CultureOne™ Supplement (100X)

Catalog Number A33202-01

Pub. No. MAN0016204 **Rev.** 3.0

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 xeno-free supplement designed to improve human pluripotent stem cell (hPSC) derived and primary neuronal cell cultures. For neuronal differentiation of hPSC-derived neural stem cells (NSCs), CultureOne™ Supplement eliminates more than 75% of contaminating neural progenitor cells as compared to conventional differentiation methods where NSCs can overgrow and become burdensome. 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.

For primary neuron cultures, CultureOne™ Supplement reduces the number of proliferating glial progenitors which can overgrow post-mitotic neurons. It can also be used to control the level of glia through modifying timing of addition.

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™ Plus Medium or Neurobasal™ Medium	A3582901 or 21103049
B-27™ Plus Supplement or B-27™ Supplement	A3582801 or 17504044
GlutaMAX™ Supplement	35030061
Ascorbic acid 2-phosphate sesquimagnesium salt hydrate	Sigma-Aldrich, A8960
Laminin Mouse Protein, Natural	23017015
StemPro™ Accutase™ Cell Dissociation Reagent	A1110501
DPBS, no calcium, no magnesium	14190144
DPBS, calcium, magnesium	14040133
Poly-D-Lysine	MLS

Item	Source
Cells	
Human Episomal iPSC Line	A18945
Primary Mouse Cortex Neurons	A15585
Primary Mouse Hippocampus Neurons	A15587
Primary Rat Cortex Neurons	A10840-01, A10840-02
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
Thermo Scientific™ Nunc™ cell culture treated plates and dishes with Nunclon™ Delta surface	
96-well	167008 or 165305
48-well	150678
24-well	142475
12-well	150628
6-well	140675

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™ Plus Medium or Neurobasal™ Medium[1]	96 mL
B-27™ Plus Supplement or B-27™ Supplement[2]	2 mL
GlutaMAX™ Supplement	1 mL
CultureOne™ Supplement (100X)[2]	1 mL
Ascorbic acid (200 mM)	100 µL

[1] Make NDMC with either Neurobasal™ Plus Medium or Neurobasal™ Medium. Do not interchange Neurobasal™ Plus Medium with Neurobasal™ Medium and vice versa.

[2] 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.

   Note: 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 Poly-D-Lysine

1. Prepare a 2-mg/mL Poly-D-Lysine stock solution in distilled water.

2. Dilute the Poly-D-Lysine stock solution 1:40 in DPBS, calcium, magnesium to prepare a 50 µg/mL working solution (i.e., 125 µL of Poly-D-Lysine stock solution into 5 mL of DPBS, calcium, magnesium).

3. Coat the surface of Nunc™ cell culture treated plate with the working solution of Poly-D-Lysine (150 µL/ cm^2, i.e., 50 µL per well for a 96-well plate).

4. Incubate the culture vessel at room temperature for 1 hour.

5. Remove the Poly-D-Lysine solution and rinse 3 times with distilled water.

   Make sure to rinse the culture vessel thoroughly, because excess Poly-D-Lysine can be toxic to the cells.

6. Leave the coated vessels uncovered in the laminar hood until the wells have completely dried.

   You may use the dry plates immediately or store them at 4°C, wrapped tightly with Parafilm™ film, for up to one week.

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™ film to prevent drying. Before using, warm up the coated plates stored at 2–8°C at room temperature for 30 minutes.

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, no calcium, no magnesium.

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 in B-27™ Supplement, or at 4×10^4 cells/cm^2 or less in B-27™ Plus Supplement and Neurobasal™ Plus Medium. See Table 1.

   Note: The optimal plating density may vary depending on NSC derivation methods and hPSC lines.

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, no calcium, no magnesium into these wells to minimize the evaporation of culture medium.

Culture primary neurons

CultureOne™ Supplement can be used with the B-27™ Plus Neuronal Culture System to suppress undesired glial cell growth, or control the level of astrocytes present in primary mouse and rat neuronal cultures.

- For full suppression of both astrocytes and oligodendrocytes, CultureOne™ Supplement should be added at a 1X concentration to complete Neurobasal™ Plus Medium at the time of plating primary neurons.
- Delay adding CultureOne™ Supplement to complete Neurobasal™ Plus Medium used for feeding after plating to achieve desired levels of astrocytes. The later CultureOne™ Supplement is added, the more astrocytes will be present in the primary neuronal culture. See Figure 2.

Note: Refer to *B-27™ Plus Neuronal Culture System User Guide* (Cat. No. A3653401, Pub. No. MAN0017319) for detailed protocol for culturing primary neurons. Some optimization of when to add CultureOne™ Supplement and final concentration may be required as glial cell conditions will vary depending on the starting cells used. CultureOne™ Supplement has been added up to a 4X concentration with no detrimental effect on primary rat and mouse neurons with the B-27™ Plus Neuronal Culture System.

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, no calcium, no magnesium at room temperature, 5 minutes per rinse.

   Note: Fixed cells in DPBS, no calcium, no magnesium can wrapped in Parafilm™ laboratory film and stored at 4°C for up to 1 week.

4. After aspirating the DPBS, no calcium, no magnesium, add appropriate amount of blocking buffer (0.1% Triton™ X-100, 1% BSA in DPBS, no calcium, no magnesium) 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, no calcium, no magnesium, 5–10 minutes per rinse at room temperature.

7. After aspirating DPBS, no calcium, no magnesium, 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, no calcium, no magnesium 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, no calcium, no magnesium 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	Surface area of Nunc™ plate	Volume of diluted laminin	NSCs/well[1]	Concentration of NSCs in NDMC	Plating volume of NSC/NDMC suspension	NDMC feed volume	Total volume of NDMC[2]
96 well	0.33 cm^2	0.05–0.06 mL/well	16,500	165,000 cells/mL	0.1 mL/well	0.1 mL/well	57.6 mL/plate
48 well	1.1 cm^2	0.1–0.15 mL/well	55,000	275,000 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	190,000 cells/mL	0.5 mL/well	0.5 mL/well	72 mL/plate
12 well	3.5 cm^2	0.5–0.6 mL/well	175,000	175,000 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	240,000 cells/mL	2 mL/well	2 mL/well	72 mL/plate

[1] Based on required plating density of 5×10^4 cells/cm^2
[2] Based on feeding every 2–3 days

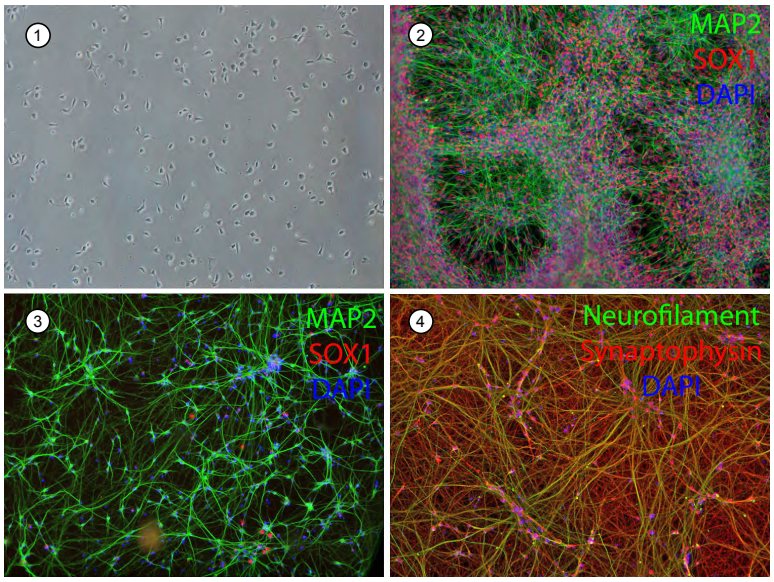

Figure 1 Treatment with CultureOne™ Supplement improves the differentiation of hPSC-derived NSCs into neurons.

① H9 ESC-derived NSCs were plated at density of 5×10^4 cells/cm^2 in B-27™ Supplement and Neurobasal™ Medium.

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).

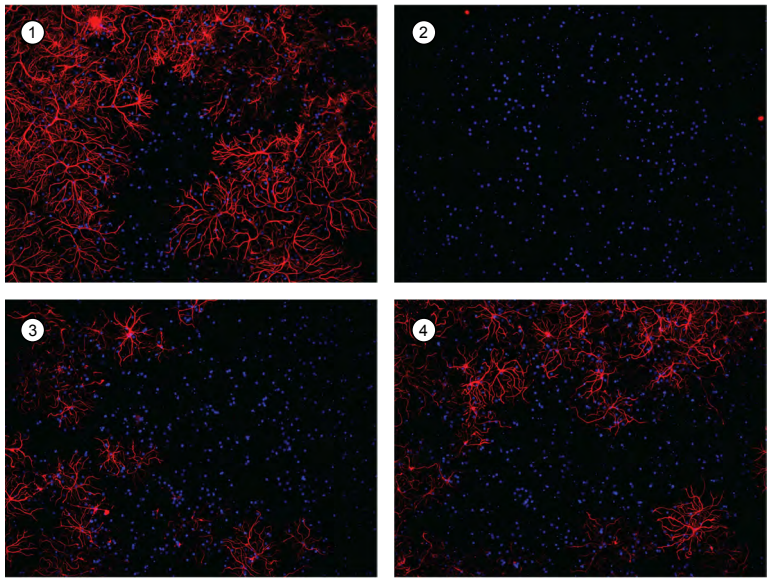

Figure 2 B-27™ Plus Neuronal Culture System with CultureOne™ Supplement can be used to control levels of astrocytes in primary rat or mouse neuronal cultures

① Rat cortical neurons were maintained for 21 days with the B-27™ Plus Neuronal Culture System. Cells were fixed at D21 and stained with the astrocyte specific antibody GFAP.

② Primary rat cortical neurons maintained with B-27™ Plus Neuronal Culture System and treated with CultureOne™ Supplement at time of plating, or starting at Day 2 results in complete suppression of astrocytes and oligodendrocytes (not pictured).

③ Delaying addition of CultureOne™ Supplement to Day 4 or 6 (pictured) results in increasing levels of astrocytes.

④ Delaying the addition of CultureOne™ Supplement treatment at Day 8 results in increasing levels of astrocytes.

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 in B-27™ Supplement and Neurobasal™ Medium or 4×10^4 cells/cm^2 in B-27™ Plus Supplement and Neurobasal™ Plus Medium. 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 plating density of NSCs and the concentration of CultureOne™ Supplement from 1X to 4X in the final Neuronal Differentiation Medium to reach optimal effect.

Limited product warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale found on Life Technologies' website at **www.thermofisher.com/us/en/home/global/terms-and-conditions.html**. If you have any questions, please contact Life Technologies at **www.thermofisher.com/support**.

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The information in this guide is subject to change without notice.