STEMdiff™ Motor Neuron Differentiation Kit
STEMdiff™ Motor Neuron Maturation Kit

Differentiation and maturation kits for generation of motor neurons from human pluripotent stem cells

Catalog #100-0871 1 Kit
Catalog #100-0872 1 Kit

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Product Description

The serum-free STEMdiff™ Motor Neuron System comprises STEMdiff™ Motor Neuron Differentiation Kit (Catalog #100-0871) and STEMdiff™ Motor Neuron Maturation Kit (Catalog #100-0872). STEMdiff™ Motor Neuron Differentiation Kit is used to generate motor neurons from human pluripotent stem cells (hPSCs) in 14 days. The resulting cells are then matured for an additional 14 or more days using STEMdiff™ Motor Neuron Maturation Kit to produce > 40% mature motor neurons, which are useful for motor neuron disease modeling and assay development. Motor neurons are also competent for co-culture with muscle cells generated using MyoCult™ Differentiation Kit (Human; Catalog #05965).

This is the Product Information Sheet (PIS) for generating motor neurons in an AggreWell™400 plate. An optional protocol using Ultra-Low Adherent Plate for Suspension Culture (Catalog #100-0083) is available upon request.

Product Information

All components listed below are sold as part of a kit (Catalog #100-0871 or 100-0872) and are not available for individual sale.

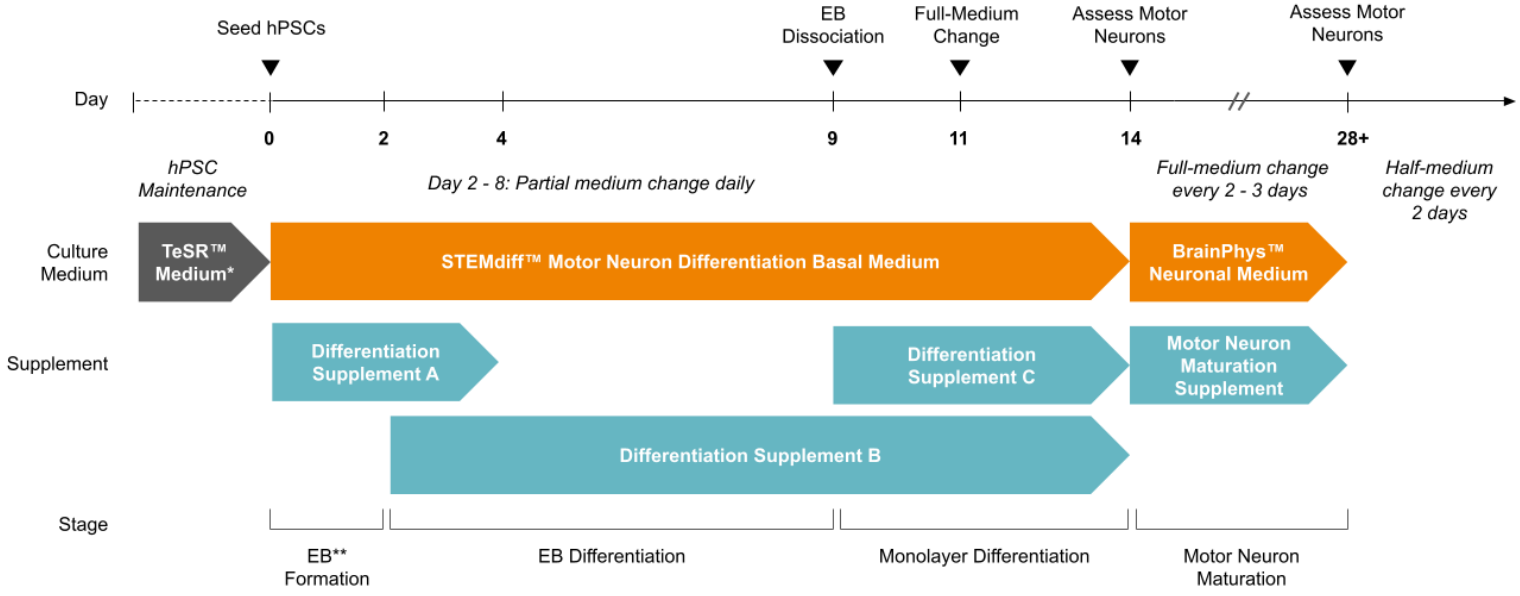
NAME	COMPONENT #	SIZE	STORAGE	SHELF LIFE
STEMdiff™ Motor Neuron Differentiation Kit (100-0871)				
STEMdiff™ Motor Neuron Differentiation Basal Medium*	100-0873	100 mL	Store at -20°C.	Stable for 2 years from date of manufacture (MFG) on label.
STEMdiff™ Motor Neuron Supplement A**	100-0874	120 µL	Store at -20°C.	Stable for 2 years from date of manufacture (MFG) on label.
STEMdiff™ Motor Neuron Supplement B**	100-0875	200 µL	Store at -20°C.	Stable for 18 months from date of manufacture (MFG) on label.
STEMdiff™ Motor Neuron Supplement C**	100-0876	120 µL	Store at -20°C.	Stable for 2 years from date of manufacture (MFG) on label.
STEMdiff™ Motor Neuron Maturation Kit (100-0872)				
BrainPhys™ Neuronal Medium***	05797	100 mL	Store at 2 - 8°C.	Stable for 2 years from date of manufacture (MFG) on label.
STEMdiff™ Motor Neuron Maturation Supplement*	100-0877	25 mL	Store at -20°C.	Stable for 2 years from date of manufacture (MFG) on label.

* This component contains material derived from human plasma. Donors have been tested and found negative for HIV-1 and -2, hepatitis B, and hepatitis C prior to donation. However, this product should be considered potentially infectious and treated in accordance with universal handling precautions.
** Please refer to the Safety Data Sheet (SDS) for hazard information. This product contains components dissolved in dimethyl sulfoxide (DMSO). DMSO is a strong solvent and skin penetrant and can transport many substances through the skin. DMSO can also penetrate some protective glove materials including latex and silicone. Extra caution should be utilized when handling this product.
*** Protect from light.

Materials Required but Not Included

PRODUCT NAME	CATALOG #
24-well flat-bottom tissue culture-treated plates	e.g. 38021
ACCUTASE™	07920
AggreWell™400 24-well plate, 1 pack OR 5 pack	34411 OR 34415
Anti-Adherence Rinsing Solution	07010
Conical tubes, 15 mL or 50 mL	e.g. 38009 or 38010
DMEM/F-12 with 15 mM HEPES	36254
D-PBS (Without Ca++ and Mg++)	37350
Laminin	Sigma L2020
Poly-L-ornithine solution	Sigma P4957
Serological pipettes, 2 mL and 5 mL	e.g. 38002 and 38003
Trypan Blue	07050
Wide-bore disposable 1 mL pipette tips	e.g. VWR CA15000-466
Y-27632 (Dihydrochloride)	72302

Protocol Diagram



*mTeSR™1 or mTeSR™ Plus

**Embryoid Body

From days 0 - 9, motor neuron progenitors develop within embryoid bodies (EB) from hPSCs using AggreWell™ 400 plates. From days 9 - 14, the resulting motor neuron precursors are differentiated to post-mitotic motor neurons, and subsequently matured during days 14 - 28.

Preparation of Reagents and Media

A. COATING CULTUREWARE WITH POLY-L-ORNITHINE AND LAMININ

1. Dilute the Poly-L-ornithine (PLO) solution in Dulbecco's phosphate-buffered saline (D-PBS) to reach a final concentration of 15 µg/mL.
   For example, add 15 mL of PLO to 85 mL of D-PBS.
2. Gently mix diluted PLO solution. Do not vortex.
3. Add PLO solution to the cultureware to cover the entire growth surface. Refer to Table 1 for recommended coating volumes.
4. Gently tilt the cultureware to spread the substrate solution evenly across the surface and incubate at 37°C and 5% CO_2 for 2 hours. Do not let the coating solution evaporate.
   NOTE: If not used immediately, cultureware must be sealed to prevent evaporation of the substrate solution (e.g. with Parafilm®). Sealed cultureware can be stored at 2 - 8°C overnight. Allow stored coated cultureware to come to room temperature (15 - 25°C) before proceeding to step 6.
5. Prepare a 10 µg/mL working solution of laminin in DMEM/F-12 with 15 mM HEPES. Refer to Table 1 for recommended coating volumes.
6. Gently tilt the PLO-coated cultureware onto one side and allow excess PLO solution to collect at the edge. Remove the excess solution using a serological pipette or by aspiration. Ensure that the coating is not scratched.
7. Wash the PLO-coated cultureware twice by pipetting D-PBS gently toward the corner of the cultureware to avoid removing the PLO coating.
8. Remove the D-PBS from the cultureware and immediately add the laminin solution to cover the entire growth surface.
9. Incubate at 37°C and 5% CO_2 for 2 hours. Do not let the laminin solution evaporate.
   NOTE: Using freshly coated cultureware is recommended. However, if not used immediately, sealed cultureware can be stored at 2 - 8°C in laminin solution for up to 4 days after coating.
10. Warm the coated cultureware to 37°C before use.
11. Gently remove the laminin solution immediately prior to seeding cells. Do not let the surface dry.
    NOTE: It is not necessary to wash cultureware after removing the laminin solution.

Table 1: Recommended Volumes for Coating Cultureware

CULTUREWARE	APPROXIMATE SURFACE AREA	VOLUME OF COATING SOLUTION
96-well plate	0.32 cm^2/well	50 µL/well
24-well plate	1.9 cm^2/well	250 µL/well
12-well plate	3.8 cm^2/well	500 µL/well
6-well plate	9.5 cm^2/well	1 mL/well
35 mm dish	9 cm^2	1 mL
60 mm dish	21 cm^2	2.5 mL

B. PREPARATION OF STEMdiff™ MOTOR NEURON MEDIA

Five medium formulations are required for the motor neuron differentiation protocol: Motor Neuron Differentiation Medium 1 (Day 0 - 1), Motor Neuron Differentiation Medium 2 (Day 2 - 3), Motor Neuron Differentiation Medium 3 (Day 4 - 8), Motor Neuron Differentiation Medium 4 (Day 9 - 13), and Motor Neuron Maturation Medium (Day 14 - 28 onwards).

Use sterile technique to prepare complete STEMdiff™ Motor Neuron media. Prepare each medium as needed in Directions for Use. Refer to Table 2 for medium components, volumes, and in-use storage and stability. The suggested volumes in Table 2 are appropriate for culturing 1 well of an AggreWell™400 24-well plate until Day 9, followed by 6 wells of a 24-well plate from Day 9 onwards. If culturing additional wells, adjust volumes accordingly.

1. Thaw Supplement(s) and STEMdiff™ Motor Neuron Differentiation Basal Medium at room temperature (15 - 25°C). Mix thoroughly.
   NOTE: If not used immediately, aliquot Supplement(s) and store at -20°C. Do not exceed the shelf life of the Supplement(s). After thawing aliquots, use immediately. Do not re-freeze.
2. Prepare media as indicated in Table 2. Mix thoroughly. Warm medium to room temperature before use.
   NOTE: If not used immediately, store media as indicated in Table 2.

Table 2. Preparation of STEMdiff™ Motor Neuron Media

MEDIUM	COMPONENT	VOLUME	IN-USE STORAGE AND STABILITY
Motor Neuron Differentiation Medium 1 (5 mL)	STEMdiff™ Motor Neuron Differentiation Basal Medium	5 mL*	Store at 2 - 8°C for up to 2 weeks.
	STEMdiff™ Motor Neuron Differentiation Supplement A	10 µL	
Motor Neuron Differentiation Medium 2 (3 mL)	Motor Neuron Differentiation Medium 1	3 mL	
	STEMdiff™ Motor Neuron Differentiation Supplement B	6 µL	
Motor Neuron Differentiation Medium 3 (14.5 mL)	STEMdiff™ Motor Neuron Differentiation Basal Medium	14.5 mL**	
	STEMdiff™ Motor Neuron Differentiation Supplement B	29 µL	
Motor Neuron Differentiation Medium 4 (7 mL)	Motor Neuron Differentiation Medium 3	7 mL	
	STEMdiff™ Motor Neuron Differentiation Supplement C	14 µL	
Motor Neuron Maturation Medium (125 mL)	BrainPhys™ Neuronal Medium	100 mL	Store at 2 - 8°C for up to 4 weeks.
	STEMdiff™ Motor Neuron Maturation Supplement	25 mL	

* Only 2 mL of Motor Neuron Differentiation Medium 1 will be required if culturing 1 well of an AggreWell™400 24-well plate. Use the remaining 3 mL to prepare Motor Neuron Differentiation Medium 2.
** Only 7.5 mL of Motor Neuron Differentiation Medium 3 will be required if culturing 1 well of an AggreWell™400 24-well plate. Use the remaining 7 mL to prepare Motor Neuron Differentiation Medium 4.

Directions for Use

Please read the entire protocol before proceeding. Use sterile technique when performing the following protocols:

A. Seeding hPSCs to Generate EBs (Day 0 - 2)
B. Differentiation of Motor Neuron Progenitor-Containing EBs (Day 2 - 9)
C. Differentiation of Motor Neuron Precursors to Motor Neurons (Day 9 - 14)
D. Maturation of Motor Neurons (Day 14 - 28+)

A. SEEDING hPSCs TO GENERATE EBs (Day 0 - 2)

This protocol is for harvesting hPSCs from a 100 mm dish and seeding into a single well of an AggreWell™400 24-well plate to form EBs. If using other cultureware, adjust volumes accordingly. Warm cultureware, media, and reagents to room temperature (15 - 25°C) before use.

hPSCs can be previously maintained in mTeSR™1 or mTeSR™ Plus; for further information on maintaining high-quality hPSCs, refer to the Technical Manuals for mTeSR™1 or mTeSR™ Plus, available at www.stemcell.com, or contact us to request a copy.

Day 0: Generating EBs Using AggreWell™400 Plates

1. Pre-treat one well of an AggreWell™400 24-well plate with Anti-Adherence Rinsing Solution as follows:
   a. Add 500 µL of Anti-Adherence Rinsing Solution to the desired microwell.
   b. Centrifuge plate at 1300 x *g* for 5 minutes in a swinging bucket rotor fitted with plate holders.
      NOTE: Plates must be well-balanced. Prepare a balance plate using a standard plate filled with water to match the weight and position of the AggreWell™ plate.
   c. Observe the plate under a microscope to ensure that bubbles have been removed from microwell. To remove any remaining bubbles, centrifuge at 1300 x *g* for an additional 5 minutes.
   d. Aspirate Anti-Adherence Rinsing Solution from the wells.
2. Warm DMEM/F-12 with 15 mM HEPES and ACCUTASE™ to room temperature (15 - 25°C). Prepare Motor Neuron Differentiation Medium 1 (see Preparation section B).
3. To prepare the Seeding Medium, add Y-27632 to 2 mL of Motor Neuron Differentiation Medium 1 to a final concentration of 10 µM and warm to room temperature.
   NOTE: The remaining 3 mL of Motor Neuron Differentiation Medium 1 will be used to prepare Motor Neuron Differentiation Medium 2. Store at 2 - 8°C until use on Day 2.
4. Add 1 mL of Seeding Medium to the pre-treated well prepared in step 1. Set the plate aside at room temperature until use.
5. Use a microscope to visually identify regions of differentiation in the hPSC culture. Mark these using a felt tip or lens marker on the bottom of the 100 mm dish. Remove regions of differentiation by scraping with a pipette tip or by aspiration.

NOTE: Removal of differentiated cells will result in improved differentiation efficiency.

6. Wash the dish once with 5 - 10 mL of room temperature D-PBS or DMEM/F-12 with 15 mM HEPES.
7. Aspirate the wash and add 3 mL of ACCUTASE™.
8. Incubate at 37°C for 6 - 8 minutes.

   NOTE: Incubation time may vary depending on the cell line and non-enzymatic cell dissociation reagent used. Monitor cell dissociation under a microscope to determine the optimal incubation time for each application.
9. Using a 1 mL pipettor, pipette the cell suspension up and down 3 - 5 times to dislodge remaining attached cells. Using a 5 mL serological pipette, transfer cell suspension to a sterile 15 or 50 mL conical tube. Ensure that any remaining cell aggregates are broken up into single cells.
10. Rinse the dish with 9 mL of DMEM/F-12 with 15 mM HEPES and add the rinse to the tube containing the cells.
11. Count viable cells using Trypan Blue and a hemocytometer.
12. Calculate the volume required to obtain 3.6 x 10^5 cells. Transfer this volume to a new conical tube and centrifuge at 300 *x g* for 5 minutes.

    NOTE: Although 300 cells/microwell (i.e. 3.6 x 10^5 cells/well) is recommended for most hPSC lines, the optimal seeding density to induce EB formation should be determined for different cell lines through performing small-scale differentiation assays (e.g. 1 well of an AggreWell™400 24-well plate). It is recommended to initially test cell densities between 200 - 400 cells/microwell (2.4 x 10^5 cells/well to 4.8 x 10^5 cells/well) and monitor EB formation rate on Day 1, EB growth between Day 2 and 4, and assess motor neuron differentiation efficiency on Day 14 (see Assessment of Motor Neuron Differentiation below).
13. Carefully aspirate the supernatant and resuspend cells in 1 mL of Seeding Medium to obtain a final concentration of 3.6 x 10^5 cells/mL.
14. Add the suspension (i.e. 3.6 x 10^5 cells) to a single well of the AggreWell™400 plate prepared in steps 1 - 4. This will result in 300 cells/microwell.

    NOTE: Ensure that newly plated cells are evenly dispersed across the entire surface of the well by gently pipetting up and down several times.
15. Centrifuge the AggreWell™400 plate at 100 x *g* for 3 minutes to capture cells in the microwells.

    NOTE: Plates must be balanced. It is recommended to balance the plate against a standard 24-well plate filled with water to match the weight and position of the AggreWell™400 plate.
16. Examine the AggreWell™400 plate under a microscope to verify that cells are evenly distributed among the microwells.
17. Incubate the cells at 37°C with 5% CO_2 and 95% humidity. Do not disturb the plate for at least 24 hours.

Day 1: Check EB Formation in AggreWell™400 Plate

18. Carefully remove the AggreWell™400 plate from the incubator, taking care not to disturb the contents.
19. Observe the cells under a microscope. Uniform EBs should be visible in the AggreWell™400 well. At least 50% of cells within the microwell should be incorporated into the EBs.
20. Proceed to section B for EB differentiation.

B. DIFFERENTIATION OF MOTOR NEURON PROGENITOR-CONTAINING EBs (Day 2 - 9)

Perform daily 75% medium changes as described below:

Day 2 - 3

1. Prepare Motor Neuron Differentiation Medium 2 using the remaining 3 mL of Motor Neuron Differentiation Medium 1 (see Preparation section B).
2. Warm 1.5 mL of Motor Neuron Differentiation Medium 2 to 37°C.
3. Using a 1 mL pipettor, slowly remove 1.5 mL of the medium from the well and discard.

   NOTE: Do not disturb the EBs. Keep the pipette tip toward the upper surface of the medium in the well while removing the medium.
4. Replace with 1.5 mL of Motor Neuron Differentiation Medium 2 by slowly pipetting down the wall of the well. Slowly dispensing the medium helps to prevent displacement of EBs/spheroids from the microwells.

   NOTE: It is important not to disturb the EBs. Do not add the medium directly onto the surface of the well. Support the pipette tip by slightly touching the side of the well at the surface level of the remaining medium inside the well. This will allow for a more controlled release of the medium. Quick release of medium will dislodge the EBs from the wells.
5. Incubate at 37°C with 5% CO_2 and 95% humidity for 24 hours.

Day 3

6. Repeat steps 2 - 5.

Day 4 - 8

7. Prepare Motor Neuron Differentiation Medium 3 (see Preparation section B).
8. Repeat steps 2 - 5 using Motor Neuron Differentiation Medium 3 until Day 8.

   NOTE: The remaining 7 mL of Motor Neuron Differentiation Medium 3 will be used to prepare Motor Neuron Differentiation Medium 4. Store at 2 - 8°C until use on Day 9.
9. Proceed to section C for motor neuron differentiation.

C. DIFFERENTIATION OF MOTOR NEURON PRECURSORS TO MOTOR NEURONS (Day 9 - 14)

Day 9: EB Dissociation

1. Coat 6 wells of a flat-bottom tissue culture-treated 24-well plate with PLO and laminin (see Preparation section A) and warm to 37°C before use. Warm DMEM/F-12 with 15 mM HEPES to room temperature (15 - 25°C).

   NOTE: It is recommended to coat and seed 3 wells of one 24-well plate and 3 wells of a second 24-well plate. This allows one set of wells to be used for the Day 14 differentiation efficiency check by immunocytochemistry (ICC), while keeping the remaining wells in culture for the maturation period until Day 28+.
2. Prepare Motor Neuron Differentiation Medium 4 using the remaining 7 mL of Motor Neuron Differentiation Medium 3 (see Preparation section B) and warm to 37°C.
3. Add Y-27632 to 4 mL of Motor Neuron Differentiation Medium 4 to a final concentration of 10 µM.
4. Using a 1 mL pipettor with a wide-bore tip:
   a. Remove 1 mL of the culture medium from the well.
   b. Dispense the medium firmly back onto the surface of the plate to dislodge the EBs from the microwells. Do not triturate.

      NOTE: It is critical to use wide-bore pipette tips to avoid damaging the EBs.
5. Using the same wide-bore tip, gently transfer the medium and dislodged EBs to a 15 mL conical tube.
6. Using a 1 mL pipettor with a wide-bore tip, dispense 1 mL of DMEM/F-12 with 15 mM HEPES across the entire surface of the well to dislodge any remaining EBs. Transfer the wash to the conical tube from step 5. Repeat this wash step 3 - 4 times and observe the well under a microscope to ensure that all EBs have been dislodged.
7. Allow the EBs to settle by gravity until all aggregates have settled to the bottom of the tubes. This should be visible by eye.
8. Carefully remove the medium, leaving behind a small amount of medium to ensure that the pellet is not disturbed.
9. Wash the EBs by adding 4 mL of DMEM/F-12 with 15 mM HEPES to the tube. Allow the EBs settle by gravity.
10. Carefully remove the medium, leaving behind a small amount of medium to ensure that the pellet is not disturbed.
11. Resuspend the EBs with 1 mL ACCUTASE™. Incubate the tube at 37°C for 8 - 10 minutes.
12. Dissociate the EBs with a regular 1 mL pipette by gently pipetting up and down no more than 10 times. There should be no clumps remaining.

    NOTE: It is absolutely critical to avoid generating bubbles when pipetting up and down.
13. Add 3 mL of DMEM/F-12 with 15 mM HEPES and centrifuge at 300 *x g* for 5 minutes
14. During centrifugation, add 0.5 mL/well of Motor Neuron Differentiation Medium 4 + 10 µM Y-27632 into 6 wells of the PLO/laminin-coated 24-well plate prepared in step 1.
15. Discard the supernatant and resuspend the cells in 1 mL of Motor Neuron Differentiation Medium 4 + 10 µM Y-27632.
16. Perform a viable cell count using Trypan Blue and a hemocytometer.
17. Calculate the required volume to obtain 5 x 10^4 cells per well (i.e. final cell denisty of 2.6 x 10^4 cells/cm^2) and add to the wells prepared in step 14.

    NOTE: The seeding density should be optimized for each cell line and application, and typically ranges from 0.5 - 1 x 10^5 cells per well of a 24-well plate (i.e. final cell density of 2.6 - 5.3 x 10^4 cells/cm^2).
18. Shake the plate in short, back-and-forth and side-to-side motions to distribute the cells evenly across the wells. Incubate at 37°C and 5% CO_2 for 2 days.

Day 11

19. Remove the medium and add 0.5 mL of warm (37°C) Motor Neuron Differentiation Medium 4 to each well in a 24-well plate.

    NOTE: Addition of Y-27632 is not required in the medium for this step, nor for the remainder of the protocol.
20. Incubate at 37°C and 5% CO_2 for 3 days.

Day 14: Assess Motor Neurons

Assess motor neuron differentiation by assessing marker expression by ICC (see Assessment of Motor Neuron Differentiation below). If marker expression meets the expected Day 14 levels outlined in Table 3, proceed to section D for motor neuron maturation.

D. MATURATION OF MOTOR NEURONS (Day 14 - 28+)

Day 14 (continued)

1. Prepare Motor Neuron Maturation Medium (see Preparation section B) and warm to 37°C.
2. Aspirate the medium and add 0.5 mL of warm (37°C) Motor Neuron Maturation Medium to each well in a 24-well plate.
3. Perform a full-medium change every 2 - 3 days until at least Day 28, at which point the neurons are considered terminally differentiated.
   NOTE: After Day 28, perform a half-medium change every other day.

Assessment of Motor Neurons

For evaluating motor neuron differentiation efficiency, marker expression may be assessed by ICC on Day 14 after labelling with the following antibodies:

- Anti-Beta-Tubulin III Antibody, Clone TUJ1 (Catalog #60052)
- Anti-islet 1 (EP4182) antibody, monoclonal (Abcam #AB109517)
- Anti-Nkx-6.1 antibody, monoclonal (DSHB #F55A10-c)
- Anti-MNR2/HB9/Mnx1 antibody, monoclonal (DSHB #81.5C10)
- Anti-choline acetyltransferase antibody, polyclonal (Sigma-Aldrich #AB144P)

Cultures may be evaluated again after the maturation period on Day 28. Refer to Table 3 below for recommended antibody dilutions and expected expression levels upon a successful differentiation experiment. Results may vary depending on the cell line used.

Table 3. Recommended Antibody Dilutions and Expected Expression Levels for Motor Neuron Assessment

ANTIBODY TARGET	RECOMMENDED ANTIBODY DILUTION	EXPECTED EXPRESSION
Class III β-tubulin	1:1000	Day 14: > 80% Day 28: > 80%
Islet 1 (ISL1)	1:500	Day 14: > 40% Day 28: > 40%
Homeobox protein NKX6.1	1:200	Day 14: > 40%
MNR2/HB9/Mnx1	1:50	Day 28: > 40%
Choline acetyltransferase	1:200	Day 28: > 40%

Related Products

For related products, including specialized cell culture and storage media, supplements, antibodies, cytokines, and small molecules, visit www.stemcell.com/hPSCNCworkflow or contact us at techsupport@stemcell.com.