EB-DISK

Embryoid Body Generation

User Guide

Contents

5524 Saint-Patrick, Office 460
Montreal, Quebec, Canada
H4E 1A8

CONTACT US AT INFO@ENUVIO.COM

v2.4

General Information

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

Storage

Uncoated EB-DISKs can be stored at room temperature. Pre-coated (ULA) EB-DISKs should be stored at 4°C.

Unpacking

To maintain sterility, it is recommended to unpack the contents in an aseptic environment (e.g. in a biological safety cabinet) using a sterile blade or scissors to cut open the plastic packaging and sterile tweezers to remove the EB-DISK. ULA-coated EB-DISKs are packaged in a 0.1 micron sterile filtered 5% Pluronic® F-127 solution (prepared in PBS without divalents) and are ready to use for seeding cells.

Reagents/equipment to reuse the EB-DISK

- 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 with microplate carrier(s) installed
- 70 % ethanol
- Steam sterilizer, autoclave or plasma cleaner (for sterilization)
- 99 % isopropyl alcohol (optional)
- Alconox®*, Tergazyme®*, or trypsin (optional)
- Ultrasonic bath (optional)

Recommended items to have on hand

(these are not included in this kit)

- Sterile tweezers to manipulate the EB-DISK (flat-tipped or wafer forceps are recommended)
- An EB collection vessel (preferably with ultra-low attachment surface; a 100 mm round culture vessel is recommended)

Required items to use the EB-DISK^{137} or EB-DISK^{360}

(these are not included in this kit)

- Sterile 6-well culture plate (for EB-DISK^{360})
- Sterile 12-well culture plate (for EB-DISK^{137})

Before Starting

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

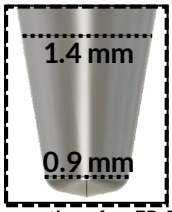
---

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

EB-DISK Specifications

Device	Number of Microwells	Disk Diameter (mm)	Microwell Volume (mm^3)	Microwell Diameter (top/bottom) (mm)
EB-DISK^{137}	137	19.5	1	1.4/0.9
EB-DISK^{360}	360	33	1	1.4/0.9
EB-DISK^{948}	948	52	1	1.4/0.9

- Microwell structure material: polydimethylsiloxane (PDMS)

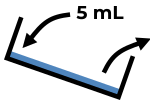


Cross section of an EB-DISK microwell. Not to scale.

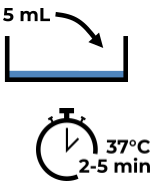
Cell Seeding Protocol

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.**

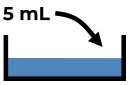
1) Unpack the EB-DISK under aseptic conditions. If starting with a **new, uncoated** EB-DISK or with an EB-DISK that is being reused, ensure it has first been coated with an anti-fouling reagent (see **Surface Coating Protocol** below). If you are using a pre-coated EB-DISK for the first time, simply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



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

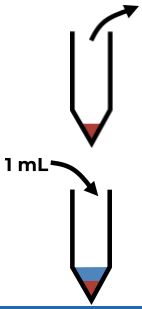


3) Add 5 mL of a gentle cell detachment solution and place the dish in a 37°C incubator for 2 to 5 minutes (or as recommended by the manufacturer). Monitor detachment progress by eye. Gentle tapping of the dish may be necessary.



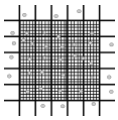
4) Stop cell detachment by adding 5 mL of media (e.g. DMEM/F12 or equivalent).

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 seeding culture media.

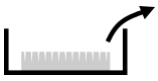
7) Gently triturate the cells to resuspend single cells in solution. This can be achieved by very gently pipetting the cells up and down (3 times) through a 1000 µL pipette tip fitted with a 200 µL pipette tip.



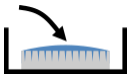
8) Count resuspended cells using a cell counter (e.g. haemocytometer or equivalent) and dilute with seeding media as required to obtain the appropriate cell density and volume required to seed the EB-DISK (see **Approximate Seeding Volumes** in the table below; also see **Example Seeding Calculation** below). Just prior to seeding, ensure that the cells are sufficiently resuspended to create a homogenous cell/media mixture.

Device	Number of Microwells	Recommended Total Media Volume (per EB-DISK)*	Approx. Seeding Volume (per EB-DISK)*
EB-DISK^{137}	137	2 – 3 mL	280 - 300 µL
EB-DISK^{360}	360	3 – 4 mL	800 - 900 µL
EB-DISK^{948}	948	5 – 6 mL	2.0 – 2.2 mL

** volumes are intended to provide a starting point; specific applications may require different volumes*

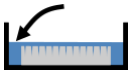


9) If coating was just completed, or if using a pre-coated EB-DISK, aspirate all Pluronic® F-127 coating solution from the device. **Washing the residual coating solution is optional.** If coating was done previously, and the EB-DISK has been allowed to dry, proceed directly with seeding.



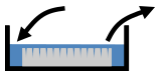
10) Add the appropriate seeding volume of cell/media mixture to each EB-DISK. Use a seeding volume of cell/media mixture such that the mixture covers the surface of the disk. Care should be taken to avoid media running down the side of the disk. Note: the homogeneity of the generated EBs is directly associated to the uniform distribution of the seeding volume across the EB-DISK.

11) Wait ~2 mins for the cells to settle on the surface of the EB-DISK. Then, using a swinging-bucket rotor with a microplate carrier, centrifuge the culture volume at 300 x *g* for 1 minute to gently collect the cells into the base of each microwell.



12) Return to a biological safety cabinet and gently add the appropriate volume of media to complete the total volume (see **Recommended Total Media Volume** in the table above). Apply the media to the sidewalls of the culture vessel to avoid disrupting the seeded cells.

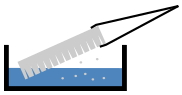
13) Place the EB-DISK in the 37°C incubator.



14) Perform media changes as needed by the culture. If air becomes trapped under the EB-DISK after exchanging media, use a pair of sterile pipet tips or tweezers to carefully lift the EB-DISK out of the media to free any trapped air. Alternatively, consider performing half-media changes more often.

15) Once the EBs have formed in the microwells and have grown to the desired size, EBs can be optionally embedded in extracellular matrix (ECM) hydrogel (see **ECM Embedding Protocol** below).

16) When EBs are ready to be removed from their microwells, prepare a new, larger and preferably ultra-low attachment vessel (e.g. 6-well plate or 100 mm dish) containing fresh, warmed culture media.



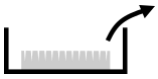
17) Using sterile forceps, carefully flip the EB-DISK over into the freshly prepared vessel. The EBs will fall from the microwells and collect in the new vessel. Gentle shaking of the disk will help release EBs. If any EBs remain in the microwells, they can be released by lifting the disk at a 30 – 45° angle and flushing the surface with media using a liquid handler and serological pipette. See https://enuvio.com/eb-disk-and-eb-plate/ for a video demonstration of this step.

18) Visualize the EB-DISK under a microscope to ensure that all EBs have been transferred. If necessary, repeat Step 17 until all EBs are released.

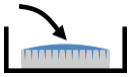
ECM Embedding Protocol (optional)

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

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**.

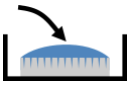


2) Remove all growth media from the EB-DISK culture vessel.



3) Add ECM hydrogel to the surface of the EB-DISK. Generally, 5 µL per microwell of matrix is sufficient for embedding.

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



5) Add 150 µL of fresh, equilibrated culture media to the EB-DISK culture vessel and place it back into the 37°C incubator.

6) Allow 16 – 24 hours of incubation before transferring EBs out of the EB-DISK into a larger vessel.

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

Reusing EB-DISKs

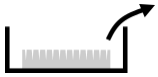
Before Starting

When preparing EB-DISKs 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 disk. It is highly recommended to test the cleaning protocol for compatibility with the intended downstream experiment. Three cleaning procedures with varying cleaning intensities are provided below. The choice of which cleaning protocol to implement for a given culture application should be empirically determined.

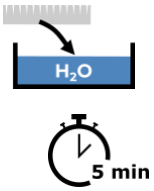
The cleaning procedure is most effective when the EB-DISK has not been allowed to dry after use. If wells do dry, place the disk in a suitable vessel, add sufficient water or buffer to completely cover the disk and centrifuge the plate at 300 x *g* for 1 minute to force liquid into the base of each well. Allow the EB-DISK 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 EB-DISK afterwards with distilled or deionized water to remove detergent residues. The addition of a 99 % isopropanol rinse serves to further remove trace amounts of detergent residue that may remain on the device.

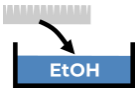
Light Cleaning Protocol



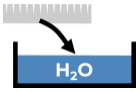
1) Aspirate all liquid from the EB-DISK culture vessel and disk surface.



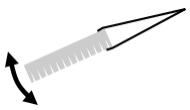
2) Rinse the disk well with distilled water. This is best achieved by submersing the disk into a clean basin containing distilled water (preferably warm; ~50°C). Allow to soak for at least 5 minutes.



3) Rinse the disk with 70 % ethanol.

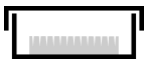


4) Re-rinse the disk with fresh distilled or deionized water (deionized is preferable to use for this step).



5) Shake the disk to remove excess water. Wrap the disk with aluminum foil or place it in an autoclavable container (e.g., glass beaker with opening covered with aluminum foil) and sterilize using a steam sterilizer or

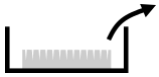
autoclave. Alternatively, sterilization can be achieved using oxygen plasma.



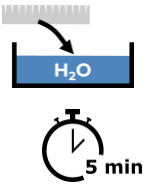
6) Once sterilized, unwrap the EB-DISK under aseptic conditions and place it into a new, appropriately sized culture vessel.

7) Follow the **Surface Coating Protocol** to prepare the EB-DISK with anti-fouling coating prior to cell seeding

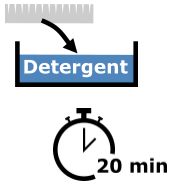
Medium Cleaning Protocol



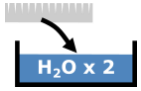
1) Aspirate all liquid from the EB-DISK culture vessel and disk surface.



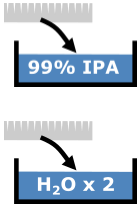
2) Rinse the disk well with distilled water. This is best achieved by submersing the disk into a clean basin containing distilled water (preferably warm; ~50°C). Allow to soak for at least 5 minutes.



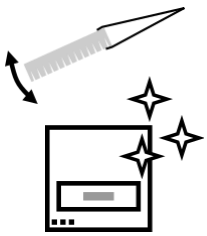
3) Submerge the disk in a 1 % solution (preferably warm; ~50°C) of Alconox® or Tergazyme®. Allow to soak for 20 minutes. Alternatively, a 0.25 % trypsin solution can be used, followed by incubation at 37°C for 20 minutes.



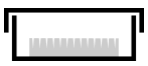
4) Submerge the disk in fresh distilled or deionized water (deionized water is preferable to use for this step). Repeat once more, for a total of 2 rinses.



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



6) Shake the disk to remove excess water. Wrap the disk with aluminum foil or place it in an autoclavable container (e.g., glass beaker with opening covered with aluminum foil) and sterilize using a steam sterilizer or autoclave. Alternatively, sterilization can be achieved using oxygen plasma.

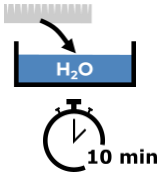


7) Once sterilized, unwrap the EB-DISK under aseptic conditions and place it into a new, appropriately sized culture vessel.

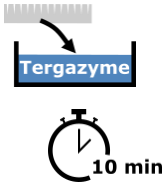
8) Follow the **Surface Coating Protocol** to prepare the EB-DISK with anti-fouling coating prior to cell seeding

Intense Cleaning Protocol

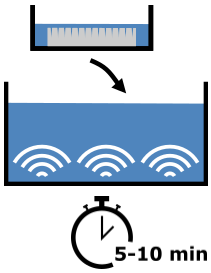
1) Aspirate all liquid from the EB-DISK culture vessel and disk surface.



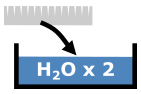
2) Rinse the disk well with distilled water. This is best achieved by submersing the disk into a clean basin containing warm (~50°C) distilled water. Allow to soak for 10 minutes.



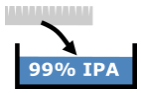
3) Submerge the disk in a warm (~50°C) 1 % solution of Tergazyme®. Allow to soak for 10 minutes.

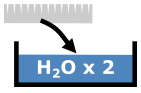


4) Place the basin with disk in an ultrasonic cleaner and sonicate for 5-10 minutes.

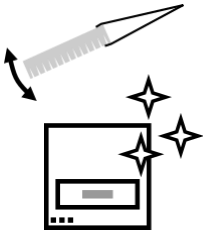


5) Rinse the disk with fresh deionized water. Repeat once more, for a total of 2 rinses.

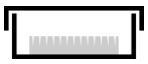




6) Rinse the disk with 99 % isopropanol. Then, rinse twice more with deionized water.



7) Shake the disk to remove excess water. Wrap the disk with aluminum foil or place it in an autoclavable container (e.g., glass beaker with opening covered with aluminum foil) and sterilize using a steam sterilizer or autoclave. Alternatively, sterilization can be achieved using oxygen plasma.



8) Once sterilized, unwrap the EB-DISK under aseptic conditions and place it into a new, appropriately sized culture vessel.

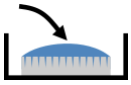
9) Follow the **Surface Coating Protocol** to prepare the EB-DISK with anti-fouling coating prior to cell seeding

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), using a sterile blade or scissors, cut open the plastic packaging of a new, uncoated EB-DISK. If reusing a sterilized EB-DISK, remove sterilization wrapping. **Pre-coated EB-DISKs do not need to be coated prior to their first seeding**.

3) For EB-DISK^{137} and EB-DISK^{360} devices, use sterile flat-tipped tweezers or another suitable tool to carefully remove the device from its package. Place each EB-DISK device, microwells facing up, in a single well of a 12-well (EB-DISK^{137}) or 6-well (EB-DISK^{360}) plate (**not included**). For EB-DISK^{948}, simply remove the included 60 mm dish containing the disk from its packaging.



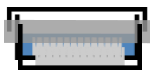
4) Add a volume of 5% Pluronic® F-127 coating solution to the surface of each disk (see **Recommended Total Loading Volume** in the table below).

It is important to use a coating volume that completely covers the surface of the disk. **EB-DISKs may float in solution: this is normal**. Using sterile tweezers or a pipet tip, gently push down on the top of the disk to ensure the disk can be completely submerged by coating solution.

Device	Number of Microwells	Recommended Total Loading Volume (per EB-DISK)*
EB-DISK^{137}	137	2 – 3 mL
EB-DISK^{360}	360	3 – 4 mL
EB-DISK^{948}	948	5 – 6 mL

** volumes are intended to provide a starting point*

5) Using a swinging-bucket rotor with a microplate carrier, centrifuge the 6-well, 12-well, or EB-DISK^{948} 60mm dish microplate carrier (w/ adapter) at 300 x *g* for 1 minute to ensure the coating solution reaches the base of each microwell.



6) Seal the edges of the culture vessel with parafilm, or place the vessel in a sterile bag, and keep at 4°C for 12 - 16 hours.

7) **Optional:** Aspirate the coating solution from the EB-DISK, wash the surface once with PBS (aspirate excess PBS), and allow the disk to air dry under a biological safety cabinet. EB-DISKs with dried coating can be

stored at room temperature or at 4°C until needed. It is recommended to use dried, coated EB-DISKs within 2 weeks.

Note: Seeding cell directly on dried, coated EB-DISKs can improve seeding homogeneity (and therefore EB uniformity) because the seeding media does not readily enter the microchannels until centrifugation. This provides the opportunity for the user to evenly distribute seeding media across the surface of the disk to cover all the microwells.

Example Cell Seeding Calculation

Hypothetical number of cells to seed per microwell in an EB-DISK^{360}: **10,000 cells/microwell**

360 microwells per EB-DISK^{360} = 10,000 cells/microwell x 360 microwells = **3,600,000 cells**

Final density in 0.9 mL volume = ~3,600,000 cells/0.9 mL = **4,000,000 cells/mL**