Mouse STEMCCA Dox-Inducible Polycistronic (OKSM) Lentivirus Reprogramming Kit

Catalog No. SCR512

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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)[1]. 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.

To address these concerns, a doxycycline (DOX)-inducible polycistronic reprogramming "stem cell cassette" or STEMCCA was generated which contains the four mouse transcription factors (OKSM) separated by the self-cleaving 2A peptide and IRES[7,11-14] sequences. The expression of the four factors are under the control of the doxycycline (DOX)-inducible tetO operator. Co-transfection with a lentivirus constitutively expressing reverse tetracycline transcriptional activator (rtTA) effectively reprogram somatic cells to an embryonic stem (ES) cell-like state when cells are cultured in the presence of DOX. This single polycistronic cassette enabled higher efficiency of reprogramming, reduced the number of viral integrations and enable subsequent shut down of the viral reprogramming transgenes upon the withdrawal of doxycycline.

EMD Millipore's Mouse STEMCCA Dox-Inducible Polycistronic (OKSM) Lentivirus Reprogramming Kit contains the following components.

- Two vials of DOX-inducible polycistronic lentivirus expressing mouse transcription factors, Oct4, Klf4, Sox2 and c-Myc.
- Two vials of lentivirus containing constitutively expressed reverse tetracycline transcriptional activator (rtTA).
- 1 vial of Polybrene transfection reagent.

Advantages to the Mouse STEMCCA Dox-Inducible Polycistronic (OKSM) system include[12]:

- Single polycistronic lentivirus cassette minimizes viral integrations. Successfully transduced cells receive all four reprogramming factors simultaneously instead of heterogeneous populations of cells containing one, two, or three factors.
- Inducible expression allows for control of the reprogramming factors to be turned on or off as necessary via addition or removal of doxycycline. The system facilitates studies involving mechanism and timing of reprogramming.
- Ability to generate 'secondary' somatic cells that contain the reprogramming factors as defined doxycycline (DOX)-inducible transgenes through the creation of chimeric mice. 'Secondary' somatic cells allows accelerated efficient production of iPS cells upon exposure to DOX, obviating the need for lentivirus infection[12].

Product Description

EMD Millipore's Mouse STEMCCA Dox-Inducible Polycistronic (OKSM) Lentivirus Reprogramming Kit contains high titer inducible polycistronic (OKSM) lentivirus, constitutive reverse tetracycline transcriptional activator (rtTA) lentivirus, and Polybrene® transfection reagent. The kit has been validated for the generation of mouse iPS cells from mouse embryonic fibroblasts (MEFs). Mouse iPS cells display characteristic ES cell-like morphology, stained positive for alkaline phosphatase, expressed the correct mouse ES cell marker phenotype (Oct-4, SSEA-1, Sox-2) and can be rapidly expanded in normal mouse ES cell culture conditions. The use of an inducible polycistronic 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 STEMCCA lentivirus has been tested to confirm the generation of iPS cells from p3 mouse embryonic 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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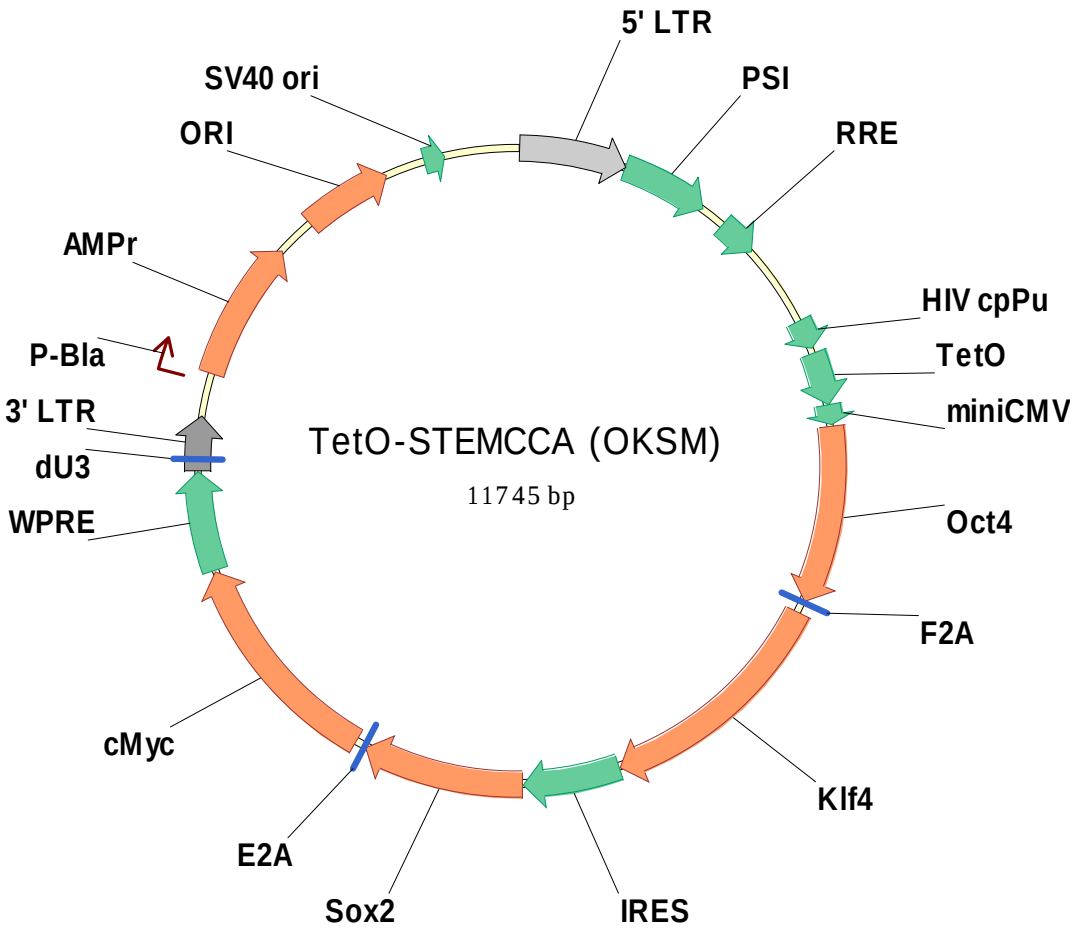

Figure 1. Schematic map of TetO-STEMCCA (OKSM) lentiviral vector.

Kit Components

Catalog number SCR512:

1. TetO-STEMCCA (OKSM) Lentivirus: (Part number CS204444) Two (2) vials, each contains 15 µL of high titer lentiviruses. For exact titer refer to the label on the front of the manual.
2. rtTA Lentivirus: (Part number CS204506) Two (2) vials, each contains 15 µL of high titer constitutively expressed reverse tetracycline transcriptional activator (rtTA) lentiviruses. For exact titer refer to the label on the front of the manual.
3. 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

Lentiviruses are 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 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. FibroGRO™ LS Complete Medium (Cat. No. SCMF002)
2. ESGRO-2i Medium (Cat. No. SF016-100 or SF016-200)
3. EmbryoMax Complete ES Cell Media w/ 15% FBS and mLIF (Cat. No. ES-101-B)
4. MEF expansion medium (see page 4)
5. Doxycycline, Hyclate (DOX) (Cat. No. 324385)
6. Rodent somatic cells or PMEF cells, not mitomycin-C treated (Cat. No. PMEF-CFL)
7. PMEF cells, growth-arrested, mitomycin-C treated (Cat. No. PMEF-CF)
8. EmbryoMax® 0.1% Gelatin Solution (Cat. No. ES-006-B)
9. ESGRO (LIF) (Cat. No. ESG1107)
10. Trypsin-EDTA Solution (0.25% Trypsin & 1 mM EDTA) (Cat. No. SM-2003-C)
11. Accutase™ Cell Dissociation Solution (Cat. No. SCR005)
12. Phosphate Buffered Saline (1X PBS) (Cat. No. BSS-1005-B)
13. 6-well plates, culture flasks, dishes (TC grade)

SECTION 1: REPROGRAMMING MOUSE EMBRYONIC FIBROBLASTS

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

Day 0: Seeding proliferating MEFs or target rodent cells

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 at room temperature 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.	EMD Millipore Cat. No.
DMEM High-Glucose Medium	44 mL	1X	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°C, 5% CO_2 incubator. It is recommended to use early passage MEFs. ***Do not use MEFs that are beyond p3.***

Day 1: Virus Infection

4. Aspirate MEF Expansion media and wash cells with 1X PBS buffer, 3 mL per well. Aspirate after the wash. Add 1 mL fresh FibroGRO™ LS Complete Medium (Cat. No. SCMF002) per well. 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. Set the plate aside in 37°C, 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 10 – 20. **Please make note of the titer as the viral titer may vary slightly from lot to lot.** Two viruses (TetO-STEMCCA (OKSM) and rtTA lentiviruses) with equal MOI will be used to infect the cells. An MOI of 10 – 20 each for TetO-STEMCCA (OKSM) and rtTA lentiviruses will typically yield 5-10 mouse iPS cell colonies from p3 MEFs. Using an MOI < 10 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:

$$\frac{1 \times 10^5 \text{ cells}}{3 \times 10^8 \text{ IFU/mL}} \times \frac{20}{1\text{ mL}} \times 1000\ \mu\text{L} = *6.6\ \mu\text{L}$$ **each** of TetO-STEMCCA (OKSM) and rtTA lentiviruses are 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 the requisite amount of vial(s) (1 vial = 15 μL) each of TetO-STEMCCA (OKSM) and rtTA lentiviruses at room temperature and quickly place the vials on ice after they are thawed. Quickly centrifuge the vials to spin down the contents. Keep the viruses on ice and proceed immediately to the next step.

7. Pre-mix the required volume of thawed viruses (TetO-STEMCCA (OKSM) and rtTA) in a sterile eppendorf tube and immediately add the viral mixture to the wells containing the attached MEFs (from Step 4). Gently rock the plate from side to side to thoroughly mix the viruses onto the MEFs. Incubate overnight in a 37°C, 5% CO_2 incubator.

Day 2: Addition of Mouse ES Cell Media containing Doxycycline (DOX)

8. Prepare stock solutions of doxycycline (10 mM) by dissolving DOX in sterile water. Filter sterilize the solution. DOX stock solutions can be stored in the dark at 4°C for up to 4 weeks without noticeable changes in activity.

a. Prepare Mouse ES Cell Media containing 2 μM DOX by adding 0.6 μL DOX (10 mM stock) to every 3 mL of Complete ES Cell Media containing 15% FBS and LIF (Cat. No. ES-101-B). Scale up medium volumes according to experimental design.

b. Exchange media in each well with 3 mL Complete ES Cell Media with 15% FBS and LIF (Cat. No. ES-101-B) containing 2 μM DOX.

***Note:** Mouse iPS Cell Boost Supplement (SCM087) should not be used as it does not exert an effect on the inducible system.*

Day 4 – Day 13: Exchange with Mouse ES Cell Media containing DOX every other day

9. Exchange the media with 3 mL fresh Complete ES Cell Media with 15% FBS and LIF (Cat. No. ES-101-B) containing 2 μM DOX every other day for a total of 10 – 13 days. Mouse iPS cell colonies start to emerge around day 7 – 10.

10. Continue to monitor the growth of the mouse iPS cell colonies daily. Look for colonies that are compact and have defined borders (refer to Figure 2). Mouse iPS cell colonies can be selected and clonally expanded (typically around Day 10 – 12) when they reach an approximate size where the colony fits into the frame of a 10X Magnification view (please refer to Figure 2C for an example of an iPS colony that can be picked). Overgrown colonies such as the ones shown in Figure 2D should not be passaged.

SECTION 2: CLONAL EXPANSION OF MOUSE iPS COLONIES

At approximately Day 11 or when the mouse iPS colonies are of sufficient size (see step 10), they can be picked for clonal expansion in serum free ESGRO-2i Medium (without DOX). Once colonies are established, it is no longer necessary to add in the DOX to the expansion medium.

***Note:** Avoid expansion media that contains serum as this may result in mixed cultures containing full and partial reprogrammed (i.e. flat morphologies) colonies.* ***Use of ESGRO-2i medium (Cat. No. SF016-100 or SF016-200) is highly recommended to obtain good ES cell-like morphology.** Good ES-cell like morphologies are typically observed after the 3rd to 5th passage in ESGRO-2i medium. If making own serum-free based media, KOSR can be used to replace FBS.*

Day before passaging

11. One day prior to passaging the iPS colonies, prepare a fresh 6-well plate with inactivated MEFs to support the expansion of the mouse iPS cells 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 4 x 10^5 cells per well of a 6-well dish. Use MEF Expansion medium to culture the cells (see page 4). Total volume per well should be 3 mL. Incubate overnight in a 37°C, 5% CO_2 incubator.

Day of passaging

12. On the day of passaging add 100 µL of 0.25% Trypsin-EDTA (prewarmed to 37°C) to a 15 mL conical tube for each mouse iPS colony to be picked. For example, for 10 iPS colonies, prepare 10 separate conical tubes containing 0.25% trypsin-EDTA. Set aside.

13. Under a dissecting microscope in a laminar flow hood, scrape and pipette up one iPS colony and deposit the pieces into the 15 mL conical tube containing 0.25% trypsin-EDTA. Repeat this step for each iPS colony to be clonally expanded, being careful to keep each iPS colony in separate conical tubes.

14. Incubate the 15 mL conical tubes at room temperature for 5-10 minutes to allow the trypsin to dissociate the cell colonies.

15. Add 5 mL fresh serum free ESGRO-2i Medium to each 15 mL conical tube containing the dissociated cell colonies.

16. Using a 5 or 10 mL pipette, slowly pipette up and down to break apart any remaining cell clumps.

17. Discard the media from the plate of inactivated MEFs (from Step 11) and add the dissociated mouse iPS colony from each 15 mL conical tube (approximately 5 mL volume) into separate wells of the 6-well dish containing inactivated MEFs. Incubate the 6-well dish in a 37°C, 5% CO_2 incubator.

Note: *It is important to avoid cross-colony contamination between mouse iPS clones. Therefore, each well of the 6-well dish should only contain dissociated cells from one mouse iPS colony.*

18. Replace with fresh serum free ESGRO-2i Medium the following day and every other day thereafter for two weeks or until the mouse iPS colonies are 80% confluent.

19. When mouse iPS clones are 80% confluent, they can be further expanded and frozen. Freezing back vials of each mouse iPS clone before proceeding for subsequent studies is strongly recommended.

Mouse iPS Cells Derived from MEF cells

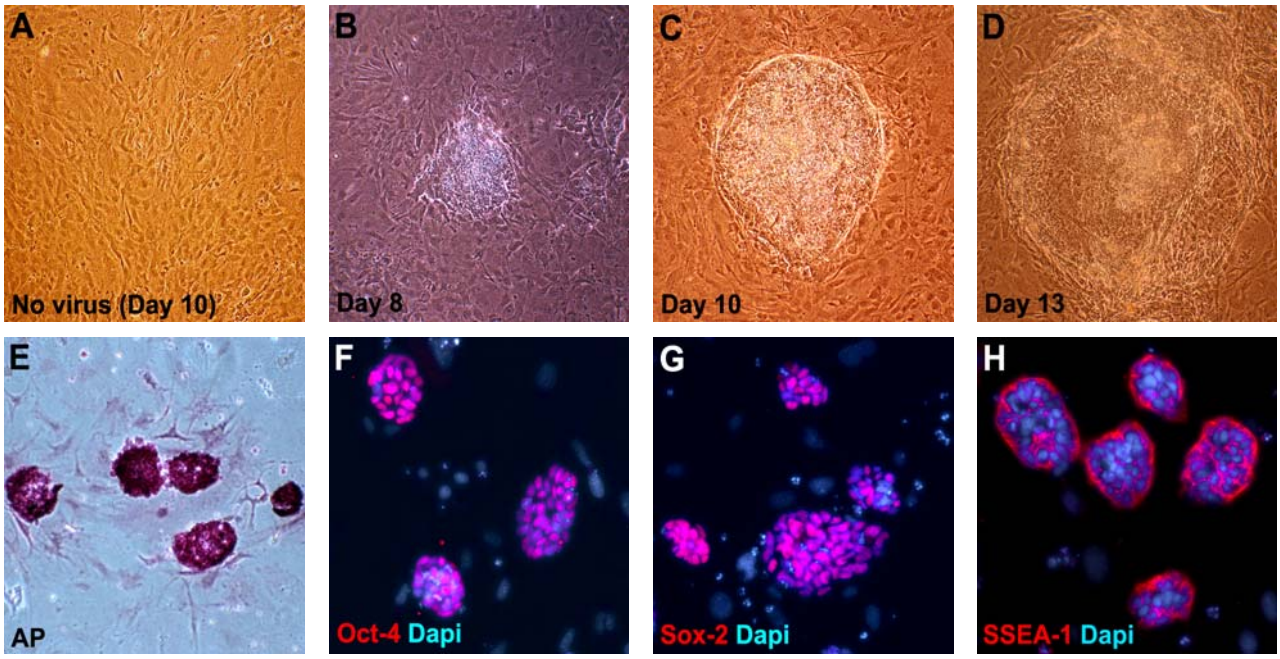

Figure 2. Mouse iPS cells derived from MEFs (passage 3) co-infected with the TetO-STEMCCA (OKSM) and rtTA lentiviruses have cell morphology and staining characteristics of mouse ES cells. Lentiviral infection was performed with an MOI of 20 and 5 µg/mL Polybrene reagent. After 10-12 days, non-infected MEFs remained in a monolayer culture with no ES cell-like colonies observed (**A**) while infected MEFs formed multilayered, tightly packed cells with defined borders (**B-D**). Four factor derived mouse iPS cells exhibited 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).

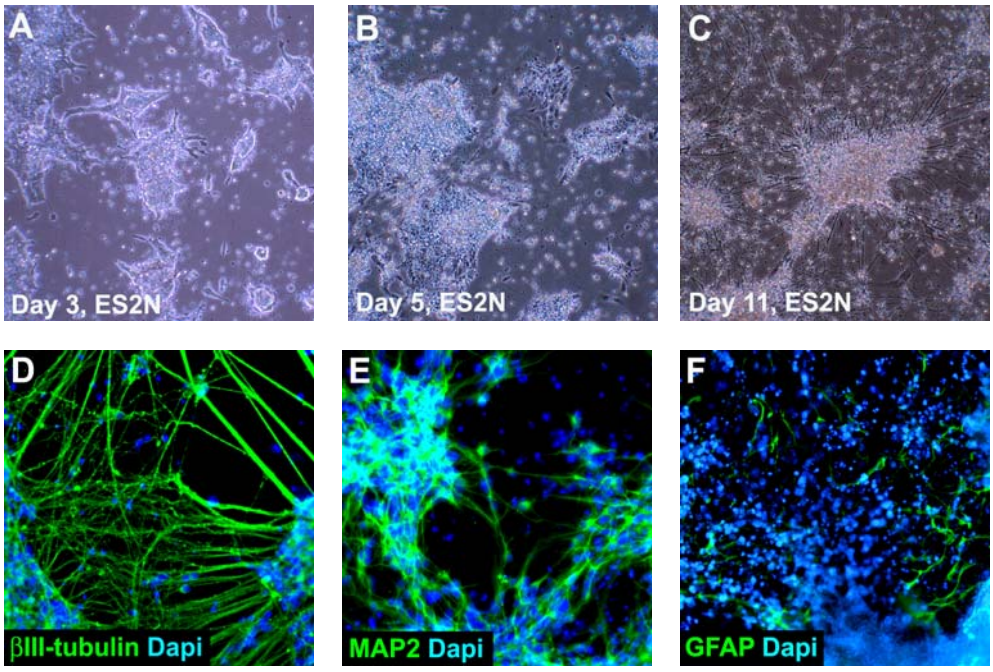

Figure 3. Directed differentiation of mouse iPS cells to mostly neuronal phenotype using serum-free ES2N media (Cat. No. SCM082). Mouse iPS cells were directly differentiated in ES2N media for 3 (**A**), 5 (**B**), and **11** (**C**) days, with media changes every other day. After 11 days of differentiation, majority of the cells expressed neuronal markers, βIII-tubulin (**D**, Cat. No. MAB1637) and MAP2 (**E**, Cat. No. MAB3418) with few GFAP-positive astrocytes (**F**, Cat. No. MAB3402) detected.

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