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Product Information

Astrocyte Culturing Information for iPSC-differentiated neurons

Catalog Numbers **AST1001, AST1002, AST1006, AST1009, AST1037, AST1039, and AST1040**
Storage Temperature –196 °C (Liquid Nitrogen, LN_2)

TECHNICAL BULLETIN

Product Description

These products contain cryo-preserved, pre-differentiated astrocyte precursors derived from a footprint-free, karyotype normal human iPSC line. They are designed for customers to generate mature astrocytes using the optimized maturation medium and supplements. Mature astrocytes can be obtained within 17 days. Shortly after seeding and recovery, the cells proliferate and need to be passaged a minimum of two times. In general, at 17 days post-seeding, the cell population will contain ≥80% Glial Fibrillary Acidic Protein (GFAP) positive astrocytes and ≤15% Tuj-1 positive neurons (neuronal class III β-tubulin).

Reagents and Equipment Required but Not Provided.

Astrocyte Medium with Supplement A, Supplement B, and Supplement C
(Catalog Numbers AST1050 [50 mL of medium] and AST1051 [250 mL of medium])

BD Matrigel™ hESC-qualified Matrix
(BD Biosciences Catalog Number 354277)

StemPro® Accutase® Cell Dissociation Reagent
(Life Technologies Catalog Number A11105-01)

DMEM, Dulbecco's Modified Eagle Medium
(Life Technologies Catalog Number 11965-084)

0.4% Trypan Blue solution
(Catalog Number T8154)

Primary antibodies:

Monoclonal anti-β-tubulin III, isotype III
clone SDL.3D10 (Catalog Number T8660)

Monoclonal Anti-Glial Fibrillary Acidic Protein (GFAP)
(Dako Catalog Number M0761)

Precautions and Disclaimer

This product is for R&D use only, not for drug, household, or other uses. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

Storage/Stability

The storage conditions for the astrocyte precursors and the required Astrocyte Medium are shown in Table 1.

A vial with cryopreserved astrocyte precursors is packed in a small Ziploc® bag, which is buried in the dry ice. Upon receiving the product, check the integrity of the package and the presence of dry ice.

Cells can be plated immediately after arrival or transferred into liquid nitrogen. Do not remove the vials from the dry ice during transportation to liquid nitrogen storage units. Transfer the cryopreserved astrocyte precursors directly to liquid nitrogen storage units from the dry ice, avoiding exposure to room temperature.

Re-freezing the Astrocyte Medium and supplements, or cryopreserving cultured astrocytes is not recommended.

Table 1.
Storage conditions of astrocyte precursor components

Component	Storage Temperature
Cell vials	–196 °C (Liquid Nitrogen, LN_2)
Astrocyte Medium	2–8 °C
Supplement A	2–8 °C
Supplement B	–20 °C
Supplement C	–20 °C

Astrocyte Maturation Procedure
This procedure has been extensively tested with the astrocyte precursors and Astrocyte Medium. The user should follow this procedure closely. The user assumes all responsibility for the failure of the experiment should there be any deviation from this procedure.

Coating Cell Culture Vessels with Matrigel
Cell culture vessels should be coated one day before or on the day of plating the cells. Please read manufacturer's manual for handling of BD Matrigel hESC-qualified Matrix.

It is extremely important that BD Matrigel hESC-qualified Matrix and all culture ware or media coming in contact with BD Matrigel hESC-qualified Matrix be pre-chilled/ice-cold since BD Matrigel hESC-qualified Matrix will start to gel >10 °C.

The dilution is calculated for each lot based on the protein concentration. Prepare aliquots according to the dilution factor provided on the BD Certificate of Analysis (use 1.5–2.0 mL tubes). The volume of the aliquots is typically between 270–350 µL.

1. Pre-chill pipettes tips and dishes at 2–8 °C.
2. Thaw an aliquot (typically between 270–350 µL) of BD Matrigel hESC-qualified Matrix at 2–8 °C (45–60 minutes).
3. Transfer the aliquot on ice into a biological safety cabinet
4. Prepare 25 mL aliquot of cold DMEM/F12 in 50 mL conical tube and keep on ice.
5. Using a micropipette and cold tips, transfer 1,000 µL of the cold DMEM/F12 from the conical tube to the tube with the Matrigel aliquot, and mix up and down several times. Transfer the Matrigel mixture back to the 50 mL conical tube containing the cold DMEM/F12 and mix several times with a serological pipette (keep on ice).
6. Immediately, coat pre-chilled culture dishes with Matrigel/DMEM F12 solution (volume 120–150 µL/cm^2), see Table 2.
7. Distribute coating matrix evenly and incubate at room temperature (15–25 °C) for at least 1 hour before use.
8. Coated dishes can be used immediately or can be stored at 2–8 °C for up to 7 days (aseptic conditions).
9. Pre-warm vessels at 37 °C before use.
10. Aspirate Matrigel just before seeding astrocyte precursors. Do not let the surface dry.

Table 2.
Recommended volumes of coating matrix for various vessels

Vessel type	Matrigel/DMEM F12 solution
96 well plate	50 µL/well
4 or 24 well plate	250 µL/well
35 mm dish	1.5 mL
60 mm dish	2.5 mL

Thawing and Culturing Cryopreserved Astrocyte Precursors
All steps described must be performed in accordance with aseptic cell culture standards. All media and vessels used for cell culture must be pre-warmed to 37 °C prior to use.

For the entire process of astrocyte maturation, only the Complete Astrocyte Medium is required (Astrocyte Medium with Supplement A, Supplement B, and Supplement C).

Thawed astrocyte precursor cells will be seeded onto Matrigel coated 35 mm dish (1.0–1.4 $\times$ 10^5 live cells/cm^2).

1. On the day of thawing the cryopreserved astrocytes, transfer an aliquot of 25 mL of Astrocyte Medium from the 50 mL bottle into a 50 mL conical tube and add supplements to prepare the Complete Astrocyte Medium, see Table 3.
   Note: It is not recommended to re-freeze supplements and medium.

Table 3.
Preparation of an aliquot of Complete Astrocyte Medium

Component	Volume
Astrocyte Medium	25 mL
Supplement A	2.25 mL
Supplement B	0.5 mL
Supplement C	25 µL

2. Transfer a 5 mL aliquot of Complete Astrocyte Medium prepared in step 1 into a 15 mL conical tube and pre-warm at 37 °C. This aliquot will be used for recovery of the precursor cells from frozen stock.

3. Prepare another 2 mL aliquot of Complete Astrocyte Medium (volume for 35 mm culture dish) and pre-warm at 37 °C. Keep remaining medium at 2–8 °C.

4. Shortly before thawing the cells, place pre-warmed medium and coated 35 mm dish in a biosafety cabinet.

5. To thaw cryopreserved astrocyte precursors, remove the vial from the liquid nitrogen storage unit and place immediately onto dry ice (the vial must be buried in the dry ice).

6. Bring the dry ice container with the vial to the site with the 37 °C water bath.

7. Immerse the vial in the water bath (up to 2/3rd of the vial) and thaw cells until only a small piece of ice is still visible (~1 minute).
   Note: Do not shake vial during thawing.

8. Immediately bring the vial to the biological cabinet, spraying it thoroughly with 70% ethanol solution and wiping it with an autoclaved paper towel.

9. Remove cells from the vial using a micropipette (or serological pipette) and transfer drop-wise while swirling into the 15 mL conical tube containing 5 mL of pre-warmed Complete Astrocyte Medium (step 2). Wash the vial with 1 mL of medium from the 15 ml conical tube and transfer it back to the tube.
   Note: Do not mix cells up and down, and avoid generating bubbles.

10. Centrifuge cells at 400 $\times$ *g* for 5 minutes at room temperature.

11. Aspirate the medium very carefully using a vacuum (or pipette if preferred), leaving only a drop of liquid in the tube. Take extra care not to remove or disturb the cell pellet during aspiration of medium.

12. Using a micropipette, add 1 mL of Complete Astrocyte Medium (step 3) into the tube and gently re-suspend cells by pipetting up and down 4–6 times.

13. Remove a 10 μL aliquot of cell suspension and mix it with 10 μL of 0.4% Trypan blue solution.

14. Count the cells.

15. Aspirate Matrigel solution from pre-warmed 35 mm cell culture dish and immediately transfer 1 mL of solution of thawed astrocyte precursors into the dish. Wash the conical tube with an additional 1 mL of Complete Astrocyte Medium and transfer to the same culture dish. Cell density should be in range of 1–1.3 $\times$ 10^5/cm^2 (1–1.3 $\times$ 10^6 cells/vial).

16. Distribute cells evenly and place the dish in the cell culture incubator (37 °C/5% CO_2/humidity control). The day of seeding cells is called **day 0**.

17. Monitor cell survival the following day (**day 1**).

18. Change Complete Astrocyte Medium at **day 2** post-seeding. Medium change should be done slowly (drop wise) pointing the pipette tip toward the wall of the cell culture vessel.
    Note: Medium should be changed every other day.

19. Monitor cell growth every day.

20. At **day 7** post thaw, the astrocytes in the 35 mm dish will reach full confluence. Passage the cells as described in the following procedure.

    a. Aspirate medium from the dish and add 1 mL of fresh pre-warmed Accutase.
    b. Keep the dish in the cell culture incubator until cells detach (3–5 minutes).
    c. Add 1 mL of pre-warmed DMEM medium into the dish and re-suspend floating cell layer with micropipette by pipetting up and down several times.
    d. Transfer astrocytes into a 15 mL conical tube containing 5 mL of pre-warmed Complete Astrocyte Medium.
    e. Centrifuge the cells at 400 $\times$ *g* for 5 minutes.
    f. Carefully aspirate liquid and gently re-suspend cell pellet in 2 mL of Complete Astrocyte Medium.
    g. Remove a 10 μL aliquot of cell suspension and mix it with 10 μL of 0.4% Trypan blue solution.
    h. Perform an accurate live cell count.

21. Remove Matrigel from pre-warmed 60 mm dish and immediately transfer 2 mL of the astrocyte solution into it. The appropriate density of cells is $8 \times 10^4 - 1 \times 10^5$ live cells/cm^2 (per 60 mm dish, $1.6 \times 10^6 - 2 \times 10^6$).

22. Monitor the cells the next day (**day 8**).

23. On **day 9**, change Complete Astrocyte Medium and keep monitoring the cells. Change the medium every other day.

24. At **day 14** post thaw, the astrocytes growing in the 60 mm dish will reach full confluence. Passage the cells as described in the following procedure.

    a. Aspirate medium from the dish and add 2 mL of fresh pre-warmed Accutase.
    b. Keep the dish in the cell culture incubator until cells detach (2–5 minutes).
    c. Add 2 mL of pre-warmed DMEM medium into the dish and re-suspend floating cell layer with micropipette by pipetting up and down several times.
    d. Transfer astrocytes into a 15 mL conical tube containing 5 mL of pre-warmed DMEM medium.
    e. Centrifuge the cells at 400 × *g* for 5 minutes.
    f. Carefully aspirate liquid and gently re-suspend cell pellet in 5 mL of Complete Astrocyte Medium.
    g. Remove a 10 μL aliquot of cell suspension and mix it with 10 μL of 0.4% Trypan blue solution
    h. Perform an accurate live cell count.
    i. Plate astrocytes in Complete Astrocyte Medium on desirable vessels coated with Matrigel with the density ranging from low to high ($5 \times 10^4 – 1 \times 10^5$ live cells/cm^2), see Table 4.

Table 4.
Recommended seeding densities for astrocytes in various types of cell culture vessels. Range: low to high.

Vessel	Surface/well	Seeding
96 well plate	0.33 cm^2	$1.6 \times 10^4 – 3.3 \times 10^4$
4 well plate	2 cm^2	$1 \times 10^5 – 2 \times 10^5$
35 mm dish	10 cm^2	$5 \times 10^5 – 1 \times 10^6$
60 mm dish	20 cm^2	$1 \times 10^6 – 2 \times 10^6$

25. Culture cells for an additional 2–3 days and use for experiments.
    Note: It is not recommended to cryopreserve cultured astrocytes.

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Results

Characterization of Mature Astrocytes

The maturation of astrocytes can be assessed by their morphology, see Figure 1, and by immunostaining using the astrocyte marker, Glial Fibrillary Acidic Protein (GFAP), see Figure 2. Percentage of astrocytes can be determined by a count of GFAP positive astrocytes divided by the total number of cells (DAPI staining of nuclei).

Figure 1.
Example of astrocyte morphology at different time points post-seeding.

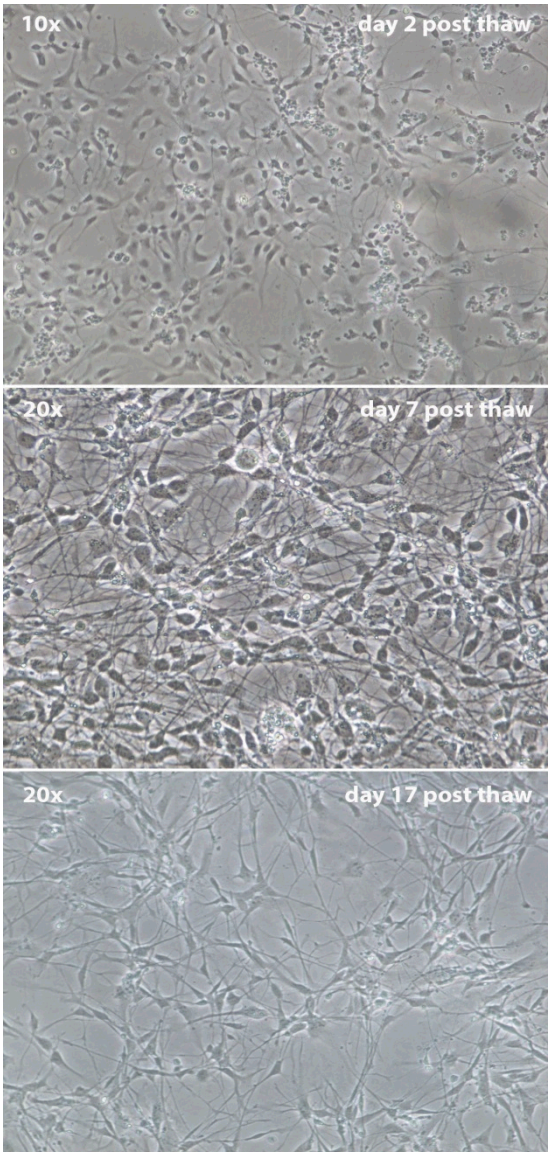

Figure 2.
Example of mature astrocytes. Immunostaining at 17 days post-seeding, showing ≥80% of total cells expressed the GFAP marker. Total nuclei count (blue) is used as the total number of cells. Less than 15% of cells express the Tuj-1 marker (neuronal class III β-tubulin).