EB-Plate

Embryoid Body Generation

User Guide

Contents

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v2.2

General Information

Thank you for purchasing eNUVIO's EB-Plate, a completely reusable embryoid body (EB) generation device. The EB-Plate contained within the plastic wrapping is packaged sterile. If the sterility of the device is suspected to be compromised, the device can be sterilized by following the cleaning protocol at the end of this user guide.

Unpacking

To maintain sterility, it is recommended to unpack the contents in an aseptic environment (e.g. in a biological safety cabinet). Use a sterile blade or scissors to cut open the plastic packaging and remove the EB-Plate.

Reagents/equipment to reuse the EB-Plate:

- Pluronic® F-127* (e.g. Sigma P2443), prepared as a 5 % w/v solution in PBS (w/o Ca^{2+} + Mg^{2+})
- Centrifuge with swinging-bucket rotor and microplate carrier(s) installed
- 70% ethanol
- 99 % isopropyl alcohol (optional)
- Alconox®, Tergazyme®, or trypsin (optional)
- Ultrasonic bath (optional)
- Steam sterilizer, autoclave or plasma cleaner (for sterilization)

Before Starting

EB-Plates are packaged sterile and are ready to be used in aseptic cell culture environments. Surface treatment of the microwells with an anti-fouling agent (e.g. Pluronic® F-127) is necessary prior to cell seeding to create an ultra-low attachment (ULA) surface. Pre-coated EB-Plates **do not require** surface treatment before using them for the first time.

Anti-Evaporation Reservoirs

The osmotic pressure, pH and nutrient concentration of the culture media is critical for maintaining a healthy culture. This can be particularly problematic when having to maintain cultures for longer periods of time (weeks or months). Microplate wells are known to be particularly prone to evaporation, especially those located around the outer border of the plate. This can lead to the loss of the culture (often to the surprise of the user) as the media gradually concentrates over time and can introduce unwanted variability in the data collected for the experiment. Unfortunately, these edge effects are so problematic that wells located along the outer edge of 96-well microplates are often left unused. To combat evaporation, all EB-Plates have fluid reservoirs built into their black outer structure. When these are filled with liquid, they serve to reduce evaporation that occurs from the outermost wells of the plate. In this way, these outer wells can become available to be used in the experiment.

* *Pluronic® is a registered trademark of BASF, Alconox® and Tergazyme® are a registered trademarks of Alconox Inc.*

EB-Plate Components

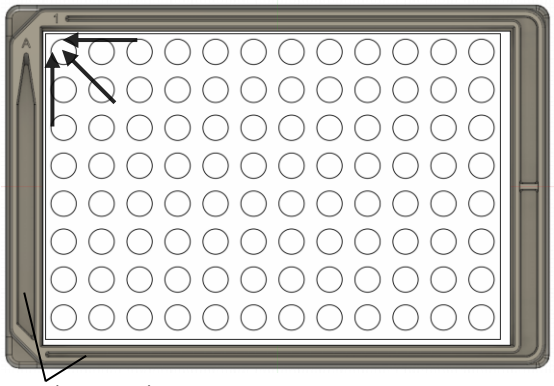
The EB-Plate is provided as three separate pieces that assemble to form the final microplate:

1) Microplate **frame** (black; polystyrene; not autoclavable)
2) Microplate **lid** (transparent; polystyrene; not autoclavable)
3) **Microwell structure** (transparent; PDMS; autoclavable)

The final EB-Plate is assembled by positioning the microplate microwells into the black frame, and then placing the lid on top of the frame. Slight adjustment can be made to the position of the microwell structure within the frame. It is recommended to position the microwell structure such that the left and top walls both are in contact with the top left corner (towards well A1; see schematic on page 3). Positioning the microplate structure in the same way will ensure consistency in the horizontal and vertical (i.e. x - y) position of the microwells between plates.

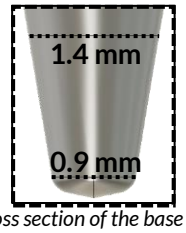
When manipulating the assembled plate, it is recommended to grasp the plate at the corners to avoid compressing the anti-evaporation reservoirs. Compression of the reservoirs will not lead to damage to the frame; however, this can cause fluids that have been added to the reservoirs to overflow.

Microplate Dimensions:
Length: 127.7 mm
Width: 85.4 mm
Height: 14.2 mm
A1 to side offset: 16.04 mm
A1 to top offset: 10.8 mm
Well spacing (center to center): 9 mm
Well diameter (top): 6 mm
Well diameter (bottom): 0.9 mm
Well bottom thickness: 1 mm
Well bottom height from frame base: 1 mm



EB-Plate Specifications

- 96 microwells/plate
- Microplate outer dimensions: 127.7 x 85.4 mm
- Microwell structure material: polydimethylsiloxane (PDMS)-biologically inert, non-degradable, non-toxic polymer
- Microwell diameter at bottom: 900 µm
- Microwell volume (max.): 170 µL
- Anti-evaporation reservoir (each; 3 total): recommended working volume: 1.6 mL (max. vol. 2 mL)

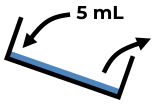


Cross section of the base of a microwell. Not to scale.

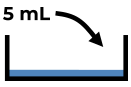
Cell Seeding Protocol

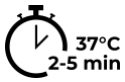
The following seeding protocol assumes the user is familiar with the basics of maintaining stem cells in culture. As protocols vary greatly between labs and target tissue type, please refer to your laboratory's internal protocols and guidelines for stem cell culture and cell seeding densities. Generally, differentiated areas of an iPSC culture should be removed prior to detachment and cultures should not surpass 80 % confluency. The wash volumes in the protocol below assume stem cell feeder cultures are being cultured in 100 mm round culture dish. **These should be adjusted accordingly to suit the user's particular situation.**

1) Unpack the EB-Plate under aseptic conditions. If starting with a new uncoated EB-Plate, or with an EB-Plate that is being reused, ensure it has first been coated with an anti-fouling reagent (see **Surface Coating Protocol**). If you are using a pre-coated EB-Plate for the first time, simply proceed to step 2. If the EB-Plate has just been freshly coated (not dried), allow the EB-Plate to equilibrate to room temperature.

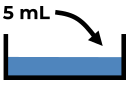


2) Wash stem cells once with 5 mL pre-warmed media (e.g. DMEM/F12 or equivalent).

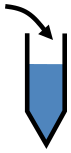




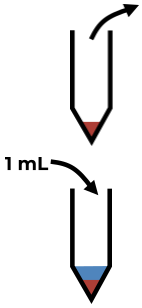
3) Add 5 mL of a gentle cell detachment solution and place the dish in a 37°C incubator for 2 to 5 minutes (or as recommended by the manufacturer). Monitor detachment progress by eye. Gentle tapping of the dish may be necessary.



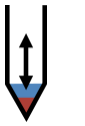
4) Stop cell detachment by adding 5 mL of media (e.g. DMEM/F12 or equivalent).



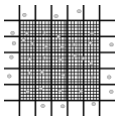
5) Collect the total volume (10 mL) of media containing the cells into a 15 mL tube. Centrifuge at 300 x *g* for 3 minutes.



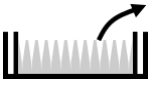
6) Remove the supernatant and add 1 mL of the desired EB seeding culture media.



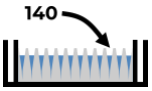
7) Gently triturate the cells to resuspend single cells in solution. This can be achieved by very gently pipetting the cells up and down (3 times) through a 1000 µL pipette tip fitted with a 200 µL pipette tip.



8) Count resuspended cells using a cell counter (e.g. haemocytometer or equivalent) and dilute with seeding media as required to obtain the appropriate cell density and total volume required to seed the EB-Plate (~15 mL per plate; adjust volumes accordingly for seeding multiple EB-Plates). Just prior to seeding, it is important to ensure that the cells are adequately resuspended to create a homogenous cell/media mixture as this will improve consistency between microwells.

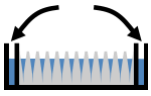


9) If necessary, remove or aspirate the Pluronic® F-127 coating solution from the device. Washing the residual solution is not necessary.



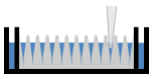
10) Add 140 µL of cell/media mixture to each well of the EB-Plate.

11) Using a swinging-bucket rotor with a microplate carrier, centrifuge the EB-Plate at 300 x *g* for 1 minute to collect cells into the base of each microwell.



12) Add sterile water, PBS, or media to the anti-evaporation tracks of the microwell plate and place the EB-Plate in the 37°C incubator. Change media as needed by the culture.

13) When embryoid bodies have formed in the microwells and have grown to the desired size, EBs can be embedded in extracellular matrix (ECM) hydrogel (see **ECM Embedding Protocol** below).

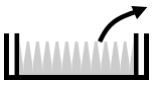


14) Once EBs have grown to their desired size, they can be transferred out of the microwells using pipet tips that have been cut to enlarge the tip opening.

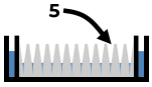
ECM Embedding Protocol (optional)

After the embryoid bodies have formed in the wells of the EB-Plate and have grown to the desired size, they can optionally be embedded in ECM hydrogel (e.g. Matrigel®, Geltrex®*, or equivalent).

1) The ECM hydrogel chosen for embedding should be handled as per the manufacturer's recommendations. In some cases, this may require overnight thawing at 4°C.

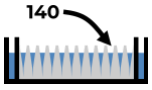


2) Remove all growth media from each well of the EB-Plate.



3) Add ECM hydrogel into each well of the EB-Plate. Generally, 5 µL per microwell of matrix is sufficient for embedding.

4) Place the EB-Plate at 37°C for 30 minutes.



5) Add 140 µL of fresh, equilibrated culture media to each well and place the device back into the 37°C incubator.

37°C

* *Matrigel® and Geltrex® are registered trademarks of Corning and Thermo Fisher Scientific, respectively*

Reusing EB-Plates

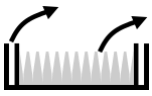
Before Starting

When preparing the EB-Plate for reuse, it is highly recommended that the well surfaces be thoroughly cleaned to properly remove molecular residues (e.g. cell debris, protein, etc.) that might remain from prior cultures. The nature and amount of residue remaining on the well surfaces depends on the culture type, conditions, reagents used, and the type of experiment that had been performed with the plates. Therefore, three cleaning procedures with varying cleaning intensity are provided below. The choice of which cleaning protocol to implement for a given culture application should be empirically determined.

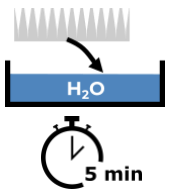
The cleaning procedure is most effective when the wells of the EB-Plate have not been allowed to dry after use. If wells do dry, add 140 µL of distilled water to each well and centrifuge the plate at 300 x *g* for 1 minute to force liquid into the base of the well. Allow this to soak for 10 – 20 minutes before starting the cleaning procedure.

When using an anionic detergent such as Alconox® or Tergazyme®, be sure to thoroughly rinse the wells afterwards with distilled or deionized water to remove detergent residues. The addition of a 99 % isopropanol rinse serves to further remove remaining detergent residue.

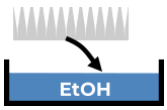
Light Cleaning Protocol



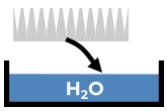
1) Aspirate all liquid from each well of the EB-Plate and anti-evaporation track. Separate the microwell structure from its frame and lid.



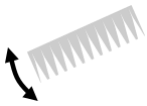
2) Rinse the microwell structure well in distilled water. This is best achieved by submersing the entire microwell structure into a clean basin containing distilled water (preferably warm; ~50°C). Allow to soak for 5 minutes.



3) Rinse the microwell structure with 70 % ethanol.

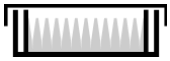


4) Rinse the microwell structure with fresh distilled or deionized water (deionized is preferable to use for this step).



5) Shake the microwell structure to remove excess water. Wrap the microwell structure with aluminum foil and sterilize using a steam sterilizer or autoclave. Alternatively, sterilization can be achieved using oxygen plasma.

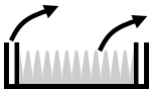
6) Sterilize the microplate frame and lid with 70 % ethanol. Place these in a biological safety cabinet to dry. **Do not use heat sterilization methods (steam or autoclave) to sterilize the microplate lid or frame.**



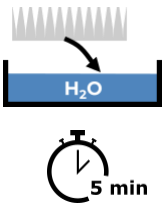
7) Once sterilized, unwrap the microwell structure under aseptic conditions and reassemble the EB-Plate with its frame and lid.

8) Follow the **Surface Coating Protocol** to prepare the microwell surface with anti-fouling coating prior to cell seeding.

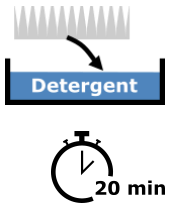
Medium Cleaning Protocol



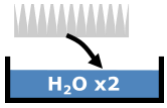
1) Aspirate all liquid from each well of the EB-Plate and anti-evaporation track. Separate the microwell structure from its frame and lid.



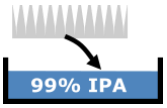
2) Rinse the microwell structure well in distilled water. This is best achieved by submersing the entire microwell structure into a clean basin containing distilled water (preferably warm; ~50°C). Allow to soak for 5 minutes.



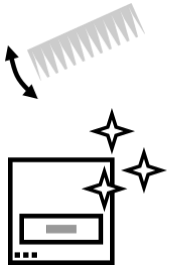
3) Submerge the microwell structure in a 1 % solution (preferably warm; ~50°C) of Alconox® or Tergazyme®. Allow to soak for 20 minutes. Alternatively, a 0.25 % trypsin solution can be added to each well, and allowed to incubate at 37°C for 20 minutes.



4) Rinse the microwell structure with fresh distilled or deionized water (deionized water is preferable to use for this step). Repeat once more, for a total of 2 rinses.



5) Rinse the microwell structure with 99 % isopropanol. Then, rinse twice more with deionized water.



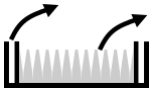
6) Shake the microwell structure to remove excess water. Wrap the microwell structure with aluminum foil and sterilize using a steam sterilizer or autoclave. Alternatively, sterilization can be achieved using oxygen plasma.

7) Sterilize the microplate frame and lid with 70 % ethanol. Place these in a biological safety cabinet to dry. **Do not use heat sterilization methods (steam or autoclave) to sterilize the microplate lid or frame.**

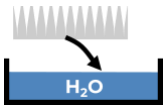
8) Once sterile, unwrap the microwell structure under aseptic conditions and reassemble the EB-Plate with its frame and lid.

9) Follow the **Surface Coating Protocol** to prepare the microwell surface with anti-fouling coating prior to cell seeding.

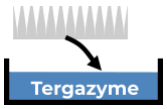
Intense Cleaning Protocol



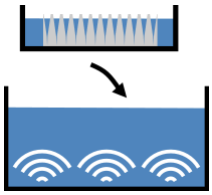
1) Aspirate all liquid from each well of the EB-Plate and anti-evaporation track. Separate the microwell structure from its frame and lid.



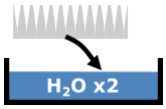
2) Rinse the microwell structure well in distilled water. This is best achieved by submersing the entire microwell structure into a clean basin containing warm (~50°C) distilled water. Allow to soak for 10 minutes.



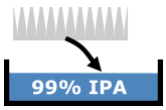
3) Submerge the microwell structure in a warm (~50°C) 1 % solution of Tergazyme®. Allow to soak for 10 minutes.



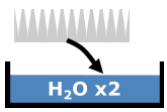
4) Place the basin in an ultrasonic cleaner and sonicate for 5-10 minutes.

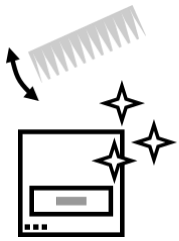


5) Rinse the microwell structure with fresh deionized water. Repeat once more, for a total of 2 rinses.



6) Rinse the microwell structure with 99 % isopropanol. Then, rinse twice more with deionized water.





7) Shake the microwell structure to remove excess water. Wrap the microwell structure with aluminum foil and sterilize using a steam sterilizer or autoclave. Alternatively, sterilization can be achieved using oxygen plasma.

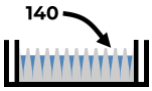
8) Sterilize the microplate frame and lid with 70 % ethanol. Place these in a biological safety cabinet to dry. **Do not use heat sterilization methods (steam or autoclave) to sterilize the microplate lid or frame.**

9) Once sterile, unwrap the microwell structure under aseptic conditions and reassemble the EB-Plate with its frame and lid.

10) Follow the **Surface Coating Protocol** to prepare the microwell surface with anti-fouling coating prior to cell seeding.

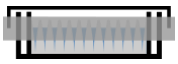
Surface Coating Protocol

1) Prepare a 5 % Pluronic® F-127 solution in PBS. Filter-sterilize the solution with a 0.2 µm filter and store at 4°C. This solution can be stored for at least 8 months at 4°C.

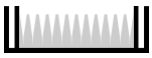


2) Under aseptic conditions (i.e. in a biological safety cabinet), add 140 µL of 5 % Pluronic® F-127 coating solution to each well of the EB-Plate. Using a multichannel/repeater pipette is recommended.

3) Using a swinging-bucket rotor with a microplate carrier, centrifuge the EB-Plate at 300 x *g* for 45 seconds to ensure the coating solution reaches the base of each microwell.



4) Seal the edges of the EB-Plate with parafilm, or place it in a sterile bag, and keep at 4°C for 12 hours.



5) **Optional:** Aspirate the coating solution from each well of the EB-Plate and allow the plate to air dry under a biological safety cabinet. EB-Plates with dried coating can be stored at room temperature until needed. However, it is recommended to use dried coated plates within 2 weeks.