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Instructions For Use

96-Well BiO Assay™ Kit

REF 655846

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Thank you for purchasing this Greiner Bio-One product. The 96-Well BiO Assay™ Kit uses NanoShuttle™-PL, a nanoparticle assembly consisting of gold, iron oxide, and poly-L-lysine to magnetise cells, at which point they can be magnetically directed. In this kit, cells in a 96-well plate are printed into rings or spheroids using a magnetic drive to aggregate cells at the bottom of the well. These printed structures will contract over time in a dose-dependent manner. NanoShuttle™-PL has to be stored sterile at room temperature.

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 96-Well BiO Assay™ Kit is for research use only. It is not approved for human or animal use.

1. MATERIALS AND SUPPLIES

Materials and Supplies Needed for High-Throughput Testing
96-Well BiO Assay™ Kit, which includes: NanoShuttle™-PL (3 x 600 µL vials); Ring Drive (1); Spheroid Drive (1); Holding Drive (1); Cell-Repellent 96-Well Plates (2); 96-Well Mixing Reservoir (1); Levitating Drive (1); Cell-Repellent 6-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 neutralisation solution to inactivate trypsin)
Microscope
Any additional supplies for the specific cell type and application

2. INSTRUCTIONS

Instructions for performing high-throughput testing using the 96-Well BiO Assay™ Kit

Overview: 600 µL of NanoShuttle™-PL will treat one T-75 flask of cells at 80 % confluence (approximately 6 million cells). At 200,000 cells/ring, or 50,000 cells/spheroid, this is typically enough to form 30 rings or 120 spheroids. Rings or spheroids to be paraffin-embedded may require more cells per structure. The 96-Well BiO Assay™ Kit works only with CELLSTAR® Cell-Repellent 6-Well and 96-Well Plates (REF 657970 and 655970, Greiner Bio-One, included in the kit).

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

2.1 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) Homogenise NanoShuttle™-PL in its vial by pipetting it up and down at least 10 times.
   b) 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 1,200 µL NanoShuttle™-PL directly to the media.
   c) Incubate cells with NanoShuttle™-PL overnight.

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

! NanoShuttle™-PL is brown in color. After incubation, the cells will appear peppered with the brown NanoShuttle™-PL (**Figure 1**).

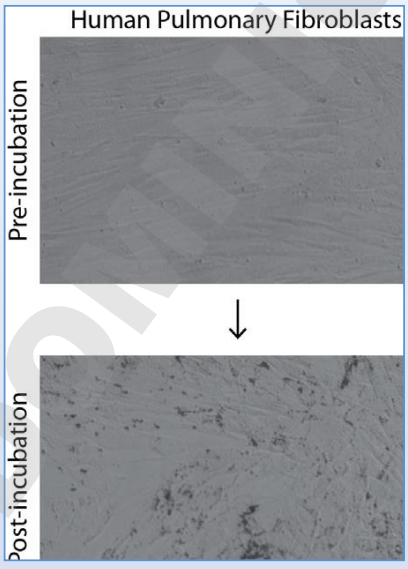

Figure 1: After incubation with NanoShuttle™-PL, cells will appear peppered with the brown nanoparticles, as demonstrated by primary human pulmonary fibroblasts. Scale bar = 100 µm[1]

2.2 Cell Detachment

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

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.

! Do not soak drives in ethanol. Lightly spray and wipe to sterilise.

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 neutralising 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 the in required amount of media (2 mL per culture).

! We recommend levitating cultures with 3.2 x 10^6 cells each (1.6 x 10^6 cells/mL), but the number of cells per culture can be different. Cultures have successfully been formed with cell numbers from 5 x 10^6 to 1.5 x 10^5. Optimise the number of cells per culture by levitating cultures with more or less cells.

2.3 Magnetic Levitation

12. Draw up the suspended cells with a sterile pipette, and dispense 2 mL of the cell suspension into the wells of a cell-repellent 6-well plate.

! Too much media in the well will bring the cells too close to the magnet, where the cells are at risk of escaping the media. Do not add more than 2 mL of media.

13. Close the plate and place the levitating drive atop the plate (**Figure 2**).

If the cells are not immediately levitating, gently agitate the plate by moving the plate back and forth, until it levitates.

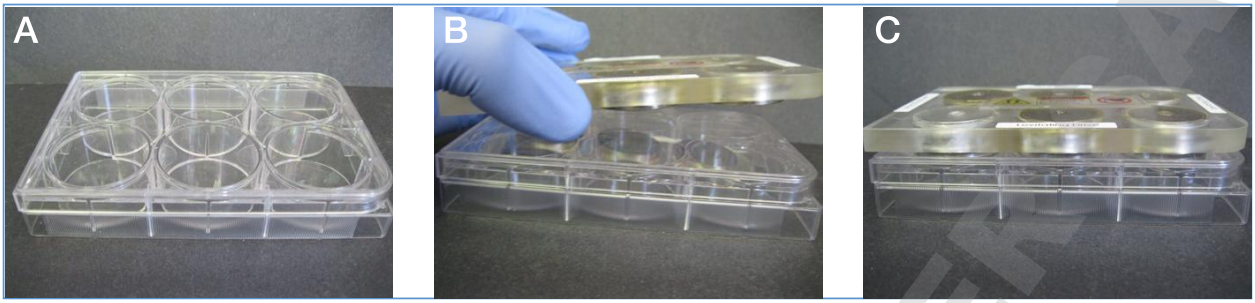

Figure 2: Take a cell-repellent 6-well plate (a) and place the levitating drive (b) atop the cell-repellent 6-well plate to levitate the cells (c).

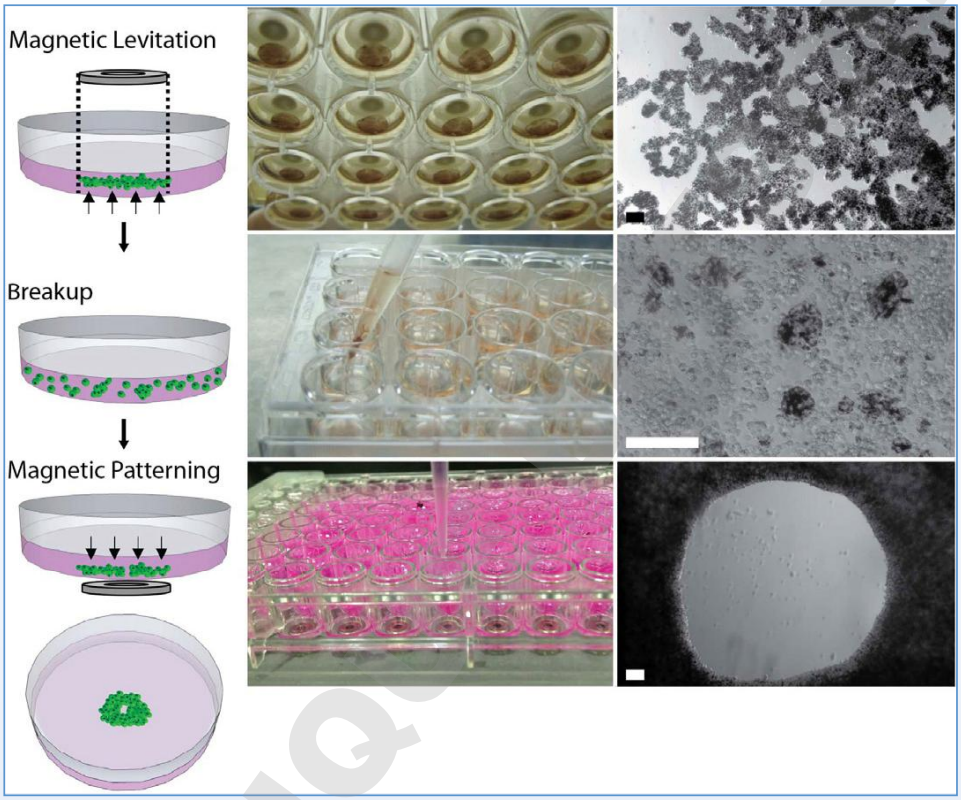

Figure 3: Magnetic 3D Bioprinting. Cells that are magnetically levitated to induce ECM formation are then broken up with pipette action and dispensed into the wells of a 96-well plate that is on either the ring drive or the spheroid drive. The cells are printed into rings or spheroids by being held there for 15 min to a few hours. Scale bar = 100 µm. Adapted from Timm et al.[2]

14. Transfer the plate to an incubator for up to 3h for fibroblasts and muscle cells or up to 24 h for other cell types. The purpose of this step is to induce the cells to generate extracellular matrix (ECM) and to mature, so when the cells are printed, they are in a representative environment. By 15 min, cells should begin to levitate and aggregate, forming a noticeably brown culture levitated within the well. The 3D cultures can be imaged under a microscope using the hole in the magnet where light will pass through.

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.

2.4 Magnetic 3D Bioprinting

15. Draw up the levitated structure with a sterile pipette, and break it up using vigorous pipette action, intaking and expelling the structure at least 10X. The resulting solution should be magnetised cells and ECM in suspension. Combine the levitated structures using the same cell type in a 15 mL tube.

16. Resuspend the cells in the required amount of media (135 µL per ring or spheroid).
    a) For rings: 200,000 cells per ring, or a concentration of 1,481,481 cells/mL.
    b) For spheroids: 50,000 cells per spheroid, or a concentration of 370,370 cells/mL.

> **!** We recommend these cell concentrations, but the number of cells per printed structure can be different. Cultures have successfully been formed with cell numbers from 1,000,000 to 20,000. Optimise that number by printing rings or spheroids with more or less cells.

17. Place a cell-repellent 96-well plate atop the ring drive or the spheroid drive (**Figure 4**).

Figure 4: Take the spheroid drive (a) and place a cell-repellent 96-well plate (b) atop the spheroid drive to print the cells into a spheroid (c).

18. Distribute the cells into the wells of the 96-well mixing plate using a volume equal to the [number of replicates] x 135 µL media (for n=3, use 3 x 135 µL = 405 µL media).

> **!** The maximum volume of a well in the mixing plate is 1.8 mL, so the max number of replicates per well is 12.

19. Add 15 µL per replicate of the compound solution to be tested at 10X the desired concentration to the cells. (for n=3, use 3 x 15 µL = 45 µL compound solution). Combined, there should be 150 µL of solution (cells + compound in media) per replicate.

> **!** The compound can be added after the cells are fully printed. In adding the compound before printing, you potentially avoid disrupting the printed ring or spheroid with the pipette. Doubly, adding the compound before printing will still yield a dose-dependent response with rapid printing times.[2] Optimise your experiment to determine whether adding the compound before or after printing cells is best.

20. Dispense the cells into the plate with 150 µL of solution per well and close the plate. The cells within the solution will aggregate at the bottom of the well plate in the shape of the magnet. Leave the plate on the spheroid drive for 15 min to a few hours, to yield a competent ring or spheroid.

! While not necessary, using a multichannel pipettor to dispense the cells would reduce time exposed to the compound before printing as well as variability between wells exposed to the compound for varying amounts of time.

! The amount of printing time depends on the experiment and cell type, and can vary. Typically these cells will form the ring/spheroid by 15 min, and longer printing times allow for cell organisation, although it will plateau. Optimise the printing time by allowing the cells to print for shorter or longer.

! While dispensing cells, bubbles may appear in the well, which will affect image analysis. During the printing time, use a pipette to carefully pop bubbles and reduce the number of bubbles.

21. Once printed, remove the plate off the drive. Ring or spheroid contraction can be imaged using a microscope or real-time imager.

2.5 Post-Culture Handling

After the rings or spheroids are done contracting, standard tissue processing techniques can be performed, such as fixation, paraffin embedding for immunohistochemistry, or RNA isolation for qRT-PCR. Use the holding drive to hold cells down while adding and removing liquids (**Figure 5**).

Figure 5: Use the holding drive to hold 3D cultures as you add and remove liquids

3. TROUBLESHOOTING

Problem	Probable Cause	Solution
NanoShuttle™-PL appears separated	NanoShuttle™-PL has settled at the bottom of the vial	Homogenise the Nano Shuttle™-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 magnetised. 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-neutralising solution in lieu of serum-containing media to stop trypsin activity. Centrifuge cells immediately after and remove trypsin solution
Magnetised cells attaching to bottom of the plate	Magnetised cells are weakly or not bound to NanoShuttle™-PL	Use cell-repellent plates to prevent cells from adhering and collect weakly magnetised cells
Ring/spheroid appears spread out	Cells have not been bioprinted for enough time	Print the cells longer and carefully monitor the formation of the ring/spheroid
Bubbles appear after printing	Bubbles dispensed when cells were added to wells	During the printing time, use a pipette to pop and reduce bubbles
3D cultures are lost or broken when removing liquids	3D culture is not held down while liquids are transferred	Use the holding drive to hold down cultures while adding and removing liquids

4. CELL TYPES

Cell types that have been successfully cultured using the procedure include:

Cell lines

- Murine Endothelial Cells
- Murine Embryonic Fibroblasts, pre-adipocytes (3T3)
- Murine Adipocytes
- Murine Melanoma
- Murine Neural Stem Cells
- Rat Hepatoma
- Human Astrocytes
- Human Glioblastoma Multiforme (LN229)
- Human Embryonic Kidney Cells (HEK293)
- Rat Vascular Smooth Muscle Cells (A10)
- Human Hepatocellular Carcinoma (HepG2)
- Human Lung Adenocarcinoma (A549)
- Human Colorectal Carcinoma (HCT116)
- Human Pancreatic Epithelioid Carcinoma (PANC-1)

Primary cells

- Human Pulmonary Microvascular Endothelial Cells
- Human Tracheal Smooth Muscle Cells
- Human Small Airway Epithelial Cells
- Human Pulmonary Fibroblasts
- Human Mesenchymal Stem Cells
- Human Bone Marrow Endothelial Cells
- Human Umbilical Vein Endothelial Cells
- Human Aortic Vascular Smooth Muscle Cells
- Human Neonatal Dermal Fibroblasts
- Murine Chondrocytes

5. REFERENCES

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

[2] Timm, D. M. et al. A high-throughput three-dimensional cell migration assay for toxicity screening with mobile device-based macroscopic image analysis. Sci. Rep. 3, 3000 (2013).

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