STEMdiff™ Spinal Cord Organoid Differentiation Kit
STEMdiff™ Neural Organoid Maintenance Kit

Catalog #100-1524 1 Kit
#100-0120 1 Kit

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Product Description

STEMdiff™ Spinal Cord Organoid Differentiation Kit is a matrix-free, serum-free cell culture medium system that enables the robust generation of human pluripotent stem cell (hPSC)-derived spinal cord organoids. The medium works with AggreWell™-generated organoids to prevent organoid fusion and enables the scalable generation of highly reproducible organoids per kit. Adapted from protocols by Sergiu Paşca (Yoon et al.; Andersen et al.), spinal cord organoids are regionalized, three dimensional in vitro models with a cellular composition and structural organization that is representative of the developing human cervical spinal cord. For extended periods of organoid culture (> 50 days), the components required for organoid maintenance are available as STEMdiff™ Neural Organoid Maintenance Kit (Catalog #100-0120).

Product Information

All components listed below are sold as part of a kit (Catalog #100-1524 or 100-0120) and are not available for individual sale.

NAME	COMPONENT #	SIZE	STORAGE	SHELF LIFE
STEMdiff™ Spinal Cord Organoid Differentiation Kit (100-1524)				
STEMdiff™ Neural Organoid Basal Medium 1	08621	20 mL	Store at -20°C.	Stable for 18 months from date of manufacture (MFG) on label.
STEMdiff™ Neural Organoid Basal Medium 2	08622	500 mL	Store at 2 - 8°C.	Stable for 18 months from date of manufacture (MFG) on label.
STEMdiff™ Neural Organoid Supplement A	08623	10 mL	Store at -20°C.	Stable for 2 years from date of manufacture (MFG) on label.
STEMdiff™ Neural Organoid Supplement C	08625	0.25 mL	Store at -20°C.	Stable for 18 months from date of manufacture (MFG) on label.
STEMdiff™ Neural Organoid Supplement M*	100-1520	0.125 mL	Store at -20°C.	Stable for 12 months from date of manufacture (MFG) on label.
STEMdiff™ Neural Organoid Supplement N	100-1521	0.125 mL	Store at -20°C.	Stable for 12 months from date of manufacture (MFG) on label.
STEMdiff™ Neural Organoid Supplement O*	100-1522	0.125 mL	Store at -20°C.	Stable for 12 months from date of manufacture (MFG) on label.
STEMdiff™ Neural Organoid Supplement P*	100-1523	0.125 mL	Store at -20°C.	Stable for 12 months from date of manufacture (MFG) on label.
STEMdiff™ Neural Organoid Maintenance Kit (100-0120)				
STEMdiff™ Neural Organoid Basal Medium 2	08622	500 mL	Store at 2 - 8°C.	Stable for 18 months from date of manufacture (MFG) on label.
STEMdiff™ Neural Organoid Supplement A	08623	10 mL	Store at -20°C.	Stable for 2 years from date of manufacture (MFG) on label.

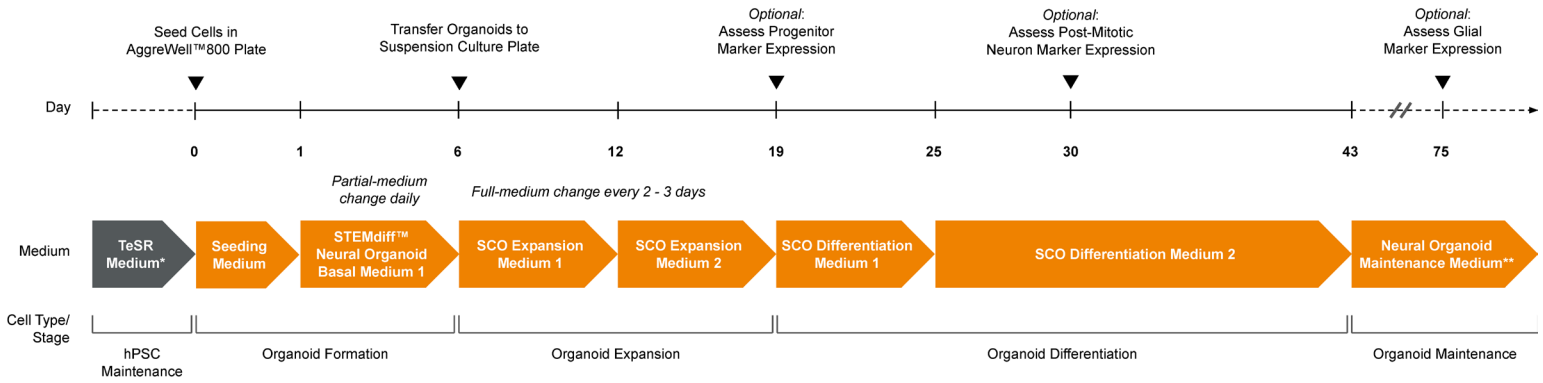
* Please refer to the Safety Data Sheet (SDS) for hazard information. This product contains components dissolved in dimethyl sulfoxide (DMSO). DMSO is a strong solvent and skin penetrant, and can transport many substances through the skin. DMSO can also penetrate some protective glove materials including latex and silicone. Extra caution should be utilized when handling this product.

All media and supplements in STEMdiff™ Spinal Cord Organoid Differentiation Kit and STEMdiff™ Neural Organoid Maintenance Kit are free of antibiotics and based on the formulation published by the Paşca lab (Andersen et al.).

Materials Required but Not Included

PRODUCT NAME	CATALOG #
ACCUTASE™	07920
AggreWell™800 24-well Plate	34811
Anti-Adherence Rinsing Solution	07010
Conical Tubes, 15 mL or 50 mL	e.g. 100-0092 or 100-0090
Culture Dish, Non-Treated, 100 mm	38045
DMEM/F-12 with 15 mM HEPES	36254
D-PBS (Without Ca++ and Mg++)	37350
Hausser Scientific™ Bright-Line Hemocytometer	100-1181
Reversible Strainer, 37 µm	27250
Serological Pipettes, 5 mL or 10 mL	e.g. 38003 or 38004
Trypan Blue	07050
Ultra-Low Adherent Plates for Suspension Cultures, 6-Well OR Tissue Culture-Treated 6-Well Flat-Bottom Plates	100-0083 OR e.g. 38015 or 38016
Wide-bore disposable pipette tips, 200 µL and 1 mL	Fisher Scientific 14-222-730 and 14-222-699
Y-27632 (Dihydrochloride)	72302

Protocol Diagram



SCO: Spinal cord organoid

*mTeSR™1 or mTeSR™ Plus

** Additional Neural Organoid Maintenance Medium is available for purchase separately (Catalog #100-0120)

Preparation of Media

Use sterile technique to prepare STEMdiff™ Spinal Cord Organoid media. Prepare each medium as needed in Directions for Use. Refer to Table 1 for medium components, volumes, and in-use storage and stability.

1. Thaw supplement(s) 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 the aliquots, use immediately. Do not re-freeze.

2. Add supplement(s) to basal medium as indicated in Table 1. Mix thoroughly. Warm medium to room temperature before use.

   NOTE: If not used immediately, store medium as indicated in Table 1. Do not exceed the shelf life of the individual components.

Table 1. Preparation of STEMdiff™ Spinal Cord Organoid Media

MEDIUM	COMPONENT	VOLUME	IN-USE STORAGE AND STABILITY
Seeding Medium (2.5 mL)	STEMdiff™ Neural Organoid Basal Medium 1	2.5 mL	Use immediately.
	Y-27632 (Dihydrochloride; 10 µM final concentration)	5 µL of 5 mM stock solution	
Spinal Cord Organoid Expansion Medium 1 (60 mL)	STEMdiff™ Neural Organoid Basal Medium 2*	58.8 mL	Store at 2 - 8°C for up to 2 weeks.
	STEMdiff™ Neural Organoid Supplement A	1.2 mL	
	STEMdiff™ Neural Organoid Supplement M	60 µL	
	STEMdiff™ Neural Organoid Supplement N	30 µL	
Spinal Cord Organoid Expansion Medium 2 (60 mL)	STEMdiff™ Neural Organoid Basal Medium 2*	58.8 mL	Store at 2 - 8°C for up to 2 weeks.
	STEMdiff™ Neural Organoid Supplement A	1.2 mL	
	STEMdiff™ Neural Organoid Supplement M	60 µL	
	STEMdiff™ Neural Organoid Supplement N	30 µL	
	STEMdiff™ Neural Organoid Supplement O	120 µL	
Spinal Cord Organoid Differentiation Medium 1 (60 mL)	STEMdiff™ Neural Organoid Basal Medium 2*	58.8 mL	Store at 2 - 8°C for up to 2 weeks.
	STEMdiff™ Neural Organoid Supplement A	1.2 mL	
	STEMdiff™ Neural Organoid Supplement C	60 µL	
	STEMdiff™ Neural Organoid Supplement P	120 µL	
Spinal Cord Organoid Differentiation Medium 2 (180 mL)	STEMdiff™ Neural Organoid Basal Medium 2*	176.4 mL	Store at 2 - 8°C for up to 2 weeks.
	STEMdiff™ Neural Organoid Supplement A	3.6 mL	
	STEMdiff™ Neural Organoid Supplement C	180 µL	
Neural Organoid Maintenance Medium (140 mL)**	STEMdiff™ Neural Organoid Basal Medium 2*	137.2 mL	Store at 2 - 8°C for up to 3 weeks.
	STEMdiff™ Neural Organoid Supplement A	2.8 mL	

* STEMdiff™ Neural Organoid Basal Medium 2 is viscous and may stick to the inside of the pipette; pipette slowly to ensure the medium is transferred effectively.
** Additional maintenance medium is available for purchase (STEMdiff™ Neural Organoid Maintenance Kit [Catalog #100-0120]).

Directions for Use

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

A. Spinal Cord Organoid Formation (Day 0 - 5)

B. Spinal Cord Organoid Expansion (Day 6 - 18)

C. Spinal Cord Organoid Differentiation (Day 19 - 42)

D. Spinal Cord Organoid Maintenance (Day 43+)

A. SPINAL CORD ORGANOID FORMATION (DAY 0 - 5)

The following instructions are for harvesting human pluripotent stem cells (hPSCs) previously cultured in mTeSR™1 (Catalog #85850) or mTeSR™ Plus (Catalog #100-0276) in a 100 mm dish, and plating the cells as a single-cell suspension into one well of an AggreWell™800 24-well plate. If using other cultureware or number of wells, adjust volumes accordingly. Warm cultureware, media, and reagents to room temperature (15 - 25°C) before use.

NOTE: hPSC cultures are ready for passage when the majority of colonies are large, compact, and have dense multi-layered centres. Passage hPSC cultures when they are no more than 70 - 80% confluent and exhibit < 10% differentiation.

Day 0: hPSC seeding

1. Pre-treat one well of an AggreWell™800 24-well plate with Anti-Adherence Rinsing Solution as follows:
   a. Add 500 µL of Anti-Adherence Rinsing Solution to one well of the AggreWell™800 24-well plate.
   b. Centrifuge the plate at 1300 x g for 5 minutes in a swinging bucket rotor fitted with plate holders.
      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™800 plate.
   c. Observe plate under a microscope to ensure that bubbles have been removed from the microwells. To remove any remaning bubbles, centrifuge the plate at 1300 x g for an additional 5 minutes.
   d. Remove the Anti-Adherence Rinsing Solution from the well and discard.
   e. Add 1 mL of of sterile D-PBS (Without Ca++ and Mg++) to the well and set the plate aside.
2. Prepare Seeding Medium (see Preparation of Media) and warm to room temperature.
3. Remove the D-PBS in the pre-treated well (prepared in step 1) and add 1 mL of Seeding Medium. Set the plate aside at room temperature until use.
4. 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.
5. Remove and discard the medium from the hPSC culture. Wash the dish with 3 - 5 mL of D-PBS.
6. Remove and discard the D-PBS wash. Add 3 mL of ACCUTASE™ and incubate at 37°C for 8 - 10 minutes.
   NOTE: Incubation time may vary when using different cell lines or other non-enzymatic cell dissociation reagents. Monitor cell dissociation under a microscope to determine the optimal incubation time for each application.
7. Using a 1 mL pipettor, gently pipette the cell supension up and down slowly 3 - 5 times to dislodge any remaining attached cells. Transfer the cell suspension to a sterile 15 mL or 50 mL conical tube.
8. Rinse the dish with 10 mL of DMEM/F-12 with 15 mM HEPES and add this rinse to the tube containing cells.
9. Count viable cells using Trypan Blue and a Hausser Scientific™ Bright-Line Hemocytometer. Calculate the volume required to obtain 4.5 x 10^6 total cells (this will be diluted in the next step to obtain a final concentration of 3 x 10^6 cells/mL).
10. Centrifuge cells at 300 x g for 5 minutes.
11. Carefully aspirate the supernatant and resuspend cells in 1.5 mL of Seeding Medium (prepared in step 2) to obtain a final concentration of 3 x 10^6 cells/mL.
12. Add 1 mL of the single-cell suspension (i.e. 3 x 10^6 cells) to the well of the AggreWell™800 plate containing Seeding Medium (prepared in step 3). This will result in 10,000 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.
13. Centrifuge the AggreWell™800 plate at 100 x g for 3 minutes. This will capture the 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™800 plate.
14. Examine the AggreWell™800 plate under a microscope to ensure that cells are evenly distributed among the microwells. Incubate at 37°C and 5% CO_2 for 24 hours.

Day 1 - 5: Partial-medium changes

NOTE: On Day 1, uniform organoids should be visible in the AggreWell™800 well.

15. Warm a sufficient volume of STEMdiff™ Neural Organoid Basal Medium 1 to room temperature (15 - 25°C).
16. Carefully remove the AggreWell™800 plate from the incubator, taking care not to disturb the contents.

    NOTE: The plate must be handled carefully to avoid organoids spilling over into other microwells, which will result in organoid fusion and lower yield.
17. Using a 1 mL pipettor, slowly remove 2 x 750 µL of medium from the well and discard.

    NOTE: To avoid disturbing the organoids, position the pipette tip toward the upper surface of the medium in the well.
18. Using a new 1 mL pipettor, **very slowly** add 2 x 750 µL of STEMdiff™ Neural Organoid Basal Medium 1 to the **side** of the well.

    CRITICAL: To avoid dislodging the organoids from the microwells, position the pipette tip at the surface level of the remaining medium in the well while slowly dispensing the medium. **DO NOT** dispense the medium directly onto the surface of the well.
19. Incubate the AggreWell™800 plate at 37°C and 5% CO_2 for 24 hours.
20. Repeat steps 15 - 19 until Day 5.

On Day 6, proceed to section B for organoid expansion.

B. SPINAL CORD ORGANOID EXPANSION (DAY 6 - 18)

The following instructions are for transferring organoids generated from one well of an AggreWell™800 plate into a 6-well Ultra-Low Adherent Plate for Suspension Cultures for organoid expansion. Organoids harvested from one well of an AggreWell™800 24-well plate can be evenly distributed into the 6-well plate. The recommended range is 25 - 40 organoids per well. STEMdiff™ Spinal Cord Organoid Differentiation Kit supports a maximum of 10 wells when used with 6-well plates. If using other cultureware, adjust volumes accordingly. Warm all cultureware, media, and reagents to room temperature (15 - 25°C) before use.

NOTE: If Ultra-Low Adherent Plates for Suspension Cultures are not available, 6-well tissue culture-treated plates pre-treated with Anti-Adherence Rinsing Solution may also be used to prevent cell attachment. Coat the plate surface with 1 mL of Anti-Adherence Rinsing Solution, then remove solution immediately from the well and discard. Add 1 mL of sterile D-PBS (Without Ca++ and Mg++) to each well and set the plate aside. Discard the D-PBS before use.

Day 6: Transfer organoids to suspension culture plate

1. Prepare Spinal Cord Organoid Expansion Medium 1 and 2 (see Preparation of Media). Warm Spinal Cord Organoid Expansion Medium 1 to room temperature and store Spinal Cord Organoid Expansion Medium 2 until use on Day 12.
2. Add 2 mL of Spinal Cord Organoid Expansion Medium 1 to each well of a 6-Well Ultra-Low Adherent Plate.
3. Place a 37 µm Reversible Strainer on top of a 50 mL conical tube. Label the tube “waste”.

   NOTE: The arrow on the reversible strainer should point upwards. Use a new strainer and a new tube for each AggreWell™800 well to be harvested.
4. Remove the medium from the organoid-containing well and firmly expel it into the well using a 1 mL pipettor with a wide-bore tip. This will dislodge the organoids from the microwells.
5. Using the same wide-bore pipette tip, transfer the suspension to the 37 µm Reversible Strainer (prepared in step 3). Organoids will remain on top of the strainer and single cells will filter through into the waste tube.
6. To collect the remaining organoids, draw up 1 mL of DMEM/F-12 and 15 mM HEPES using a new wide-bore pipette tip and firmly expel it into the same AggreWell™800 well. While the remaining organoids are in suspension, quickly transfer the suspension into the strainer from step 5.
7. Repeat step 6 until all organoids are removed from the AggreWell™800 well. One or two washes should be sufficient to dislodge all organoids. Examine the well under a microscope to ensure that all organoids have been collected.
8. Invert the strainer over a new 50 mL conical tube and add 2 mL of Spinal Cord Organoid Expansion Medium 1 onto the strainer to collect all the organoids in the tube.

   NOTE: Spinal Cord Organoid Expansion Medium 1 is viscous, thus a high pipetting force may be required to efficiently collect organoids from strainer.
9. Gently swirl the tube of organoids to create an even suspension and use a wide-bore tip to transfer 25 - 40 organoids into each well of the 6-well plate (prepared in step 2).

   NOTE: **Do not exceed 40 organoids per well of a 6-well plate**. Controlling the number of organoids in the well is critical to avoid loss in yield due to organoid fusion.
10. Gently rock the plate in short, back-and-forth and side-to-side motions to distribute the organoids across the wells. Visually inspect plate to ensure minimal contact between organoids.
11. Carefully place the 6-well plate on a **level surface** in a 37°C and 5% CO_2 incubator. Incubate for 2 days.

Day 8 - 18: Full-medium changes

12. On **Day 8**, warm a sufficient volume of Spinal Cord Organoid Expansion Medium 1 to room temperature (15 - 25°C).
13. Gently tilt the 6-well plate and allow the organoids to sink to the bottom of the wells (~15 - 30 seconds).
14. Carefully level the plate and use a 1 mL pipettor to remove the medium from the top portion of each well.

    NOTE: < 500 µL of medium may be left in the well in order to avoid disturbing the organoids. This will not affect performance.
15. Add 2 mL of fresh Spinal Cord Organoid Expansion Medium 1 to each well of the 6-well plate.
16. Immediately before incubating, gently shake the plate in short, back-and-forth and side-to-side motions to evenly distribute the organoids across the wells. Visually inspect the plate to ensure minimal contact between organoids.
17. Carefully place the plate on a **level surface** in a 37°C and 5% CO_2 incubator. Do not disturb the plate for 2 - 3 days.

    NOTE: If the plate is disturbed, repeat step 16 to evenly distribute the organoids across the wells.
18. Perform full-medium changes (steps 12 - 17) using Spinal Cord Organoid Expansion Medium 1 every 2 - 3 days until Day 11.
19. On **Day 12**, warm a sufficient volume of Spinal Cord Organoid Expansion Medium 2 (prepared in step 1) to room temperature.
20. Perform full-medium changes (steps 12 - 17) using Spinal Cord Organoid Expansion Medium 2 every 2 - 3 days until Day 18.

On Day 19, proceed to section C for organoid differentiation.

C. SPINAL CORD ORGANOID DIFFERENTIATION (DAY 19 - 42)

NOTE: Organoids may be assessed for progenitor marker expression on Day 19 and post-mitotic neuron marker expression on Day 30+ by immunofluorescence staining (see Assessment of Spinal Cord Organoids).

1. Prepare Spinal Cord Organoid Differentiation Media 1 and 2 (see Preparation of Media). Store Spinal Cord Organoid Differentiation Medium 2 until use on Day 25.
2. On **Day 19**, warm a sufficient volume of Spinal Cord Organoid Differentiation Medium 1 to room temperature (15 - 25°C).
3. Gently tilt the 6-well plate and allow the organoids to sink to the bottom of the wells (~15 - 30 seconds).
4. Carefully level the plate and use a 1 mL pipettor to aspirate medium from the top portion of each well.

    NOTE: < 500 µL of medium may be left in the well in order to avoid disturbing the organoids. This will not affect performance.
5. Add 2 mL of fresh Spinal Cord Organoid Differentiation Medium 1 to each well. Incubate at 37°C and 5% CO_2 for 2 - 3 days.
6. Perform full-medium changes (steps 2 - 5) every 2 - 3 days until Day 24.

    NOTE: If the medium becomes very acidic (bright yellow) on feed days, culture fewer organoids per well (e.g. add < 25 per well of a 6-well plate).
7. On **Day 25**, warm a sufficient volume of Spinal Cord Organoid Differentiation Medium 2 (prepared in step 1) to room temperature.
8. Perform full-medium changes (steps 2 - 5) using Spinal Cord Organoid Differentiation Medium 2 every 2 - 3 days until Day 42.

On Day 43, proceed to section D for organoid maintenance.

D. SPINAL CORD ORGANOID MAINTENANCE (DAY 43+)

Components to prepare additional Neural Organoid Maintenance Medium (i.e. STEMdiff™ Neural Organoid Basal Medium 2 and STEMdiff™ Neural Organoid Supplement A) are available in STEMdiff™ Neural Organoid Maintenance Kit (Catalog #100-0120), available for purchase separately.

1. Prepare Neural Organoid Maintenance Medium (see Preparation of Media) and warm to room temperature (15 - 25°C).
2. Gently tilt the 6-well plate and wait for organoids to sink to the bottom of the wells (~15 - 30 seconds).
3. Carefully level the plate and use a 1 mL pipettor to aspirate medium from the top portion of each well.
4. Add 2 mL of fresh Neural Organoid Maintenance Medium to each well.
5. Perform a full-medium change every 2 - 3 days.

    NOTE: If the spent medium appears very acidic (i.e. bright yellow) on feed days, culture fewer organoids per well (e.g. use < 10 per well of a 6-well plate). Once the organoids grow larger (i.e. Day 50+), increase the feed volume to 3 mL/well to ensure they are fully submerged in maintenance medium.

NOTE: Organoids may be assessed for glial marker expression on Day 75+ by immunofluorescence staining (see Assessment of Spinal Cord Organoids).

For related protocols, visit www.stemcell.com/NeuralCultureProtocols.

Assessment of Spinal Cord Organoids

Marker expression of spinal cord organoids can be assessed by immunofluorescence staining on Days 19, 30, and 75. For instructions on how to prepare neural organoids for immunofluorescence staining, refer to the protocol for Cryogenic Tissue Processing and Section Immunofluorescence of Neural Organoids, available at www.stemcell.com.

NOTE: For optimal binding of primary antibodies, antigen retrieval (outlined in the protocol linked above) is highly recommended.

Day 19 spinal cord organoids display interior expression of progenitor marker PAX6 and exterior expression of ventral progenitor markers NKX6.1, OLIG2, and NKX2.2, which can be verified using the following antibodies:

- Anti-PAX6 antibody, rabbit polyclonal (BioLegend #901301),
- Anti-OLIG2 antibody, rabbit polyclonal (MilliporeSigma AB9610),
- Anti-NKX6.1 antibody, mouse monoclonal (DSHB #F55A10-c), and
- Anti-NKX2.2 antibody, mouse monoclonal (DSHB #74.5A5)

Day 30+ spinal cord organoids express motor neuron markers MNX1, ISLET1, FOXP1, and CHAT, as well as V2a glutamatergic interneuron marker CHX10, which can be verified using the following antibodies:

- Anti-MNR2/HB9/MNX1 antibody, mouse monoclonal (DSHB #81.5C10),
- Anti-ISLET1 (EP4182) antibody, rabbit monoclonal (Abcam #AB109517),
- Anti-FOXP1 antibody, mouse monoclonal (RND Systems #mab45341),
- Anti-choline acetyltransferase (CHAT) antibody, goat polyclonal (Sigma-Aldrich #AB144P), and
- Anti-CHX10 antibody, sheep polyclonal (Exalpha Biologicals #X1180P)

Day 75+ spinal cord organoids express oligodendrocyte marker MBP and astrocyte markers S100β and GFAP, which can be verified using the following antibodies:

- Anti-MBP antibody, rabbit polyclonal (MilliporeSigma #AB980),
- Anti-S100β antibody, rabbit polyclonal (Agilent #GA50461-2), and
- Anti-GFAP antibody, rabbit polyclonal (Catalog #60128) or Anti-GFAP antibody, chicken polyclonal (Aves Labs #GFAP)

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.

References

Andersen, J et al. (2020) Generation of Functional Human 3D Cortico-Motor Assembloids. Cell. 183(7):1913-1929.e26.

Yoon SJ et al. (2018) Reliability of human cortical organoid generation. Nature Methods 16: 75–8.