n3D Biosciences, Inc.

greiner bio-one

Instruction Manual
24-Well Bio-Assembler™ Kit

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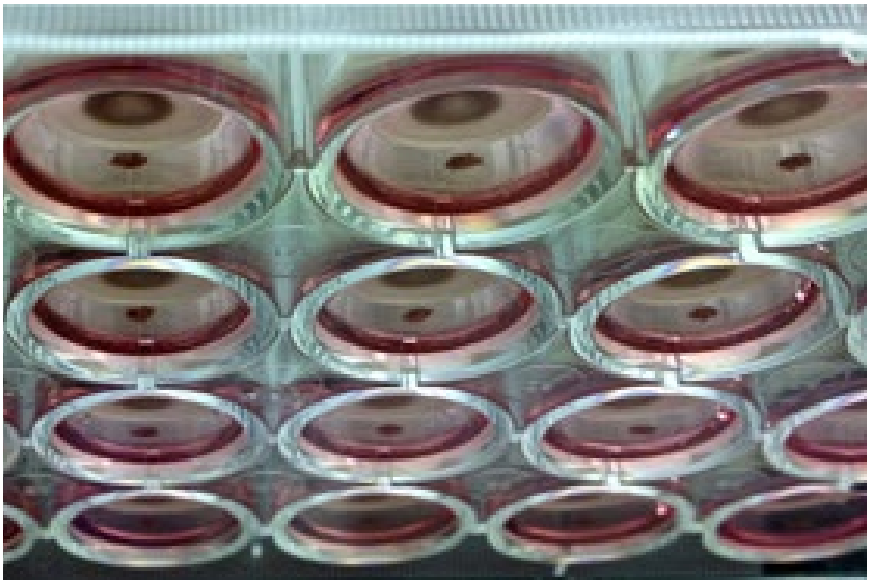

Introduction

24-Well Bio-Assembler™ Kit Instruction Manual

Thank you for purchasing our product for 3D cell culturing. The **24-Well Bio-Assembler™ Kit** uses **NanoShuttle™-PL,** a nanoparticle assembly consisting of gold, iron oxide, and poly-L-lysine to magnetize cells, at which point they can be magnetically directed. In this kit, cells are levitated in 24-well plates with a magnetic drive above the plate to levitate them off the bottom, where they are aggregated at the air-liquid interface to form larger 3D cultures. **NanoShuttle™-PL** should be stored at 4°C.

Caution

The magnets in this kit are strong, can damage electronics, and cause injury if not handled correctly. **DO NOT** remove the magnets from the protective covers. **DO NOT** autoclave. **DO NOT** store near metal surfaces. Read the attached instructions carefully on how to handle the magnets.

Product Use

The **24-Well Bio-Assembler™ Kit** is for research use only. It is not approved for human or animal use.

Materials and Supplies

Materials and Supplies Needed to Levitate Cells
24-Well Bio-Assembler™ Kit, which includes: NanoShuttle™-PL (2 600 µL vials); 24-Well Levitating Drive (1); 24-Well Concentrating Drive (1); Custom Lid (1); Cell-Repellent 24-Well Plates (2).
Other Materials Provided by User:
70% Ethanol
Phosphate Buffered Saline (PBS, Calcium and Magnesium free)
0.25% Trypsin/EDTA Solution or the recommended detaching solution for your cell type
Pipettes, flasks, other general tissue culture supplies and tools
Cells (in suspension or monolayer)
Medium (use typical media for 2D culture, if serum-free, use trypsin neutralization solution to inactivate trypsin)
Microscope
Any additional supplies for the specific cell type and application

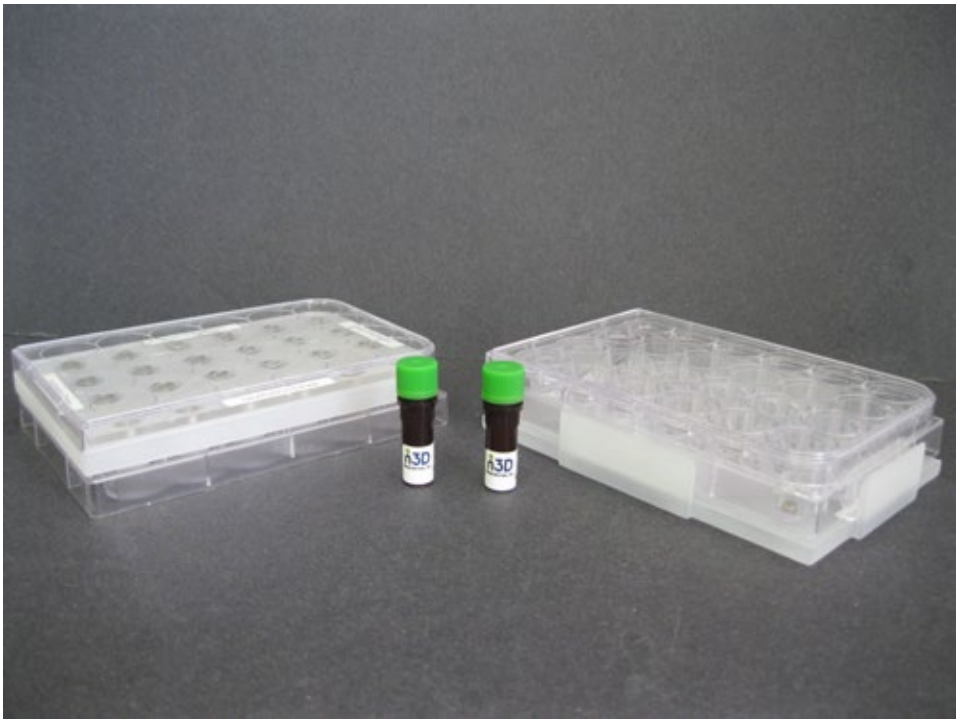

Fig. 1: 24-Well Bio-Assembler™ Kit

Instructions for magnetically levitating cells in the 24-Well Bio-Assembler™ Kit

Overview: 600 µL of **NanoShuttle™-PL** will treat one T-75 flask of cells at 80% confluence (approximately 6 million cells). At 2.5 x 10^5 cells/ structure, this is enough to levitate 24 3D cultures. 3D cultures to be paraffin-embedded may require more cells per culture. The **24-well Bio-Assembler™ Kit** works best with CELLSTAR® Cell-Repellent 24-Well Plates (662970, Greiner Bio-One, included in the kit).

Optimization may be required for different cell types or specific experimental aims.

Treating Cells with NanoShuttle™-PL

1. Culture cells to 80% confluence in a T-25, T-75, or T-150 culture flask using standard procedures in your laboratory for your specific cell type.

2. Treat cells with **NanoShuttle™-PL** as follows:

a) Remove **NanoShuttle™-PL** from refrigeration and let it stand at room temperature for at least 15 minutes.

b) Homogenize **NanoShuttle™-PL** in its vial by pipetting it up and down at least 10 times.

c) For a **T-25 flask add 200 µL NanoShuttle™-PL**, or for a **T-75 flask add 600 µL NanoShuttle™-PL**, or for a **T-150 flask add 1200 µL Nanoshuttle™-PL** directly to the media.

d) Incubate cells with **NanoShuttle™-PL** overnight.

Note: The amount of **NanoShuttle™-PL** added can be optimized to use more or less volume for specific cell types. Optimize the volume before experimentation by forming 3D cultures with more or less **Nanoshuttle™-PL** added. A benchmark concentration is 1 µL/10,000 cells.

Note: NanoShuttle™-PL is brown in color. After incubation, the cells will appear peppered with the brown **NanoShuttle™-PL** (Fig. 2).

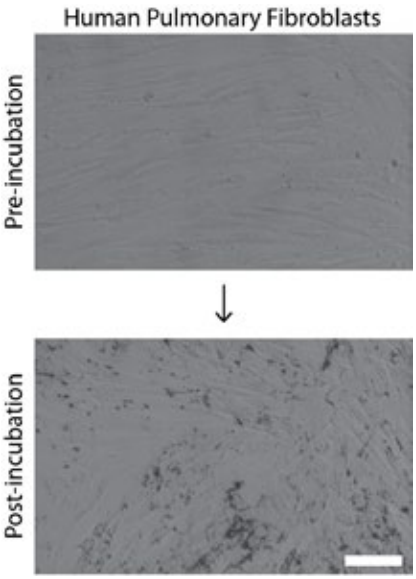

Fig. 2: After incubation with NanoShuttle™-PL, cells will appear peppered with the brown nanoparticles, as demonstrated by primary human pulmonary fibroblasts. Scale bar = 100 µm.[1]

Cell Detachment

3. After incubation, warm/thaw Trypsin/EDTA solution, PBS, and media in a water bath to 37°C.

4. In a sterile hood, aspirate all media (including excess **NanoShuttle™-PL**) from the flask.

5. Wash cells to remove any remaining media and excess **NanoShuttle™-PL** by adding PBS to the flask and gently agitating. We recommend **2 mL of PBS for a T-25 flask**, **5 mL for a T-75 flask**, and **10 mL for a T-150 flask.**

6. Aspirate PBS and add Trypsin/EDTA solution to the flask. Add enough Trypsin/EDTA solution to cover the cell monolayer, about **1 mL to a T-25 flask**, **2 mL to a T-75 flask**, or **4 mL to a T-150 flask**. Follow your laboratory's cell-specific detachment protocols.

7. Place the flask in an incubator for approximately 3-5 minutes or for a time prescribed by your standard protocol for detaching cells. Check for detachment under a microscope.

8. While waiting for cells to detach, clean the magnetic drives that you will use by wiping them with 70 % ethanol. Keep the magnetic drives sterile.

Note: Do not soak drives in ethanol. Lightly spray and wipe to sterilize.

9. Remove flask from incubator and check under a microscope that the cells are detached from the surface. Excess exposure to Trypsin/EDTA will adversely affect cell health, so proceed to the next step quickly.

10. Deactivate Trypsin/EDTA by adding 37°C media with serum. The amount of media with serum added should at least match the original volume of Trypsin/EDTA added. If cells are sensitive to serum, either use trypsin neutralizing solution, or immediately centrifuge cells (at least 100 G for 5 min) and aspirate the trypsin.

11. Count the cells using a hemacytometer or Coulter counter. Centrifuge cells and resuspend them in the required amount of media (400 µL per culture).

Note: We recommend levitating cultures with 2.5 x 10^5 cells each (6.25 x 10^5 cells/mL), but the number of cells per culture can be different. Cultures have successfully been formed with cell numbers from 1 x 10^5 to 6.25 x 10^3. Optimize the number of cells per culture by levitating cultures with more or less cells.

Magnetic Levitation

12. Draw up the suspended cells with a sterile pipette, and dispense 400 µL of the cell suspension into the wells of the cell-repellent 24-well plate.

Note: Too much media in the dish will bring the cells too close to the magnet, where the cells are at risk of escaping the media. **Do not add more than 400 µL of media.**

13. Close the plate with the custom lid, place the 24-well levitating drive atop the custom lid, place the regular lid atop the levitating drive, transfer the plate and drive together to an inbubator, and leave them overnight (Fig. 3).

Note: If the cells are not immediately levitating, gently agitate the plate by moving the plate back and forth, until they levitate.

Fig. 3: Take a cell-repellent 24-well plate, place the custom lid atop the cell-repellent 24-well plate, (a) place a 24-well levitating drive atop the custom lid, (b) and place the regular lid atop the 24-well levitating drive to levitate the cells (c).

14. The next day, remove the levitating drive and the custom lid from the plate, close the plate with the regular lid, place the plate on the 24-well concentrating drive, transfer the plate and drive together to an incubator, and leave them overnight (Fig. 4).

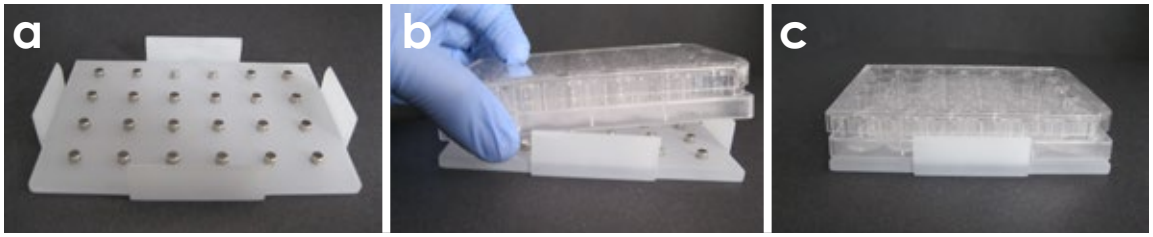

Fig. 4: Take a 24-well concentrating drive (a) and place a cell-repellent 24-well plate (b) atop the 24-well concentrating drive to aggregate the cells (c).

15. The next day, remove the plate from the concentrating drive and place the custom lid and the levitating drive atop the plate (Fig. 3).

Note: If the 3D structures are not immediately levitating, gently agitate the plate by moving the plate back and forth, until it levitates.

16. Transfer the plate to an incubator for the length of the experiment. By 15 min - 1 hr, cells should begin to levitate and aggregate, forming a noticably brown culture levitated within the well. The 3D cultures can be imaged under a microscope using the hole in the magnet where light can pass through. If media exchange is necessary, use the concentrating drive to hold the 3D cultures down while aspirating liquids (Fig. 4).

Note: When moving the plate, keep the plate flat at all times. Tilting the plate could bring the 3D culture close to the magnet, where it could escape the media.

Note: If the 3D culture is not competent enough, repeat steps 14-16 . This can be done at any time during your experiment for however long is necessary to form a 3D culture.

Post-Culture Handling

After culturing, standard tissue processing techniques can be performed on the 3D cultures, such as fixation, paraffin embedding for immunohistochemistry, or RNA isolation for qRT-PCR. Use the concentrating drive to hold the culture down while adding and removing liquids (Fig. 4).

Troubleshooting

Problem	*Probable Cause*	*Solution*
NanoShuttle™-PL appears separated	**NanoShuttle™-PL** has settled at the bottom of the vial	Homogenize the **NanoShuttle™-PL** before use by pipetting up and down 10X
NanoShuttle™-PL do not appear to fully bind with cells, floating in medium	Binding with **NanoShuttle™-PL** varies in efficiency among cell types	**NanoShuttle™-PL** will appear peppered on cells and some will float, but the cells are still magnetized. Add less **NanoShuttle™-PL** if too excessive
	Cells were incubated with **NanoShuttle™-PL** too long	Incubate cells with **NanoShuttle™-PL** overnight at most
Cells taking longer than usual to detach	Cells strongly adhered to substrate	Before adding trypsin, wash flask with PBS 1-2X
NanoShuttle™-PL sparsely attached to cells	Too many cells	Increase **NanoShuttle™-PL** volume added to each well to yield an ideal concentration of 1 µL/10,000 cells
Cells are sensitive to serum	Cells may undergo unwanted differentiation with serum	Use a trypsin-neutralizing solution in lieu of serum-contained media to stop trypsin activity. Centrifuge cells immediately after and remove trypsin solution
Magnetized cells attaching to bottom of the plate	Magnetized cells are weakly or not bound to **NanoShuttle™-PL**	Use cell-repellent plates to prevent cells from adhering and collect weakly magnetized cells
Levitated cells are escaping the medium and attaching to the lid insert	Too much medium	Only add a maximum of 400 µL per well
	Plate tilted too far	Always keep the plate flat when moving
Levitated cells appear spread out	Cells have not been levitated for enough time	Levitate the cells longer and carefully monitor the formation of the 3D culture
3D cultures are lost or broken when removing liquids	3D culture is not held down while liquids are transferred	Use the 24-well concentrating drive to hold down cultures while adding and removing liquids

Cell Types

Some of the cell types that have been successfully cultured using the procedure include:

Cell lines

- Murine Endothelial
- Murine Embryonic Fibroblasts, pre-adipocytes (3T3)
- Murine Adipocyte
- Murine Melanoma Murine Neural Stem Cells
- Rat Hepatoma
- Human Astrocytes
- Human Glioblastoma Multiforme (GBM) LN 229
- Human Embryonic Kidney (HEK293)
- Rat Vascular Smooth Muscle (A10)
- Human Hepatocellular Carcinoma Cells (HepG2)
- Human Lung Adenocarcinoma Cells (A549)
- Human Colorectal Carcinoma Cells (HCT116)
- Human Pancreatic Epithelioid Carcinoma (PANC-1)

Primary cells

- Human Pulmonary Microvascular Endothelial Cells (HPMEC)
- Human Tracheal Smooth Muscle Cells (HTSMC)
- Human Small Airway Epithelial Cells (HSAEpiC)
- Human Pulmonary Fibroblasts (HPF)
- Human Mesenchymal Stem Cells (HMSC)
- Human Bone Marrow Endothelial Cells (HBMEC)
- Human Umbilical Vein Endothelial Cells (HUVEC)
- Human Aortic Vascular Smooth Muscle (HASMC)
- Human Neonatal Dermal Fibroblasts (HDFn)
- Murine Chondrocytes

References

1. Haisler, W. L. et al. Three-dimensional cell culturing by magnetic levitation. Nat. Protoc. 8, 1940–9 (2013).

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