Biosciences, Inc.

Contact information

Nano3D Biosciences, Inc.
University Tower Center
7000 Fannin Street
Suite 2140
Houston, TX 77030

Phone: 713-790-1833
info@n3dbio.com

Distributed in Austria, Germany and Switzerland by PELOBIOTECH GmbH

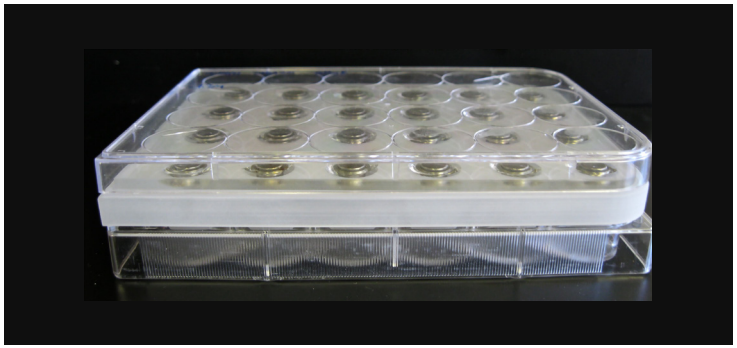
PELOBIOTECH GmbH
Am Klopferspitz 19
82152 Planegg / Germany

Contact us:
(P) +49 (0)89 517 28 59-0
(F) +49 (0)89 517 286 59-88
Email:info@pelobiotech.com
www.pelobiotech.com

Biosciences, Inc.

Instruction Sheet

Instruction Sheet for the
Bio-Assembler™
24-well Configuration with
Nanoshuttle™-PL



Instruction Sheet for the Bio-Assembler™ 24-well Configuration with Nanoshuttle™-PL

Thank you for purchasing this Nano3D, Biosciences product. The **Bio-Assembler™** uses magnetic levitation to enable fast and simple three-dimensional cell culturing without artificial scaffolds or matrices. **Nanoshuttle™-PL** is an animal-origin-free, polylysine-based nano-assembly that delivers magnetic nanoparticles to cells so that they can be manipulated with magnetic fields to manipulate cells. **Nanoshuttle™-PL** should be stored at 4°C.

Caution

Magnets are strong and can damage electronics and cause injury if not handled correctly. **DO NOT** remove magnets from the protective covers. Read the attached magnet-handling instructions carefully.

Product Use

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

Materials and Supplies Needed for One 24-well Experiment
24-well Bio-Assembler™ magnetic drive and special white lid
200 mL **Nanoshuttle™-PL**
24-well tissue culture plate (see below for recommendations)
70% Ethanol
PBS Buffer (Calcium and Magnesium free)
0.25% Trypsin/EDTA Solution or the recommended detaching solution for your cell type
Pipettes
T-25 tissue culture flask (25 cm^2)
Cells
Cell Media*
Inverted Microscope
Additional supplies may be needed for specific applications

*Use the media type that is typically used to culture your cells of interest in 2D. (If it does not contain serum, you will also need serum to inactivate the Trypsin/EDTA.)

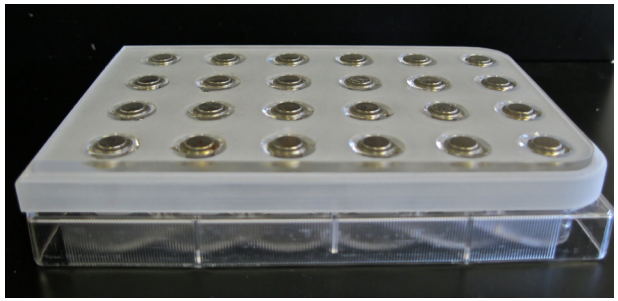


Figure 1. 24-well Bio-Assembler™ magnetic drive on top of standard 24-well tissue culture plate.

Instruction for culturing cells with the Bio-Assembler™ 24-well configuration starting with surface-attached cells:

Overview: 200 µl of **Nanoshuttle™-PL** will treat one T-25 flasks of cells at 80% confluence (approximately 1 million cells). This is typically enough cells for seeding all the wells of a 24-well tissue culture plate. Experiments requiring large tissue samples, such as paraffin tissue embedding, may require twice as many cells per well. The 24-well Bio-Assembler™ works best with 300 µl media volume per well, which yields 4.5 mm from the magnet bottom to the bottom of the media meniscus where most of the levitating cells reside. This system is designed to be used with 24-well Ultra Low Cluster Plate from Corning (Costar 3473; it can be purchased at www.n3DBio.com) provided with the kit (2 plates).

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

1. Culture cells to 80% confluence in a T-25 or T-75 culture flask using standard procedures in your laboratory for your specific cell type. 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** directly to the media.
d) Gently agitate flask to evenly distribute **Nanoshuttle™-PL**.

2. Incubate treated cells in their flask for at least 5 hours or overnight using your laboratory's standard cell culture incubation conditions. Overnight incubation is acceptable.
Note: Nanoshuttle™-PL has a darkened coloration. The cells will display this coloration after being treated with **Nanoshuttle™-PL**.

3. After the incubation period is complete, warm/thaw Trypsin/EDTA, PBS buffer, and Media in a water bath to about 37°C.

4. Remove and discard all media (including excess of **Nanoshuttle™-PL**) from the culture flask with a sterile pipette.

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

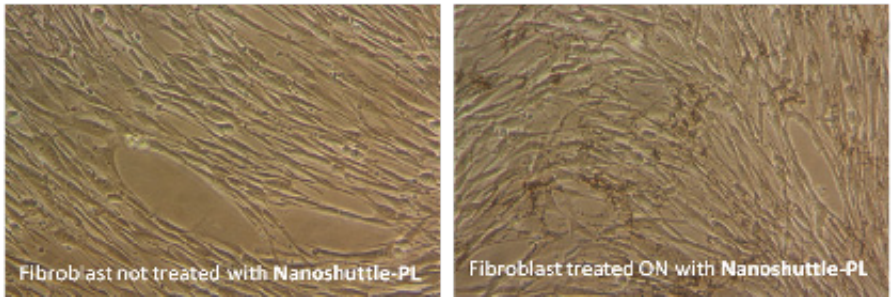


Figure 2. Human fibroblast cells plated in a T-25 flask before treatment (left) and after treatment with Nanoshuttle™-PL, incubation overnight, and application of PBS (right). Magnification is 10x.

6. Remove PBS with a pipette and discard it. To begin detaching cells, add enough Trypsin/EDTA solution to cover the adhering cell layer. Detachment protocols are cell specific. If your laboratory has an established protocol, we suggest you follow it. If not, we suggest 0.5 - 1.0 mL of Trypsin/EDTA solution. Contact n3D for additional help. Optimization may be required.

7. Place the vessel in an incubator for approximately 3 minutes or for a time prescribed by your standard protocol for detaching cells.

8. While waiting for cells to detach, clean the magnetic drives that you will use by wiping them with 70 % ethanol
and
spray special white lid with 70% EtOH for sterilization and let it dry under sterile conditions. (Make sure lid is dry before step 13.d)

9. Remove flask from incubator and check with an inverted microscope to be sure that the cells are adequately 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 serum or media containing serum. The volume added must be at least 10x larger than the volume of Trypsin/EDTA used. We suggest 5 mL of 10% serum/media for a T-25 flask or 10 mL of 10% serum/media for a T-75 flask.

11. Draw the cell suspension with a sterile pipette. Expel the suspension back into the flask to rinse the inside surfaces. Repeat several times. Check again under a microscope to ensure that cells are in suspension. Redraw the entire cell suspension into the pipette. Cells should then be counted using a hemacytometer or Coulter counter.

12. If needed, dilute solution with media to a desired density to facilitate adding a specific number of cells to each culture vessel. For optimum results with levitated cultures, seed approximately 30,000 cells per well.
Note: we recommend evaluating different cell seeding number for determining optimum experimental outcome. For example: for rapid formation of large structures, seed more than 50,000 cells; for smaller 3D structures, seed less than 50,000 cells. (size and structure of 3D cultures may vary for different cell types)

13. Load and levitate cells as follows (Fig. 4):
a) Ensure there is no settling of cells in the pipette by expelling the suspension into a suitable container and redrawing. Repeat several times

b) Add desired amount of cell suspension and additional media if needed to each well. Total media in well should be 310 - 400 µl (Fig. 4.2)

c) Make sure special white lid is dry from EtOH spraying process in 8.)

d) Place lid on 24-well plates carrying cells (Fig. 4. 3 and 4)

e) Place magnet driver on special white lid (Fig. 4. 5 and 6)

f) Place original 24-well lid on top of magnet driver (Fig. 4. 7 and 8)

g) Label wells as needed (Fig. 4. 9)

Note: The time between adding cell suspension to wells and placing the magnetic drives should be kept short (less than a few minutes) and consistent for optimal and reproducible levitation results.

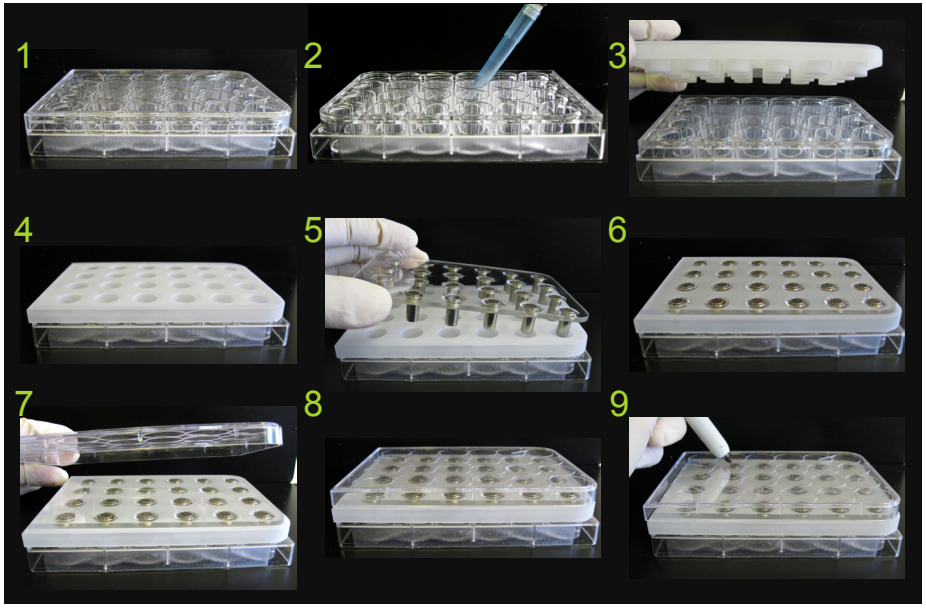


Figure 4. Steps for assembly of 24-well plate and magnetic drive.

14. Gently agitate the 24-well plate holder for 3 seconds (gentle side-to-side motion). Ensure that media does not come close to magnets. Failure to do so may lead to the deposition of cells on the underside of the plate cover.

15. Gently place 24-well plate with magnetic drive in an incubator and incubate according to your standard protocols for 2D cell culturing. Take care while moving plate to keep it level to ensure that media does not come close to magnets.

16. Cells may be removed from the incubator for diagnostics or the addition of new reagents. Care should be taken to minimize the agitation of the plate and to keep it level as described above. Agita-

tion may disturb the formation of large-scale tissue structure, especially during the first day of culturing. The magnetic drive may be removed, and the cells will typically drop to the bottom of the well. They will re-levitate when the magnetic drive is reapplied, but this may disturb the formation of large-scale tissue structure. After several a couple days of culturing, most cell types form large-scale tissue structure that is robust against agitation and removal of the magnetic drive.

17. Cells can be visualized using a standard inverted optical microscope without removing magnet driver. Position the 24-well plate with magnetic drive on the microscope stage and pass light though the opening in the center of the magnet over the well of interest. Using 4x, 10x, or 20x magnification, it should be possible to visualize levitating 3D cell cultures. If higher magnification is needed, remove the magnetic drive. Wait approximately 1 minute 30 seconds for cells to drop to the bottom of the wells for improved imaging. After imaging, replace the magnetic drive on top of the plate cover. The cells should readily re-levitate.

18. If necessary, feed 3D cultures after 2-3 days by adding or replacing media. New media should be fresh and 37°C before adding it to wells. Ideally, media volume should be kept at 300 to 400 microliter.

Caution: If replacing media, carefully remove the old media with a pipette so the 3D structures are not lost. The safest way to change media is to place the magnetic drive under the bottom of the 24-well plate before doing any pipetting. The exposed sides of the magnets should now be oriented up. The cells will be magnetically held on the bottom of the plate while you change the media.

Note: If desired, after 48 hours of levitation, levitating 3D cell structures can be transferred to a new 24-well plate containing fresh media. To do so, carefully pipette the 3D structures from each well into the new plate.

Post-culture handling of 3D structures:
After cells are cultured, remove magnetic drive and wait for cells to drop to the bottom of the 24-well plate. Standard laboratory procedures for cell fixation, paraffin embedding or freezing, sectioning, and staining may then be used.

Cell Types
All cell types that have been tested with the **Bio-Assembler™** using the procedure described below have been cultured successfully. This includes:

Cell lines
- Murine Endothelial
- 3T3 Fibroblasts, pre-adipocytes
- Murine Adipocyte
- Murine Melanoma
- Murine Neural Stem Cells
- Rattus Norvegicus Hepatoma
- Human Astrocytes
- Human Glioblastoma Multiforme (GBM) LN 229
- Human Embryonic Kidney 293 (HEK 293)

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)
- Murine Chondrocytes

Nano3D Biosciences believes that the technology is broadly applicable to essentially all cell types. If you would like to discuss other cell types with our technical support, please call: *713-790-1833*.