CultureOne™ Supplement (100X)

For neuronal differentiation of hPSC-derived NSCs

Catalog Number A33202-01

Pub. No. MAN0016204 **Rev.** 1.0

WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from **thermofisher.com/support**.

Product description

Gibco™ CultureOne™ Supplement is a chemically-defined, serum-free supplement designed to significantly improve the differentiation of neural stem cells (NSCs) to neurons. As compared to conventional differentiation methods where NSCs can overgrow and become burdensome, CultureOne™ Supplement eliminates more than 75% of contaminating neural progenitor cells with minimal cell death and no effect on other kinase mediated pathways. The resulting superior neuronal cell cultures of evenly distributed, differentiated neurons enable improved downstream assays, accelerated neuronal maturation, and seamless maintenance for 5 weeks or more. Each vial of CultureOne™ Supplement contains a 100X, 5 mL liquid reagent that can be easily added to any conventional neuronal differentiation medium, such as Neurobasal™ Medium with B-27™ Supplement.

Contents and storage

Contents	Amount	Storage
CultureOne™ Supplement (100X)	5 mL	–20°C to –5°C

Required materials not supplied

Unless otherwise indicated, all materials are available through **thermofisher.com**. MLS: Fisher Scientific (**fisherscientific.com**) or other major laboratory supplier.

Item	Source
Reagents	
Neurobasal™ Medium	21103049
B-27™ Supplement	17504044
GlutaMAX™ Supplement	35050061
Ascorbic acid 2-phosphate sesquimagnesium salt hydrate	Sigma-Aldrich A8960
Mouse laminin	23017015
StemPro™ Accutase™ Cell Dissociation Reagent	A1110501
DPBS, no calcium, no magnesium	14190144
Equipment	
37°C humidified cell culture incubator with 5% CO_2	MLS
Centrifuge and 37°C water bath	MLS
Consumables	
15-mL and 50-mL sterile polypropylene conical tubes	MLS
0.22-µm filter	MLS
5, 10, 25, and 50-mL sterile pipettes	MLS
Poly-D-Lysine coated plates	
96-well	Fisher Scientific 08-774-255
48-well	Fisher Scientific 08-774-288
24-well	Fisher Scientific 08-774-271
12-well	Fisher Scientific 08-774-270
6-well	Fisher Scientific 08-774-268

Before you begin

Prepare Neuronal Differentiation Medium with CultureOne™ Supplement (NDMC)

1. Prepare 200 mM ascorbic acid:
   a. Dissolve 1 g ascorbic acid 2-phosphate sesquimagnesium salt hydrate in 17.3 ml distilled water.
   b. Filter through a 0.22-µm filter.

   Note: If not using solution right away aliquot 100–200 µL into sterile tubes, and store at –5°C to –20°C in the dark for up to 6 months.
2. Prepare NDMC by mixing the following components:

Reagent	Volume
Neurobasal™ Medium	96 mL
B-27™ Supplement[1]	2 mL
GlutaMAX™ Supplement	1 mL
CultureOne™ Supplement (100X)[1]	1 mL
200 mM ascorbic acid (200 mM)	100 µL

[1] Supplement can be thawed at 2°C to 8°C overnight or quickly in a 37°C water bath for about 5 minutes, and then aliquoted and frozen at –5°C to –20°C to allow for the preparation of smaller volumes of complete medium. Avoid repeated thawing and freezing.

   NDMC can be stored at 2–8°C in the dark for up to 2 weeks. Warm media in a 37°C water bath for 5–10 minutes before using. Do not warm media in a 37°C water bath for >10 minutes, as this may cause degradation of the media.
3. (*Optional*) Add growth factors such as 10–20 ng/mL glial cell-derived neurotrophic factor and 10–20 ng/mL brain-derived neurotrophic factor into NDMC to improve neuron survival.
4. (*Optional*) Add antibiotics such as Gentamicin into NDMC.

Coat culture plates with laminin

1. Thaw a vial of laminin stored at –80°C at room temperature. .

   Note: Thawed laminin can be aliquoted and stored at –80°C. Avoid repeated thawing and freezing. Storage of laminin at –5 to –20°C compromises laminin performance.
2. To create a working solution, dilute the thawed laminin solution 1:100 with sterile distilled water.
3. Add laminin solution into Poly-D-Lysine coated plates to cover the whole surface, and incubate in a 37°C, 5% CO_2 incubator for 1 hour. See Table 1.

4. Culture plates can now be used. Just prior to use, aspirate the laminin solution from each wells. Cells can be plated directly onto the laminin-coated plates without rinsing. Coated plates can also be stored at 2–8°C for up to one week. When storing, seal culture plates with Parafilm™ laboratory film to prevent drying. Before using, warm up the coated plates stored at 2–8°C at room temperature for 30 minutes.

   Note: If culture plates are not Poly-D-Lysine coated, coat plates with Poly-D-Lysine first and then with laminin.

Plate and differentiate NSCs

1. Dissociate expanded hPSC-derived NSCs in culture with StemPro™ Accutase™ Cell Dissociation Reagent or thaw frozen hPSC-derived NSCs.
2. Re-suspend dissociated or thawed NSCs with 5–10 mL DPBS.
3. Centrifuge the cells at 300 × g for 5 minutes and aspirate the supernatant.
4. Resuspend NSCs in 1–2 mL of pre-warmed NDMC depending on the number of NSCs.
5. Determine the concentration of viable cells using your preferred method.
6. Dilute the NSC suspension with pre-warmed NDMC to an appropriate concentration.
7. Aspirate the laminin solution from Poly-D-Lysine and laminin - coated plates.
8. Gently shake the tube containing NSCs and add an appropriate amount of diluted NSC/NDMC suspension into each well of culture plates to plate NSCs at a density of 5×10^4 cells/cm^2 or less. See Table 1.
9. Move the culture plates in several quick back-and-forth and side-to-side motions to disperse NSCs across the surface and place them gently in a 37°C CO_2 incubator.
10. 2–3 days after NSC plating, add the same volume of pre-warmed NDMC into each well of plates and return them into a 37°C CO_2 incubator.
11. Change spent medium every 2–3 days thereafter. When changing medium, remove half spent medium from each well and add the same volume of pre-warmed fresh NDMC into each well of plates and return them into a 37°C CO_2 incubator.
12. Maintain neurons differentiated with CultureOne™ Supplement for 1–5 weeks or longer depending on NSC lines and the purpose of experiments. See Figure 1.

Note:

- Differentiating neurons detach easily. When removing spent medium, do not touch cells with pipette tips. Also, add fresh medium gently toward the wall of culture plates.
- At 1–2 weeks after NSC differentiation, CultureOne™ Supplement can be withdrawn by adding fresh Neuronal Differentiation Medium without the supplement into each well of plates when changing spent medium. However, withdrawal of CultureOne™ Supplement may increase the chance of cell clumps reforming in the culture due to proliferating progenitor cells.
- If some wells of the culture plate do not have cells, add DPBS into these wells to minimize the evaporation of culture medium.

Characterize neurons by immunocytochemical staining

1. At the end of neuronal differentiation, add the same volume of 4% paraformaldehyde (PFA) into each well of plates to fix cells for 5 minutes at room temperature.
2. Aspirate solution from each culture vessel, add appropriate amount of 4% PFA (such as 0.5 mL for each well of a 24-well plate or 0.1 ml for each well of a 96-well plate) to fix cells for 15 minutes at room temperature.

   Note: Do not touch cells when aspirating to avoid cell detachment.
3. Aspirate PFA solution from each culture vessel and rinse the cells 2–3 times with DPBS at room temperature, 5 minutes per rinse.

   Note: Fixed cells in DPBS can wrapped in Parafilm™ laboratory film and stored at 4°C for up to 1 week.
4. After aspirating the DPBS, add appropriate amount of blocking buffer (0.1% Triton™ X-100, 1% BSA in DPBS) to cover whole surface and incubate for 30 minutes at room temperature.
5. Aspirate the blocking buffer, then add appropriate amount of primary antibody/antibodies diluted in blocking buffer to cover whole surface and incubate at 4°C overnight.
6. Rinse the cells 3 times with DPBS, 5–10 minutes per rinse at room temperature.
7. After aspirating DPBS, add appropriate amount of secondary antibody/antibodies diluted in blocking buffer to cover whole surface and incubate at room temperature for 30 minutes.
8. Rinse once with DPBS and add the appropriate amount of DAPI solution to cover whole surface and incubate at room temperature for 5 minutes.
9. After rinsing with DPBS 2–3 times, images can be taken with a fluorescence microscope. See Figure 1.

   Note: Differentiated neurons detach easily. Do not touch cells when aspirating and add solutions toward the wall of culture plates throughout the whole staining procedure to avoid cell detachment.

Supplemental information

Table 1 Two week differentiation of NSCs to neurons using Neuronal Differentiation Medium with CultureOne™ Supplement (NDMC)

Culture plates[1]	Well surface area	Volume of diluted laminin	NSCs/well[2]	Concentration of NSCs in NDMC	Plating volume of NSC/NDMC suspension	NDMC feed volume	Total volume of NDMC[3]
96 well	0.32 cm^2	0.05– 0.06 mL/well	16,000	160 cells/mL	0.1 mL/well	0.1 mL/well	57.6 mL/plate
48 well	0.95 cm^2	0.1–0.15 mL/well	47,500	238 cells/mL	0.2 mL/well	0.2 mL/well	57.6 mL/plate
24 well	1.9 cm^2	0.3–0.4 mL/well	95,000	190 cells/mL	0.5 mL/well	0.5 mL/well	72 mL/plate
12 well	3.8 cm^2	0.5–0.6 mL/well	190,000	190 cells/mL	1 mL/well	1 mL/well	72 mL/plate
6 well	9.6 cm^2	1.0–1.5 mL/well	480,000	240 cells/mL	2 mL/well	2 mL/well	72 mL/plate

[1] Poly-D-Lysine coated

[2] Based on required plating density of 5×10^4 cells/cm^2

[3] Based on feeding every 2–3 days

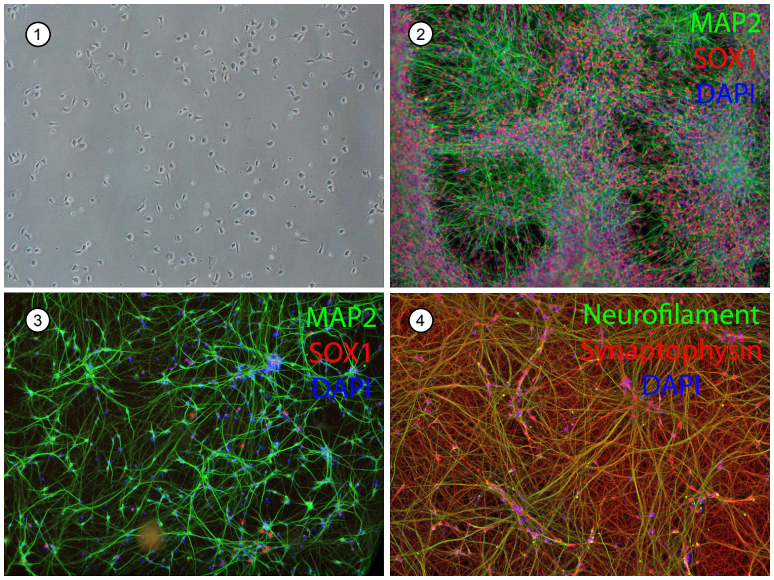

Fig. 1 Treatment with CultureOne™ Supplement improves the differentiation of hPSC-derived NSCs into neurons.

① H9 ESC-derived NSCs were plated at density of $5x10^4$ cells/cm^2.

② Without CultureOne™ Supplement, cells at 2 weeks of differentiation were highly dense, formed cell clumps, and contained MAP2 positive neurons and a significant number of SOX1 positive NSCs.

③ At 2 weeks of differentiation, cultures treated with CultureOne™ Supplement had an even distribution of MAP2 positive neurons with minimal SOX1 positive NSCs and no cell clumps.

④ At 5 weeks of differentiation, differentiated cells treated with CultureOne™ Supplement expressed mature neuronal markers Neurofilament and Synaptophysin. Cell nuclei were counter stained with DAPI (blue).

Troubleshooting

Observation	Possible cause	Recommended action
Cells detach during differentiation	Inappropriate coating of culture plates	Check whether culture plates are coated with poly-D-lysine and laminin.
	Mechanical cell detachment	Do not touch cells with pipette tips when changing spent medium and add fresh medium gently toward the wall of the culture plate.
Differentiating neurons form cell clumps	High plating cell density	Check whether NSC plating density is higher than $5×10^4$ cells/cm^2. If higher NSC plating density is required for your experiment, the concentration of CultureOne™ Supplement can be increased to 2–4X in the final Neuronal Differentiation Medium without toxicity to neurons. However, increasing the CultureOne™ Supplement concentration may not completely eliminate cell clump formation for NSCs plated at higher than $5×10^4$ cells/cm^2.
		Use laminin stored at –80°C to coat culture plates.
Non-neural cells in culture after NSC diffentiation	Contamination of other cell types in NSCs	Check the purity of derived NSCs from hPSCs.
Varying effects of CultureOne™ Supplement on different lines of NSCs	Insensitive NSCs from specific hPSC lines.	The efficiency of CultureOne™ Supplement treatment varies for NSCs derived from different hPSC lines. Adjust the concentration of CultureOne™ Supplement from 1X to 4X in the final Neuronal Differentiation Medium to reach optimal effect.

Limited product warranty

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