STEMdiff™ Choroid Plexus Organoid Differentiation Kit and STEMdiff™ Choroid Plexus Organoid Maturation Kit

Culture medium kits for establishment and maturation of human choroid plexus organoids

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Product Description

STEMdiff™ Choroid Plexus Organoid Differentiation Kit (Catalog #100-0824) is a defined, serum-free cell culture medium that enables the robust generation of human pluripotent stem cell (hPSC)-derived choroid plexus organoids in a simple five-stage protocol. Choroid plexus organoids are three-dimensional in vitro models with a cellular composition and structural organization that is representative of developing human blood-cerebrospinal fluid (CSF) barrier. STEMdiff™ Choroid Plexus Organoid Kit has been optimized to increase efficiency and reproducibility of organoid formation based on the formulation published by Pellegrini et al., which was awarded a 3Rs Prize for its breakthroughs in animal reduction, replacement, and refinement.[1] Choroid plexus organoid formation is initiated through an intermediate embryoid body (EB) formation step followed by expansion of neuroepithelia and patterning to choroid plexus-like epithelium. After a period of maturation, organoids generated using this kit feature cystic structures filled with a fluid resembling CSF and surrounded by an epithelial layer expressing ependymal choroid plexus-specific markers (TTR, CLIC6, AQP1). For extended periods of organoid culture (> 40 days), the components required for organoid maturation can be purchased as STEMdiff™ Choroid Plexus Organoid Maturation Kit (Catalog #100-0825).

Product Information

All components listed below are sold as part of a kit (Catalog #100-0824 or 100-0825) and are not available for individual sale.

NAME	COMPONENT #	SIZE	STORAGE	SHELF LIFE
STEMdiff™ Choroid Plexus Organoid Differentiation Kit (100-0824)				
STEMdiff™ Neural Organoid Basal Medium 3	100-0826	100 mL	Store at 2 - 8°C.	Stable for 2 years from date of manufacture (MFG) on label.
STEMdiff™ Neural Organoid Basal Medium 4	100-0827	250 mL	Store at 2 - 8°C.	Stable for 2 years from date of manufacture (MFG) on label.
STEMdiff™ Neural Organoid Supplement E*	100-0828	10 mL	Store at -20°C.	Stable for 2 years from date of manufacture (MFG) on label.
STEMdiff™ Neural Organoid Supplement F*	100-0829	0.5 mL	Store at -20°C.	Stable for 2 years from date of manufacture (MFG) on label.
STEMdiff™ Neural Organoid Supplement G**	100-0830	0.25 mL	Store at -20°C.	Stable for 2 years from date of manufacture (MFG) on label.
STEMdiff™ Neural Organoid Supplement H*	100-0831	0.5 mL	Store at -20°C.	Stable for 2 years from date of manufacture (MFG) on label.
STEMdiff™ Neural Organoid Supplement I*	100-0832	4.5 mL	Store at -20°C.	Stable for 2 years from date of manufacture (MFG) on label.
STEMdiff™ Neural Organoid Supplement J*	100-0833	0.5 mL	Store at -20°C.	Stable for 12 months from date of manufacture (MFG) on label.
STEMdiff™ Choroid Plexus Organoid Maturation Kit (100-0825)				
STEMdiff™ Neural Organoid Basal Medium 4	100-0827	250 mL	Store at 2 - 8°C.	Stable for 2 years from date of manufacture (MFG) on label.
STEMdiff™ Neural Organoid Supplement I*	100-0832	4.5 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.

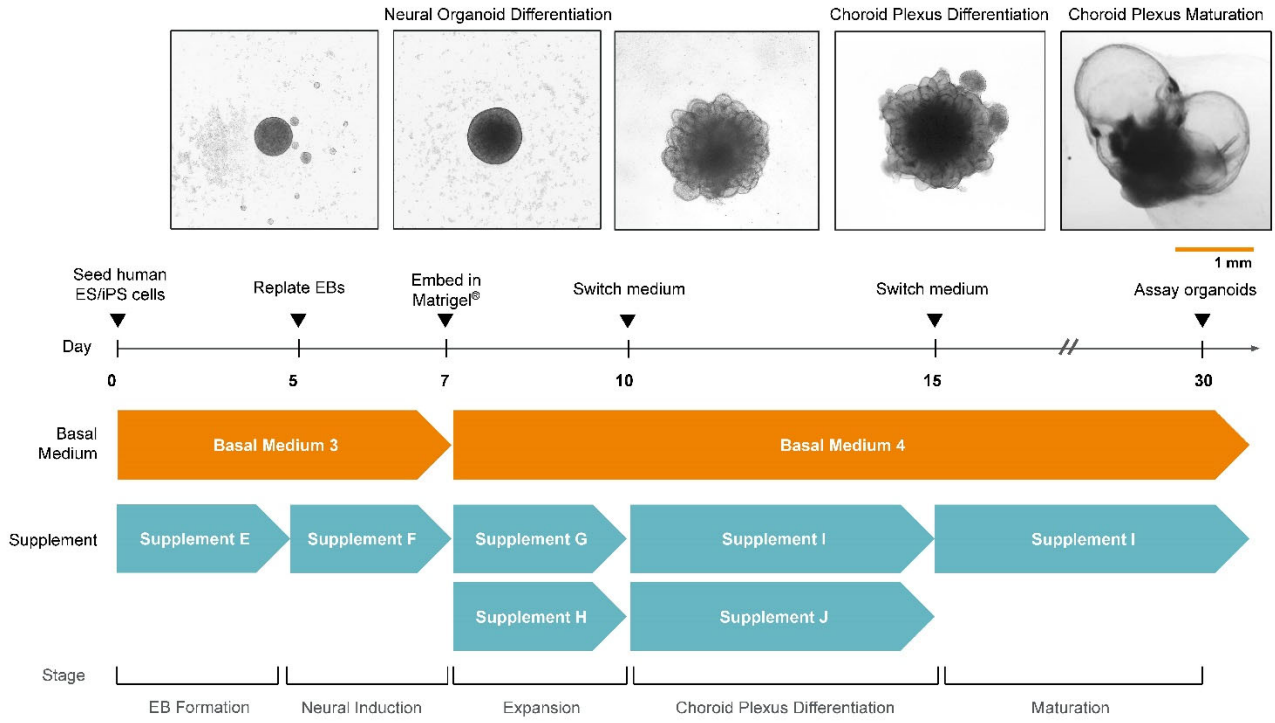
Materials Required But Not Included

PRODUCT NAME	CATALOG #
D-PBS (Without Ca++ and Mg++) (PBS)	37350
Gentle Cell Dissociation Reagent	100-0485
Y-27632	72302
Trypan Blue	07050
Corning® Matrigel® hESC-Qualified Matrix	Corning 354277
Costar® 24-Well Flat-Bottom Plate, Tissue Culture-Treated	38017
Costar® 24-well ultra-low attachment plate	Corning 3473
Corning® 96-well round-bottom ultra-low attachment microplate	Corning 7007
6-Well Ultra-Low Adherent Plates for Suspension Cultures	100-0083
Axygen® 200 µL wide bore universal pipette tips	Fisher Scientific 14-222-730
100 mm Dish, Non-Treated	38045
Conical tubes, 50 mL	e.g. 38010
Serological Pipettes, 5 mL or 10 mL	e.g. 38003 or 38004

Equipment Required

- Sterile forceps
- Embedding surface (e.g. Organoid Embedding Sheet [Catalog #08579] or Parafilm®)
- Orbital shaker + sticky mat (e.g. INFORS HT Celltron; Catalog #69455)

Protocol Diagram



EB: Embryoid body

Preparation of Media

Use sterile technique to prepare STEMdiff™ choroid plexus organoid media. Prepare each medium as indicated 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 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.

Table 1. Preparation of STEMdiff™ Choroid Plexus Organoid Media

MEDIUM	COMPONENT	VOLUME	IN-USE STORAGE AND STABILITY
EB Formation Medium (50 mL)	STEMdiff™ Neural Organoid Basal Medium 3	40 mL	Store at 2 - 8°C for up to 2 weeks.
	STEMdiff™ Neural Organoid Supplement E	10 mL	
Induction Medium (50 mL)	STEMdiff™ Neural Organoid Basal Medium 3	49.5 mL	Store at 2 - 8°C for up to 2 weeks.
	STEMdiff™ Neural Organoid Supplement F	0.5 mL	
Expansion Medium (25 mL)	STEMdiff™ Neural Organoid Basal Medium 4	24.25 mL	Store at 2 - 8°C for up to 2 weeks.
	STEMdiff™ Neural Organoid Supplement G	0.25 mL	
	STEMdiff™ Neural Organoid Supplement H	0.5 mL	
Choroid Plexus Differentiation Medium (50 mL)	STEMdiff™ Neural Organoid Basal Medium 4	48.5 mL	Store at 2 - 8°C for up to 2 weeks.
	STEMdiff™ Neural Organoid Supplement I	1 mL	
	STEMdiff™ Neural Organoid Supplement J	0.5 mL	
Maturation Medium (100 mL)	STEMdiff™ Neural Organoid Basal Medium 4	98 mL	Store at 2 - 8°C for up to 2 weeks.
	STEMdiff™ Neural Organoid Supplement I	2 mL	

Directions for Use

Please read the entire protocol before proceeding.

Use sterile technique when performing the following protocols:

A. EB Formation (Day 0 - 5)
B. Induction (Day 5 - 7)
C. Expansion (Day 7 - 10)
D. Choroid Plexus Differentiation (Day 10 - 15)
E. Choroid Plexus Organoid Maturation (Day 15 - 30+)

A. EB FORMATION (Day 0 - 5)

This protocol is for the formation of EBs from an hPSC culture in a single well of a 6-well plate. For other cultureware, 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

1. Prepare EB Formation Medium (see Preparation of Media) and warm to room temperature.
2. Prepare EB Seeding Medium as follows: Add 30 µL of 5 mM Y-27632 to 15 mL of EB Formation Medium (final concentration 10 µM).
3. Use a microscope to visually identify regions of differentiation in the hPSC culture. Remove regions of differentiation by scraping with a pipette tip or by aspiration.
4. Aspirate medium from hPSC culture and wash the well with 1 mL of sterile phosphate-buffered saline (PBS).
5. Aspirate PBS and add 1 mL of Gentle Cell Dissociation Reagent.

6. 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.
7. Using a 1 mL pipettor, gently resuspend cells by pipetting up and down slowly 3 - 5 times. Transfer cell suspension to a sterile 50 mL conical tube.
8. Rinse the well with an additional 1 mL of EB Seeding Medium and add this rinse to the tube containing cells.
9. Centrifuge cells at 300 x *g* for 5 minutes.
10. Remove and discard supernatant. Add 1 - 2 mL of EB Seeding Medium to resuspend cells.
11. Count cells using Trypan Blue and a hemocytometer.
12. Calculate the volume of cells required to obtain 90,000 cells/mL; add this volume of cells to an appropriate volume of EB Seeding Medium.
13. Add 100 µL of cell suspension from step 12 into each well of a 96-well round-bottom ultra-low attachment plate (9000 cells/well).

    NOTE: To improve efficiency and reproducibility of EB formation, a multi-channel pipettor is recommended for this step.
14. Incubate 96-well plate at 37°C. Do not disturb plate for at least 24 hours.
15. Observe plate under microscope. Small EBs (100 - 200 µm) will be observed with a layer of unincorporated cells around the central EB.

Day 2 - 5

16. On day 2 and day 4, gently add 100 µL of EB Formation Medium per well. A multi-channel pipettor is recommended for this step. Incubate at 37°C.
17. On day 5, observe EBs under microscope. EBs should reach a diameter of > 300 µm (typically 400 - 600 µm) and exhibit round and smooth edges (see image in Protocol Diagram).
18. Proceed to section B (Induction).

B. INDUCTION (Day 5 - 7)

NOTE: Warm cultureware, medium, and reagents to room temperature (15 - 25°C) before use.

NOTE: If ultra-low attachment plates are not available, tissue culture-treated cultureware can be used if it is pre-treated with Anti-Adherence Rinsing Solution (Catalog #07010) to prevent cell attachment.

Day 5

1. Prepare Induction Medium (see Preparation of Media) and warm to room temperature.
2. Add 0.5 mL of Induction Medium to each well of a 24-well ultra-low attachment plate.
3. Add 1 - 2 EBs to each well of the 24-well plate as follows:
   a. Using a wide-bore 200 µL pipette tip, draw up 50 µL from one well of the 96-well plate from section A to obtain EB(s).
   b. Remove most of the medium by carefully ejecting it back into the well, retaining EB(s) in the pipette tip.
   c. Dispense EB(s) into one well of the 24-well plate containing Induction Medium.

   NOTE: Ensure that EBs are evenly distributed in the well by shaking the plate back and forth 3 - 4 times in incubator. EBs that touch are more likely to merge. If a high number of EBs merge, transfer only a single EB per well.
4. Incubate plate at 37°C for 48 hours. EBs will maintain smooth edges and develop optically translucent edges (see image in Protocol Diagram).
5. Proceed to section C (Expansion).

C. EXPANSION (Day 7 - 10)

Day 7

1. Thaw Matrigel® on ice at 2 - 8°C for 1 - 2 hours.

   NOTE: Thaw enough Matrigel® to have 15 µL/EB (e.g. 96 wells x 15 µL/well = 1.44 mL Matrigel®).

   NOTE: Keep Matrigel® on ice to prevent premature polymerization. All plasticware that comes in contact with Matrigel® should be chilled at -20°C for at least 30 minutes prior to use.
2. Prepare Expansion Medium (see Preparation of Media) and warm to room temperature.
3. Place embedding surface (e.g. Organoid Embedding Sheet or Parafilm®) into an empty, sterile, 100 mm dish.
4. Using a wide-bore 200 µL pipette tip, draw up 25 - 50 µL of medium + EB from one well of the 24-well plate and transfer to embedding surface. Repeat this step until 12 - 16 EBs are collected on the embedding surface.

   NOTE: Embed no more than 12 - 16 EBs at a time; this will prevent the EBs from drying out and the Matrigel® from prematurely polymerizing.

5. Remove excess medium from each EB by carefully drawing up medium with a standard 200 µL pipette tip. Position the opening of the tip so that it is pointing away from the EB to avoid drawing it up.
6. Using a pipettor with a cold 200 µL standard pipette tip, add 15 µL of Matrigel® dropwise onto each EB.
7. Using a new cold 200 µL pipette tip, reposition the EB to the center of the droplet.
8. Place the plate in an incubator at 37°C for 30 minutes to polymerize Matrigel®.
9. Use sterile forceps to grasp embedding surface containing Matrigel® droplets.
10. Position sheet directly above one well of a 6-well ultra-low adherent plate. Using a 1 mL pipettor, draw up Expansion Medium and gently wash Matrigel® droplets off the sheet and into the well. Use 3 mL of Expansion Medium/well. Repeat until all 12 - 16 Matrigel® droplets are in the well.
11. Incubate at 37°C for 3 days. Embedded organoids will develop expanded neuroepithelia as evidenced by budding of the EB surface .
12. Proceed to section D (Choroid Plexus Differentiation).

D. CHOROID PLEXUS DIFFERENTIATION (Day 10 - 15)

Day 10

1. Prepare Choroid Plexus Differentiation Medium (see Preparation of Media) and warm to room temperature.
2. Using a 5 mL or 10 mL serological pipette at the slowest setting, carefully remove all medium from wells containing organoids. Do not disturb Matrigel®-embedded organoids.
3. Replace medium with 3 mL/well of Maturation Medium.
4. Place plate of organoids on an orbital shaker in a 37°C incubator. For the INFORS HT Celltron orbital shaker, set the shaker speed (rpm) according to Table 2. For other orbital shaker models, calculate the rpm using the equation in Figure 1. Incubate for 3 days.

Day 13

5. Perform a full-medium change with fresh Choroid Plexus Differentiation Medium, as follows:
   a. Tilt the cultureware.
   b. Using a 5 mL serological pipette at the slowest setting, slowly remove medium.
   c. Add 3 mL/well of fresh Maturation Medium.
   d. Return plate to the orbital shaker in a 37°C incubator and incubate for 2 days.
6. The budding of the EB surface will expand as choroid plexus epithelium is formed (see image in Protocol Diagram).
7. Proceed to section E (Organoid Maturation).

E. CHOROID PLEXUS ORGANOID MATURATION (Day 15 - 30+)

Day 15

1. Prepare Maturation Medium (see Preparation of Media) and warm to room temperature.
2. Using a 5 mL or 10 mL serological pipette at the slowest setting, carefully remove all medium from wells containing organoids. Do not disturb Matrigel®-embedded organoids.
3. Replace medium with 3 mL/well of Maturation Medium.
4. Place plate of organoids on an orbital shaker in a 37°C incubator. For the INFORS HT Celltron orbital shaker, set the shaker speed (rpm) according to Table 2. For other orbital shaker models, calculate the rpm using the equation in Figure 1.

Table 2. Recommended Shaker Speeds for INFORS HT Celltron Orbital Shaker for Various Cultureware

CULTUREWARE*	VOLUME OF MEDIUM (mL)	RECOMMENDED SHAKER SPEED (rpm)	RELATIVE CENTRIFUGAL FORCE (RCF)† (*g*)
6-well plate or 60 mm dish	3	65	0.11808
12-well plate	1.5	85	0.20194
24-well plate	1	100	0.27950

*6-well and 12-well plates are optimal cultureware for organoid culture.
†Calculated using throw (shaking diameter) of 25 mm for the INFORS HT Celltron.

$$rpm = \sqrt{\frac{RCF}{throw \times 1.118}} \times 1000$$

Where:
rpm = shaker speed (revolutions per minute)
RCF = relative centrifugal force (*g*), provided in Table 2 for various cultureware
throw = shaking diameter (mm), as specified by manufacturer

Figure 1. Conversion of RCF to rpm

5. Perform a full-medium change every 3 - 4 days as follows:
   a. Tilt the cultureware.
   b. Using a 5 mL serological pipette at the slowest setting, slowly remove medium.
   c. Add 3 mL/well of fresh Maturation Medium.
   d. Return plate to the orbital shaker in a 37°C incubator.

Day 30+

By day 30, organoids will exhibit cystic structures forming around a dense core, and can be used for analysis (see image in Protocol Diagram). There is a > 70% success rate for obtaining organoids with cystic structures by day 30; this rate will vary with different cell lines. Organoids with cystic structures can be grown beyond day 30 whereby the cyst may continue to balloon and enlarge; however, heterogeneous morphologies will be apparent.

6. Assay organoids by cryosectioning/immunolabeling and/or by RT-qPCR. The following are marker proteins for immunolabeling:
   - Choroid plexus markers: TTR, CLIC6, AQP1
   - CSF markers: CLU, IGF2
   - Cortical plate/pre-plate/neuron markers: PAX6, MAP2, FOXG1
7. Choroid plexus organoids can continue to be cultured beyond day 30 using STEMdiff™ Choroid Plexus Organoid Maturation Kit (Catalog #100-0825).

   NOTE: For applications requiring transferring cystic organoids, use a 1 mL pipette tip cut to a bore size of ~3 - 5 mm. Transfer the organoids gently, as cysts may be prone to shearing if not handled carefully.

Troubleshooting

STAGE	PROBLEM	POSSIBLE CAUSE	RECOMMENDED ACTION
EB Formation (Day 0 - 5)	Many small EBs do not form into a large central EB	Starting hPSCs were of poor quality	• Check pluripotency by observing morphology and labeling pluripotency markers OCT3/4 and TRA-1-60 to ensure hPSCs are of high quality. If OCT3/4 and TRA-1-60 are low (< 90%), restart with high-quality hPSCs. • Start with high-quality hPSCs from an earlier passage number • Passage cells when they are no more than 70 - 80% confluent
		96-well plate was disturbed during culture	• Ensure 96-well plate is not disturbed for at least 24 hours after seeding (section A, step 14)
	EB surface has significant outgrowth or does not have brightening along edges	Starting hPSCs were of poor quality	• Check pluripotency by observing morphology and staining for pluripotency markers OCT3/4 and TRA-1-60 to ensure hPSCs are of high quality. If OCT3/4 and TRA-1-60 are low (< 90%), restart with high-quality hPSCs. • Start with high-quality hPSCs from an earlier passage number • Passage cells when they are no more than 70 - 80% confluent
Induction (Day 5 - 7)	EBs exhibit large balls of outgrowth	Starting hPSCs were of poor quality	• Do not continue with the experiment; restart with high-quality hPSCs
		Too much EB Formation Medium was transferred	Transfer only a small amount of EB Formation Medium when transferring EBs (~10 - 20 µL in 500 µL)
	Large amount of cell debris in well	Too much EB Formation Medium was transferred	Transfer only a small amount of EB Formation Medium when transferring EBs (~10 - 20 µL in 500 µL)
Expansion (Day 7 - 10)	No expansion or increase in surface area, as indicated by bubbling of the EB surface.	Matrigel® lot was sub-optimal	Use alternative lot of Matrigel®
		Improper timing of Matrigel® embedding	The time required for appearance of the neuroepithelium may vary depending on cell line. Continue Induction phase beyond 7 days (up to day 10 - 11) if sub-optimal expansion continues to occur.
		Starting hPSCs were of poor quality	Do not continue with this experiment; restart with high-quality hPSCs

STAGE	PROBLEM	POSSIBLE CAUSE	RECOMMENDED ACTION
Differentiation (Day 10 - 15)	No increase in bubbling of the EB surface	Failure of differentiation medium	Confirm that supplement J was added to differentiation medium (Table 1). Check that supplements were stored correctly and not freeze-thawed more than once.
		Starting hPSCs were of poor quality	Do not continue with this experiment; restart with high-quality hPSCs
Maturation (Day 15 - 30+)	No cyst formation	Failure of differentiation medium	Confirm that supplement J was added to differentiation medium (Table 1). Check that supplements were stored correctly and not freeze-thawed more than once.
		Starting hPSCs were of poor quality	Do not continue with this experiment; restart with high-quality hPSCs

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

1. Pellegrini L et al. (2020) Human CNS barrier-formaing organoids with cerebrospinal fluid production. Science 369(6500): 5626.