Manchester BIOGEL Protocol

How to perform 3D cell culture

This protocol describes the 3D encapsulation of cells into PeptiGels®, and the subsequent media changing process to ensure pH neutralization of the cell-containing construct. This protocol is designed for making 50–100 µL hydrogel constructs in 24-well inserts* (insert sitting inside a 24-well plate) or 150-300 µL in 48-well plates. *Note: media changing is not required for PeptiGel® Alpha4 and PeptiGel® Alpha5.*

**suggested brand for inserts: Greiner-Bio-One, with insert membrane pore size of 1.0 µm.*

This protocol also describes the use of a LIVE/DEAD™ assay (L3224, ThermoFisher Scientific) coupled with fluorescence imaging to assess viability of cells *in-situ* inside the hydrogel.

Cell culture

1. Warm the hydrogel to room temperature or 37 °C, then centrifuge (3000 rpm) for 1 minute to remove air bubbles from the hydrogel.
2. Warm up the culture media and other relevant cell culture reagents (e.g. trypsin) to room temperature or 37 °C.
3. Trypsinize cells and perform cell counting. Then, prepare a cell suspension that's 6 times more concentrated than the final cell concentration required (e.g. for 4 million cell/mL of hydrogel, prepare a 24 million cell/mL cell suspension).
4. Using a positive pipette, transfer 1 mL of the hydrogel into a 1.7 mL Eppendorf/14 mL Falcon centrifuge tube.
5. Pipette 0.2 mL of the prepared cell suspension using a positive pipette or a micro-pipette with a cut-end tip (cut with sterile scissors) and insert the pipette tip into the bottom of the hydrogel. Then, release the cell suspension slowly, while pulling the pipette upwards, in a stirring motion, towards surface of the hydrogel. Make sure the pipette tip does not leave the hydrogel.
6. With the pipette tip now close to the surface of the hydrogel, refill the tip with the top section of the hydrogel-cell mixture, and move the tip down to the bottom again. Release the hydrogel-cell mixture using the same method as before; pulling the pipette upwards, in a stirring motion, towards surface of the hydrogel, making sure the pipette tip doesn't leave the hydrogel.
7. Follow Step 6 for at least 10 times to obtain a homogenous hydrogel-cell mixture.
8. Using either a positive pipette, or a pipette with a cut-end tip, dispense 50–100 µL aliquots of above hydrogel-cell mixture into 24-well inserts or 150-300 µl into 48-well plates. It is recommended to spread the hydrogel-cell mixture to cover the bottom surface of the insert or the well.
9. Place 50–100 µL of culture media to cover the surface of the hydrogel in the insert, followed by another 900–950 µL of culture media to fill the well containing the insert. For 48-well plates, gently pipette 300–600 µL of culture media onto the surface of the gel and ensure the hydrogel-cell mixture is covered.
10. After 10 minutes, remove the culture media, and replace with fresh culture media. Repeat this step until the culture media is neutral after a 10 minute soak, typically 3 repeats. The

colour of culture media in the well inserts can be monitored for pH neutralization. *This step is not required for PeptiGel® Alpha4 and PeptiGel® Alpha5..*

11. The following day, change the culture media and then every other day (in general).

Viability assessment

1. Warm the LIVE/DEAD™ reagents (Calcein AM and Ethidium homodimer-1) to room temperature.
2. Pipette 20 µL of Calcein-AM into 10 mL of phosphate buffered saline (PBS), vortex to ensure homogeneity.
3. Pipette 5 µL of Ethidium homodimer-1 (EthD-1) into the above solution, and further vortex to obtain a homogenous solution.
4. Remove the culture media from the 24-well inserts (including media in the wells containing the inserts) or 48-well plates.
5. Place 300 µL of the assay solution into the insert or well containing the PeptiGel®-cell constructs and incubate for 1–2 hours, depending on volume of the hydrogel.
6. Place the PeptiGel® onto a glass slide, and cover with a coverslip. Image the cells using a fluorescence microscope at an excitation wavelength of 495 nm and two emission wavelengths; 515 and 635 nm, respectively.
   Note: gentle pressure can be applied to flatten the hydrogel in order to aid imaging.
   Note: to assess the cell distribution within the hydrogel, confocal microscopy can be used. With this study purpose, the application of any pressure is not recommended.

If you have any questions or queries when working with PeptiGels®, please contact our technical support team on +44 (0) 1625 238 800 or email info@manchesterbiogel.com.