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Product Information

ENDO.4M GFP Endothelial Cells
(Mouse ES cells derived) 1M with Medium

Catalog Number **AXIO0052**

TECHNICAL BULLETIN

Product Description
ENDO.4M GFP Endothelial Cells 1M are endothelial cells derived from transgenic mouse embryonic stem (mES) cells. These cells are puromycin resistant and have the green fluorescent protein (GFP) reporter gene controlled by the endothelial cell-specific *Flt1* promoter.

ENDO.4M cells are produced by *in vitro* differentiation of mouse embryonic stem (ES) cells and puromycin selection of endothelial precursor cells. When cultured in an endothelial cell growth medium, they express specific markers like PECAM and VE-cadherin, and are sensitive to known antiangiogenetic substances like fumagillin and suramin. When cultured in an ECM (extracellular matrix) preparation (e.g., protein mixture secreted by Engelbreth-Holm-Swarm mouse sarcoma cells), they show tube formation similar to HUVEC cells.

Component

ENDO.4M GFP Endothelial Cells (Mouse ES cells derived) 1M (ENDO.4M GFP-eE-1M) 1×10^6 mouse ES cells derived cells Catalog Number AXIO0033	1 vial
ENDO.4M Endothelial Cell Culture Medium (ENDO.4M Culture Medium) Catalog Number AXIO0074	250 mL

Reagents and Equipment Required but Not Provided.
- PBS without Ca^{2+} and Mg^{2+}
- Disposable Neubauer Hemacytometer
- 0.4% Trypan Blue solution, Catalog Number 93595
- Biological safety cabinet certified for Level I handling of biological materials
- Incubator with humidity and gas control to maintain 37 °C and 95% humidity in an atmosphere of 7% CO_2 in air
- 37 °C water bath
- Sterile 50 mL polypropylene (PP) tubes
- Centrifuge with rotor for 50 mL PP tubes
- 8 channel or 12 channel micropipette
- Sterile pipette tips
- BD BioCoat™ Fibronectin-coated dishes (Catalog Number 354403 for 60 mm dishes or Catalog Number 354451 for 10 cm dishes)
- Fibronectin from bovine plasma (1 mg/mL solution, Catalog Number F1141) for coating alternate sized dishes and plates
- 1× Trypsin-EDTA solution (Catalog Number T3924)
- Fetal Bovine Serum (FBS, Catalog Number F2442) (for passaging of cells only)
- Antiangiogenetic compound (e.g., Fumagillin, Catalog Number F6771 or Suramin, Catalog Number S2671) as positive control (toxicity testing only)
- Neutral Red Solution (Catalog Number N6264)
- PBS with Ca^{2+} and Mg^{2+} (Neutral Red Test only)

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

Storage/Stability
AXIO0033 is shipped in liquid nitrogen dry shippers and stored at –196 °C.

AXIO0074 is shipped on dry ice and storage at –20 °C is recommended.

Procedure
ENDO.4M cells are genetically modified mouse cells and should be handled according to local directives (Typically Biosafety level 1).

The endothelial cells should be cultured using sterile cell culture techniques and good laboratory practices.

Cells can be inactivated by autoclaving at 121 °C for 20 minutes.

Day 0 – Medium Preparation

1. Thaw the ENDO.4M Endothelial Cell Culture Medium, Catalog Number AXIO0074, at 4 °C and mix carefully after thawing. Store the medium at 4 °C in the dark until use.
   Note: Avoid foaming when mixing the medium.

2. Thaw the FBS at 4 °C. Mix well after thawing and prepare 10 mL aliquots for further use. Store aliquots at –20 °C.

3. Pre-warm the required amount of culture medium in a 37 °C water bath.

Day 0 – Fibronectin-Coated Dishes
BD BioCoat Fibronectin-coated dishes (Catalog Number 354403 for 60 mm dishes or Catalog Number 354451 for 10 cm dishes) are recommended for the culture of the endothelial cells. Prior to plating the cells onto the BD BioCoat fibronectin-coated dishes, hydrate the dish by adding the appropriate volume (see Table 1) of the culture medium (Catalog Number AXIO0074) into the dish and place the dish into the 37 °C incubator for 1 hour.

For coating alternate sized dishes and plates (e.g., 96 well or other multiwell plates), use of Fibronectin from bovine plasma (1 mg/mL solution, Catalog Number F1141) is recommended.

1. Dilute the 1 mg/mL fibronectin solution 1:100 with PBS **without** Ca^{2+} and Mg^{2+}.

2. Add the appropriate volume of fibronectin solution into the cultureware according to Table 1.

3. Place the freshly coated cultureware into the 37 °C incubator for at least 3 hours. If the dishes are not immediately used, they can be stored for up to 3 days at 4 °C.

4. Remove the remaining fibronectin solution immediately before seeding the endothelial cells using a slow-running suction pump or a multichannel pipette.
   Notes: Do not allow the surface of the cultureware to become dry after fibronectin coating. **Do not** remove the fibronectin solution until ready to plate the cells.

   Fibronectin is highly susceptible to shear stress. Avoid vigorous pipetting and do not vortex the fibronectin solution. For additional handling instructions, consult the Product Display page on the Sigma website.

Table 1.
Plate Parameters

Dish/Multiwell Plate	Surface Area (cm^2)	Volume of Fibronectin Solution for Coating (µL)	Endothelial Cells per Well ($\times 10^6$)*	Volume (Medium Change or Cell Suspension, mL)
10 cm dish	78.5	3,000	2	10
60 mm dish	21.29	1,500	0.5	5
1 well of a 6 well plate	9.62	750	0.25	3–4
1 well of a 12 well plate	3.80	300	0.1	2
1 well of a 24 well plate	2.00	200	0.05	1
1 well of a 48 well plate	0.75	100	0.02	0.5
1 well of a 96 well plate	0.32	50	0.01	0.2–0.25

* ~2.5×10^4 viable cells per cm^2 of the fibronectin-coated cultureware

Day 0 – Washing & Counting Cells

1. Add 50 μL of Trypan Blue solution to 40 μL of PBS **without** Ca^{2+} and Mg^{2+} in a microcentrifuge tube.

2. Prepare the hemacytometer by cleaning the chambers and coverslip with isopropanol. Wipe the hemacytometer dry using lint-free tissue.

3. Remove the vial(s) containing the endothelial cells from the shipment box or storage. Immediately place the vial(s) in a 37 °C water bath for two minutes. Remove the vial(s) from the water bath and wipe with 70% isopropanol or ethanol to sterilize.

4. Immediately pipette the cell suspension into the second 50 mL tube containing 8 mL of thawed culture medium to wash the cells.

5. Use 1 mL of thawed culture medium to rinse the vial, ensuring any cells remaining in the vial are also recovered. Add the 1 mL of cells to the 50 mL tube (there should now be ~9 mL of cell suspension in the tube).

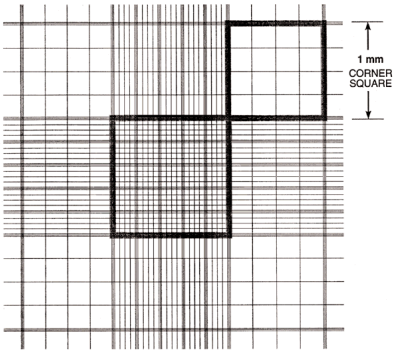
6. Centrifuge cells at 200 × *g* for 5 minutes at room temperature.

7. Aspirate the supernatant, leaving the cell pellet intact. In preparation for cell counting, resuspend the cells in 1 mL of culture medium.

8. Pipette 10 μL of the cell suspension into the prepared Trypan Blue/PBS solution, mix well, and incubate for 2 minutes. This is a 10-fold dilution of the cells.

9. After 5 minutes, fill the Neubauer hemacytometer with the cell suspension (step 8), ensuring the chambers are not underfilled or overfilled (use 10 μL of cells/Trypan Blue/PBS suspension). Count both the clear, refractile cells and the blue colored cells in the 4 large outer quadrants. This is the **total number of cells (N)** in the four large, outer quadrants (see Figure 1).

Figure 1.
Neubauer Hemacytometer – dimensions of each square.



10. Subsequently, count the **number of blue cells (D)** in the same four, large outer quadrants. These are the non-viable cells that have incorporated the Trypan Blue dye. Because of the characteristic decrease in cell membrane integrity in non-viable cells, the Trypan Blue dye is able to pass through the cell membrane.

11. Calculate the average number of total cells (A_N) per square by dividing N by 4 (N/4) and the average number of non-viable cells (A_D) per square by dividing D by 4 (D/4).

$$A_N = N \div 4$$
$$A_D = D \div 4$$

12. Calculate the **absolute number of viable cells (V)** using the formula:

$$V = (A_N - A_D) \times 10 \times 10^4 \times 0.5 \text{ mL}$$

Cell count per mL = Average cell count per square × Dilution factor × 10^4

Day 0 – Plating Cells

1. Adjust the cell concentration with an appropriate volume of culture medium for plating ~2.5 × 10^4 viable cells per cm^2 of the fibronectin-coated cultureware (see Table 1).

2. Plate the cells on the fibronectin-coated culture dishes, ensuring the cells uniformly cover the bottom surface area of the dish to form a monolayer of cells.

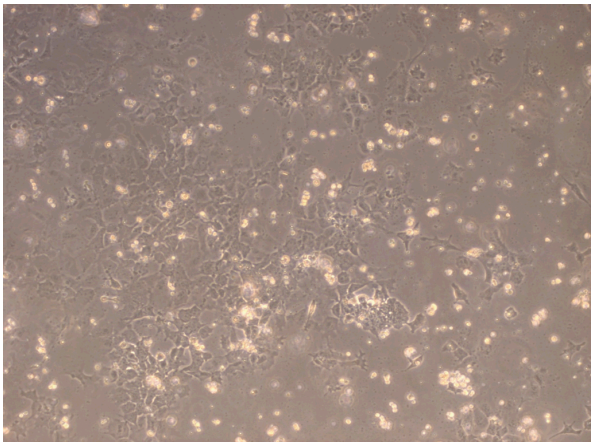
3. Place the dish into the 37 °C incubator and culture the cells at 37 °C with 7% CO_2 and 95% humidity.

Medium Changes

1. Observe the growing cells (see Figures 1a and 1b) and change the culture medium if the medium starts changing color or if a number of dead cells are floating in the medium (24–48 hours).

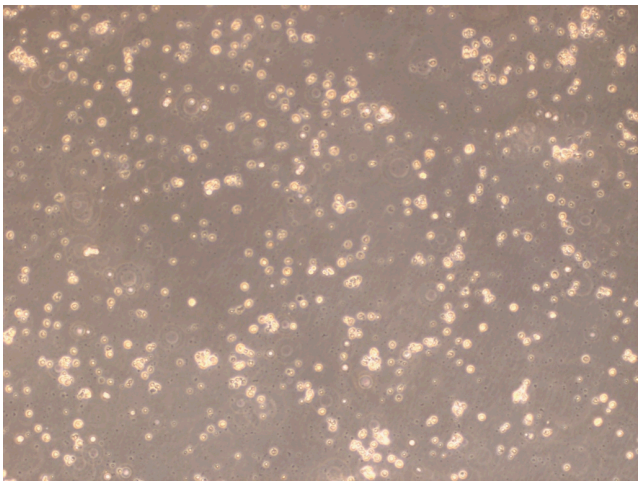
Figures 1a and 1b.
Microphotographs of Sub-confluent Culture of Endothelial Cells

1a.
Microphotograph focused on adherent cells on the surface of the dish.



When checking the cells with the microscope, make sure to identify the adherent cells on the culture surface.

1b.
Microphotograph focused on floating cells.



When ES cell derived endothelial cells are cultured as described, floating dead or non-adherent cells may appear in the culture. These dead cells will be removed with every subsequent medium change.

2. After the initial medium change, continue observing the growing cells and change the culture medium if it starts changing color or if a number of dead cells are floating in the medium (every 2–3 days).

Passage and Splitting of the Culture

As soon as the cells on the surface of the dish are nearly confluent, the culture can be passaged and split.

1. Thaw a 10 mL aliquot of FBS (prepared on day 0).

2. Remove the culture medium and rinse the dish twice with PBS **without** Ca^{2+} and Mg^{2+}.

3. Incubate the cells with an appropriate volume of pre-warmed standard 1× Trypsin-EDTA solution for 3 minutes at 37 °C. Check dish under microscope, make sure all cells are detached from the surface. If necessary, prolong incubation for another minute.

4. Stop trypsin digestion by adding to the cells a volume of culture medium **with** 10% FBS equal to 4× the volume of the 1× Trypsin-EDTA solution (step 3), resuspend cell suspension carefully, and transfer the suspension to a 50 mL centrifuge tube.

5. Repeat steps 6–12 (Washing & Counting Cells) and steps 1–3 (Plating Cells), or use cells for functional experiments.

Figure 2.
Layout to assess antiangiogenetic properties of compounds

1	2	3	4	5	6	7	8	9	10	11	12
A	Compound 1					Comound 2					
B	control	Suramin 1e-6M	Suramin 1e-5M	Suramin 1e-4M	control	control	Fumagillin 1e-9M	Fumagillin 1e-8M	Fumagillin 1e-7M	control	
C											
D											
E											
F											
G											
H											

Seeding of 96 Well Plates for Toxicity Tests
For toxicity tests, the endothelial cells can be seeded in 96 well plates either directly, or after the first or second passage, using the previously described procedures with the following modifications:

1. For fibronectin coating, use 50 µL per well of the fibronectin solution.

2. Seed 20,000 cells in 200 µL of medium per well of a fibronectin-coated 96 well plate using a multichannel pipette. It is strongly recommended to seed only the inner 60 wells of the plate and to fill the outer wells with culture medium or PBS to avoid evaporation (see Figure 2).

3. Place the dish into the 37 °C incubator and culture the cells at 37 °C with 7% CO_2 and 95% humidity for 24 hours.

4. After 24 hours, remove the culture medium and replace with 200 µL each of appropriate test compound dilutions (see Figure 2). It is recommended to use suramin and/or sumagillin as a positive control.

5. After 48–72 hours of treatment, viability of the endothelial cells can be determined by a standard Neutral Red Uptake test.

Quantification of cytotoxicity using the Neutral Red Uptake Test[1]

1. Dilute 0.2 mL of the Neutral Red Solution (Catalog Number N6264) with 20 mL of warm culture medium in a 50 mL tube. Pass the solution through a 0.2 µm filter.
   Note: Do not prepare more than 20 mL of dye solution at once.

2. Remove the 96 well plate from the incubator. Gently aspirate the medium.

3. Carefully add 200 µL of PBS **with** Ca^{2+} and Mg^{2+} to each well of the plate using a multichannel pipette at slow speed. Incubate with the PBS for 2 minutes, then gently remove the PBS using a slow-running suction pump or a multichannel pipette.

4. Add 100 µL of the prepared Neutral Red solution to **each** well using a multichannel pipette at slow speed.

5. Incubate the plate at 37 °C (with 7% CO_2 and 95% humidity) for 3 hours. Check cells of control wells for neutral red uptake after 30 minutes under the microscope.

6. 15 minutes before the end of the incubation time, prepare Desorb Solution (49:50:1, ultrapure water:ethanol:glacial acetic acid) For one plate, prepare 15 mL of Desorb Solution (7.35 mL of water, 7.5 mL of ethanol, and 0.15 mL of glacial acetic acid).

7. After the 3 hour incubation, gently aspirate the Neutral Red solution.

8. Carefully add 200 µL of PBS **with** Ca^{2+} and Mg^{2+} to each well of the plate using a multichannel pipette at slow speed. Incubate with the PBS for 2 minutes, then gently remove the PBS using a slow-running suction pump or a multichannel pipette.
   Notes: It is extremely important the tips do not touch the bottom of the wells during aspiration. Make sure to remove all PBS after the washing step without destroying the cell layer.

9. Add 150 µL of Desorb Solution to each well. Incubate plate on a plate shaker for 10 minutes at maximal speed.

10. Measure absorbance at 540 nm with a reference wavelength of 690 nm.

Seeding on ECM for Tube Formation Assay

The endothelial cells can be used for tube formation assays on ECM-coated plates after 2 days of culture on the fibronectin-coated cultureware as previously described. The cells are harvested and seeded on the ECM-coated plates

1. Prepare ECM-coated tissue culture plates (e.g., 24 well plates) following the protocol provided by the manufacturer. ECM preparations are highly susceptible to shear stress and temperature changes. Strictly follow the instructions for good results.

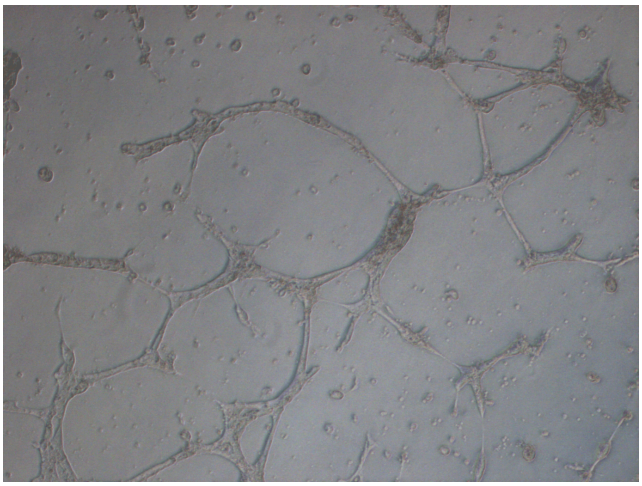
2. After 2 days of culture, perform steps 1–3 (Passage and Splitting of the Culture) and steps 6–12 (Washing & Counting Cells).

3. Resuspend the remaining cells in culture medium and plate the cells on ECM-coated tissue culture plates at a density of ~60,000 cells per cm^2 in an appropriate volume of medium (e.g., 1 mL per 24 well; 0.5 mL per 48 well).

4. Incubate the cells overnight at 37 °C with 7% CO_2 and 95% humidity.

5. Next day, microscopically examine the endothelial cells for tube formation (see Figure 3).

Figure 3.
Tube Formation of Endothelial Cells on ECM after overnight incubation



References

1. Borenfreund, E., and Puerner, J.A., Toxicity determined *in vitro* by morphological alterations and neutral red absorption. Toxicol. Lett., **24**(2-3), 119-24 (1985).

AXIOGENESIS Label License

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