirus Reprogramming Kit (Cat. No. SCR548).

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% 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 x 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 x 10^4 cells per well.

If using FibroGRO Human Foreskin Fibroblasts (Cat. No. SCC058), seed 1 x 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 20 used to transduce 1 x 10^4 Human Foreskin Fibroblasts in the presence of the Human iPS Cell Boost Supplement II will typically yield 20-50 human iPS cell colonies (~ 0.25% 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 20, then the volume of virus required is:

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

4. Thaw the requisite amount of vial(s) (1 vial = 15 µL) of EF1α – hSTEMCCA-LoxP (OKS/L-Myc) 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. 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.

Day 4

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 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 5

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

a. Make up 250 mL Human ESC Medium. Sterile filter with 0.22 µm filter. Set aside 100 mL to supplement with the Human iPS Cell Boost Supplement II (Part No. SCM094). 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
bFGF	10 ng/ml final	Millipore	GF003

b. To 100 mL Human ESC Medium, 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 Medium
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 (refer to 12b) per well. 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 Medium containing the Human iPS Cell Boost Supplement II (see Step 12b).

j. Count the number of cells using a hemocytometer.

k. Seed approximately 1 x 10^4 to 5 x 10^4 of the virus-infected cells (from Step 12i) onto the 6-well plate containing inactivated MEFs (from Step 12c). Total volume per well should be 3 mL. 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.

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 Medium containing Human iPS Cell Boost Supplement II (see Step 12b). Use extreme care to prevent the feeder layer from peeling off.

15. Exchange with 3 mL per well of fresh Human ESC Medium 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. 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 15 – Day 20

17. Continue to monitor the growth of the human iPS cell colonies daily. Colonies should appear and be 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. 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 FibroGRO Human Foreskin Fibroblasts (Cat. No. SCC058), colonies may become large enough to be manually passaged anytime between Day 16 – Day 20.**

18. 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).

19. 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 18). Wash the plate once with 2 mL 1X PBS. Aspirate and add in 3 mL fresh Human ESC Medium to each well of inactivated MEFs. **Note: Human iPS Cell Boost Supplement II is no longer required from this time onward.** Set the plate in a 37°C, 5% CO2 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 containing inactivated MEFs (from step 19). 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.

21. 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.

22. DO NOT EXCHANGE MEDIA one day after passaging.

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

24. Replace daily with 3 mL (for 6-well plates) or 0.5 mL (for 4-well plates) fresh Human ESC Medium for 5-7 days before the next passage. After colonies are picked, they can be cultured similarly to human ES cells.

Representative Results

Time-course of human iPS colony formation

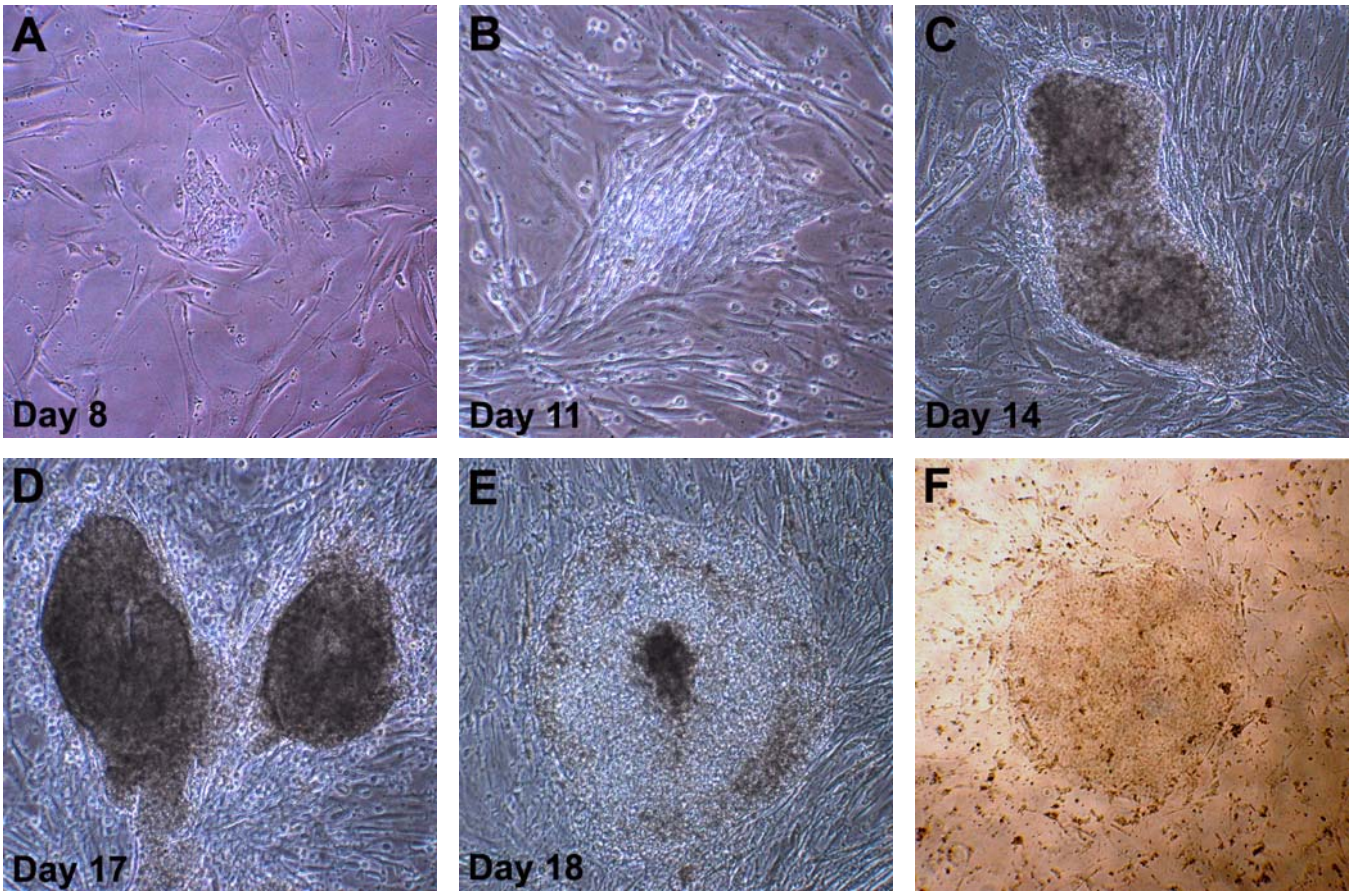

Figure 4. Time-course of human iPS colony formation. At Day 5, virus-infected human foreskin fibroblasts were dissociated into a single cell suspension and replated at a density of 1 x 10^4 to 5 x 10^4 cells to each well of a 6-well plate containing irradiated MEFs. Morphology and approximate density 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)**.

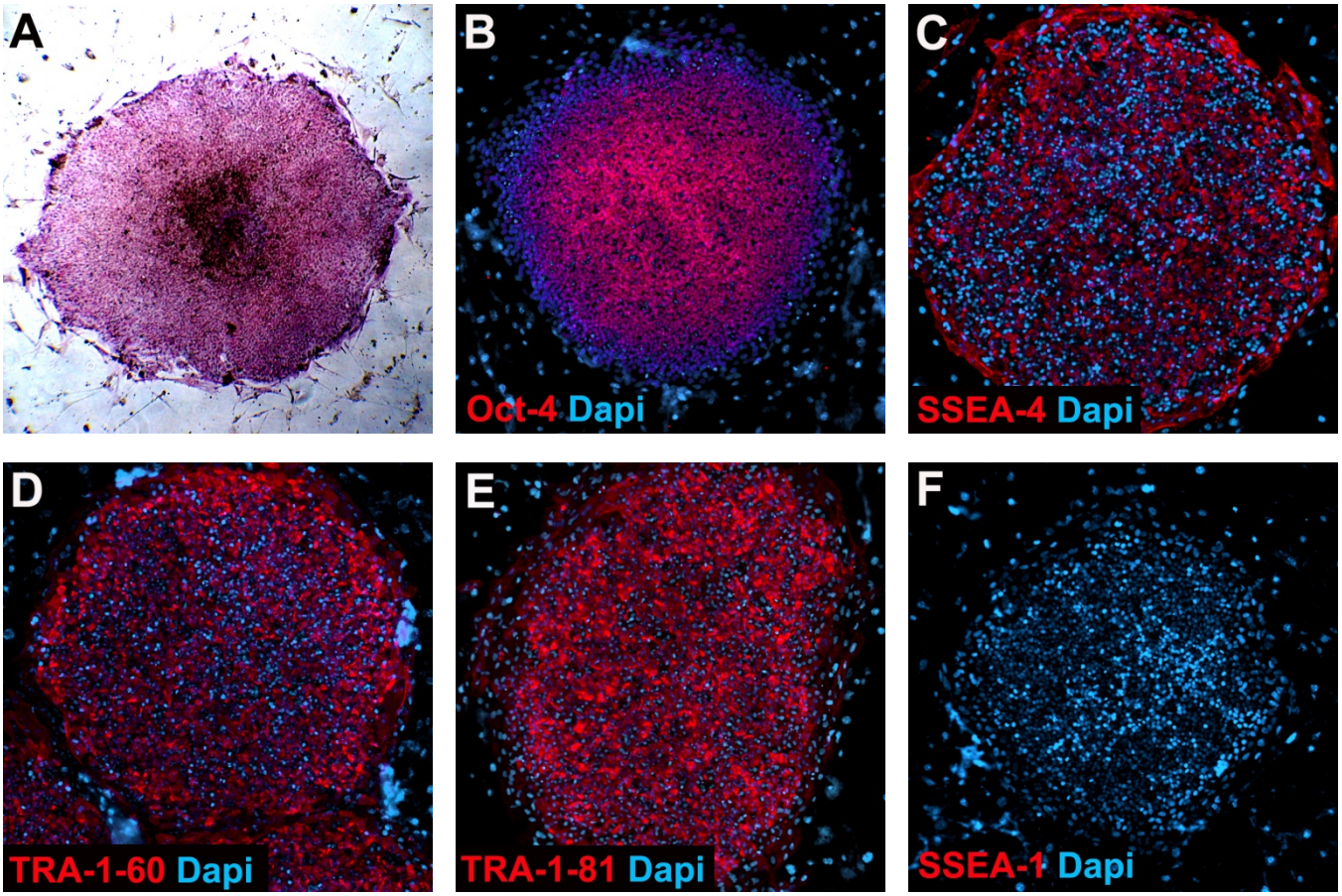

Figure 5. Passages 3 **(A)** and 4 **(B-F)** human iPS colonies generated using Human STEMCCA Cre-Excisable Constitutive Polycistronic (OKS/L-Myc) Lentivirus Reprogramming Kit (Cat. No. SCR548). Human iPS cells express human pluripotent markers including alkaline phosphatase **(A)**, Oct-4 **(B)**, SSEA-4 **(C)**, TRA-1-60 **(D)** and TRA-1-81 **(E)**. Human iPS cells do not express SSEA-1 **(F)**.

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