STEMdiff™ Midbrain Organoid Differentiation Kit
STEMdiff™ Neural Organoid Maintenance Kit

Catalog #100-1096 1 Kit
Catalog #100-0120 1 Kit

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Product Description

STEMdiff™ Midbrain Organoid Differentiation Kit is a matrix-free, serum-free cell culture medium that enables the robust generation of human pluripotent stem cell-derived midbrain organoids. The medium works with AggreWell™-generated aggregates to prevent organoid fusion and enables the scalable generation of over 500 highly reproducible organoids per kit. Adapted from protocols by Sergiu Paşca (Yoon et al.), midbrain organoids are regionalized, three-dimensional in vitro models with a cellular composition and structural organization that is representative of the developing human midbrain. 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).

Ordering Information

PRODUCT NAME	CATALOG #	SIZE	KIT COMPONENTS
STEMdiff™ Midbrain Organoid Differentiation Kit	100-1096	1 Kit	• STEMdiff™ Neural Organoid Basal Medium 1 • STEMdiff™ Neural Organoid Basal Medium 2 • STEMdiff™ Neural Organoid Supplement A • STEMdiff™ Neural Organoid Supplement C • STEMdiff™ Neural Organoid Supplement K • STEMdiff™ Neural Organoid Supplement L
STEMdiff™ Neural Organoid Maintenance Kit	100-0120	1 Kit	• STEMdiff™ Neural Organoid Basal Medium 2 • STEMdiff™ Neural Organoid Supplement A

Component Storage and Stability

The following components are sold as part of a kit (Catalog #100-1096) and are not available for individual sale.

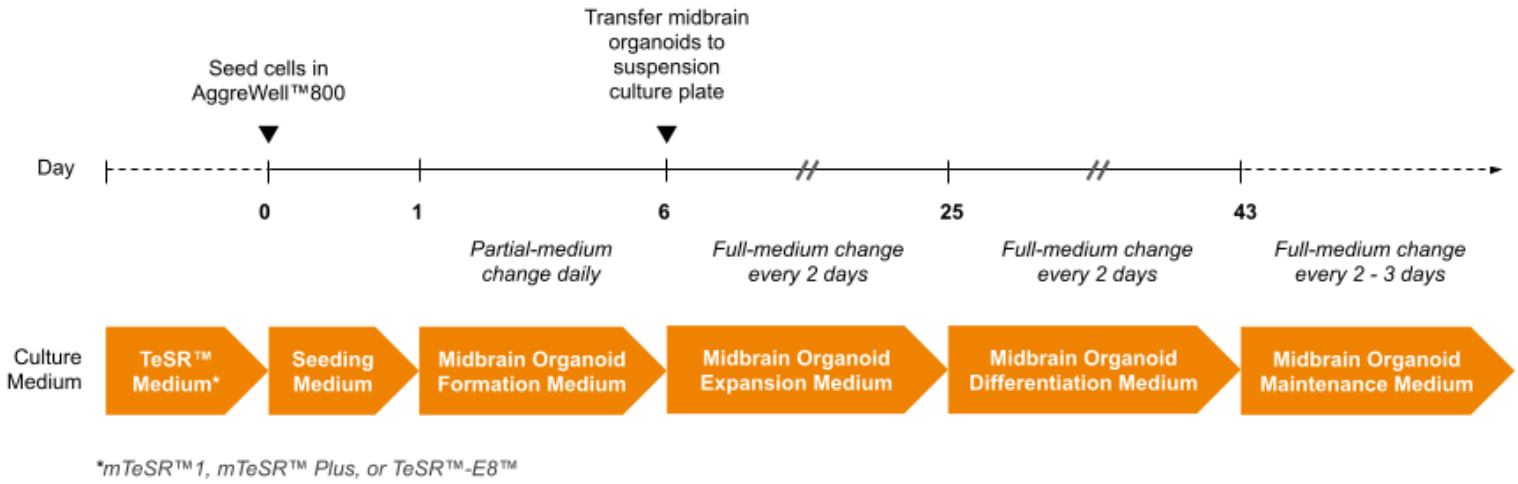
COMPONENT NAME	COMPONENT #	SIZE	STORAGE	SHELF LIFE
STEMdiff™ Neural Organoid Basal Medium 1	08621	20 mL	Store at -20°C.	Stable for 18 months from date of manufacture (MFG) on label.
STEMdiff™ Neural Organoid Basal Medium 2	08622	500 mL	Store at 2 - 8°C.	Stable for 18 months from date of manufacture (MFG) 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 K	100-1094	0.5 mL	Store at -20°C.	Stable for 12 months from date of manufacture (MFG) on label.
STEMdiff™ Neural Organoid Supplement L*	100-1095	0.5 mL	Store at -20°C.	Stable for 12 months 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.

Materials Required but Not Included

PRODUCT NAME	CATALOG #
AggreWell™800 (1 x 24-well plate)	34811
Anti-Adherence Rinsing Solution	07010
Culture Dish, Non-Treated, 100 mm	38045
DMEM/F-12 with 15 mM HEPES	36254
D-PBS (Without Ca++ and Mg++)	37350
Falcon® Conical Tubes, 15 mL or 50 mL	100-0092 (15 mL) OR 100-0090 (50 mL)
Falcon® Serological Pipettes, 5 mL or 10 mL	38003 OR 38004
Gentle Cell Dissociation Reagent	100-0485
Hausser Scientific™ Bright-Line Hemocytometer	100-1181
Reversible Strainer, 37 µm	27215
Trypan Blue	07050
Ultra-Low Adherent Plates for Suspension Cultures, 6-Well	100-0083
Wide-bore disposable pipette tips, 200 µL and 1 mL	Fisher Scientific 14-222-730 (200 µL) and 14-222-699 (1 mL)
Y-27632 (Dihydrochloride)	72302

Protocol Diagram



Preparation of Media

Use sterile technique to prepare STEMdiff™ Midbrain 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 aliquots, use immediately. Do not re-freeze.

2. Combine components as indicated in Table 1. Mix thoroughly. Warm to room temperature before use.

   NOTE: If not used immediately, store media as indicated in Table 1.

Table 1. Preparation of STEMdiff™ Midbrain Organoid Media

MEDIUM	COMPONENT	VOLUME	IN-USE STORAGE AND STABILITY
Midbrain Organoid Formation Medium (20 mL)	STEMdiff™ Neural Organoid Basal Medium 1	20 mL	Store at 2 - 8°C for up to 3 weeks. Do not exceed the shelf life of the basal medium.
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	
Midbrain Organoid Expansion Medium (250 mL)	STEMdiff™ Neural Organoid Basal Medium 2*	245 mL	Store at 2 - 8°C for up to 3 weeks. Do not exceed the shelf life of the basal medium or supplements.
	STEMdiff™ Neural Organoid Supplement A	5 mL	
	STEMdiff™ Neural Organoid Supplement K	0.5 mL	
	STEMdiff™ Neural Organoid Supplement L	0.5 mL	
Midbrain Organoid Differentiation Medium (250 mL)	STEMdiff™ Neural Organoid Basal Medium 2*	245 mL	
	STEMdiff™ Neural Organoid Supplement A	5 mL	
	STEMdiff™ Neural Organoid Supplement C	0.25 mL	
Midbrain Organoid Maintenance Medium (100 mL)**	STEMdiff™ Neural Organoid Basal Medium 2*	98 mL	
	STEMdiff™ Neural Organoid Supplement A	2 mL	

*This medium is viscous; pipette slowly to ensure 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. Midbrain Organoid Formation (Day 0 - 6)
B. Midbrain Organoid Expansion (Day 6 - 25)
C. Midbrain Organoid Differentiation (Day 25 - 43)
D. Midbrain Organoid Maintenance (Day 43+)

A. MIDBRAIN ORGANOID FORMATION (Day 0 - 6)

The following instructions are for generating a single-cell suspension of human embryonic stem (ES) or induced pluripotent stem (iPS) cells previously cultured in mTeSR™1, mTeSR™ Plus, or TeSR™-E8™ in a 100 mm dish and then plating cells into one well of an AggreWell™800 24-well plate. If using other cultureware, adjust volumes accordingly. Warm cultureware, media, and reagents to room temperature (15 - 25°C) before use.

NOTE: Human pluripotent stem cells (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 an AggreWell™800 24-well plate:
   a. Add 500 µL of Anti-Adherence Rinsing Solution to each well to be used.
   b. Centrifuge 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 microwells. If bubbles remain trapped in any microwells, centrifuge at 1300 x *g* for an additional 5 minutes.
   d. Aspirate Anti-Adherence Rinsing Solution from the wells.
2. Prepare Seeding Medium (see Preparation of Media) and warm to room temperature.
3. Add 1 mL Seeding Medium to 1 well of the plate prepared in step 1. Set the plate aside.
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. Aspirate medium from hPSC culture and wash the well with 3 - 5 mL sterile phosphate-buffered saline (PBS).

6. Aspirate PBS and add 3 mL of Gentle Cell Dissociation Reagent. 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 15 mL or 50 mL conical tube.
8. Rinse the well with an additional 5 - 10 mL of DMEM/F-12 and add this rinse to the tube containing cells.
9. Count viable cells using Trypan Blue and a hemocytometer. Calculate 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 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, 5% CO_2.

Day 1 - 5: Partial-Medium Change

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

15. 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 having organoids come out of the well, which will result in premature fusion and lower yield.
16. Gently remove 2 x 750 µL of medium from the well using a 1 mL pipettor and discard.

    NOTE: Do not disturb the organoids. Keep the pipette tip toward the upper surface of the medium in the well while removing the medium.
17. Slowly add 2 x 750 µL of Midbrain Organoid Formation Medium to the well using a 1 mL pipettor.

    NOTE: It is important not to disturb the organoids. 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. Release the medium very slowly into the well; quick release of medium will dislodge the organoids from the wells.
18. Incubate at 37°C, 5% CO_2 for 24 hours.
19. Repeat steps 15 - 18 on Days 2 - 5.
20. On Day 6, proceed to section B for organoid expansion.

B. MIDBRAIN ORGANOID EXPANSION (Day 6 - 25)

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 to prevent cell attachment. Add 1 mL of Anti-Adherence Rinsing Solution to each well, then remove solution immediately from the well and discard.

Day 6

1. Prepare Midbrain Organoid Expansion Medium (see Preparation of Media) and warm to room temperature.
2. Add 2 mL of Midbrain Organoid Expansion Medium to each well of a 6-Well Ultra-Low Adherent Plate.

   NOTE: Organoids from one well of an AggreWell™800 24-well plate can be evenly distributed into the 6-well plate. Do not exceed 40 organoids per well; the recommended range is 25 - 40 organoids per well. Controlling the number of organoids in the well is critical to avoid loss in yield through premature fusion events.
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 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 well.

5. Using the same wide-bore tip, aspirate the suspension and filter it through the 37 µm Reversible Strainer. Organoids will remain on top of the strainer and single cells will flow through into the waste tube.
6. Draw up 1 mL of DMEM/F-12 using the wide-bore tip and firmly expel it into the same AggreWell™800 well. While 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 well. One or two repeats should be sufficient to dislodge all organoids. Examine the well under a microscope to ensure that all organoids have been removed.
8. Invert the strainer over a new 50 mL conical tube and add 2 mL of Midbrain Organoid Expansion Medium onto the strainer to collect all the organoids in the tube.

   NOTE: Expansion Medium is viscous, thus a high pipetting force might be required to efficiently collect organoids from strainer.
9. Gently swirl the organoids to create a suspension and use a wide-bore tip to transfer organoids into the 6-well plate. **Do not exceed 40 organoids per well of a 6-well plate**.
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 plate on a level surface in a 37°C, 5% CO_2 incubator. Incubate for 2 days.

Day 8 - 25: Full-Medium Change

12. Warm Midbrain Organoid Expansion Medium to room temperature.
13. Gently tilt the 6-well plate and wait for organoids to sink to the bottom of the well (~15 - 30 seconds).
14. Carefully level the plate and use a 1 mL pipettor to remove medium from the top portion of each well.
15. Add 2 mL of fresh Midbrain Organoid Expansion Medium to each well. Immediately before incubating, gently shake the plate in short, back-and-forth and side-to-side motions to distribute the organoids across the wells. Visually inspect the plate to ensure minimal contact between organoids. Carefully place the plate on a **level surface** in a 37°C, 5% CO_2 incubator and ensure the plate is not disturbed. The shaking step must be completed any time the plate is moved.
16. Perform a full-medium change (steps 12 - 15) **every 2 days** on Day 8 - 25.
17. On Day 25, proceed to section C for organoid differentiation.

C. MIDBRAIN ORGANOID DIFFERENTIATION (Day 25 - 43)

1. Prepare Midbrain Organoid Differentiation 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 Midbrain Organoid Differentiation Medium to each well. Incubate at 37°C, 5% CO_2.
5. Perform a full-medium change (steps 1 - 4) every 2 days on Day 27 - 43.

   NOTE: If 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).
6. On Day 43, proceed to section D for organoid maintenance.

D. MIDBRAIN ORGANOID MAINTENANCE (Day 43+)

1. Prepare Midbrain 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 Midbrain Organoid Maintenance Medium to each well.
5. Perform a full-medium change every 2 - 3 days.

   NOTE: If medium becomes 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 are larger (i.e. Day 50+), increase feed volume to 3 mL per well to ensure they are covered by medium.

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

Assessment of Midbrain Cells

Assessment of midbrain cells can be verified on Day 50 by immunostaining. By Day 50, markers specific to midbrain cell phenotypes can be detected in the peripheral areas of midbrain organoids. These markers include FOXA2+ and LMX1A+ midbrain progenitor cells, TH+ dopaminergic neurons, and GIRK2+ dopaminergic cells that are found in substantia nigra in vivo.

- Anti-human tyrosine hydroxylase (TH) antibody (Pel-Freeze, P40101-150)
- Anti-human LMX1A antibody
- Anti-human HNF-3 beta/FoxA2 antibody (R&D Systems, AF2400)
- Anti-human GIRK2 antibody (Alomone labs, APC-006)

Midbrain organoids can continue to be cultured beyond Day 50 using STEMdiff™ Neural Organoid Maintenance Kit (Catalog #100-0120).

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

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