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PM-PD-SOP2 Encapsulating cells in PeptiMatrix hydrogels	1.5	
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1. BACKGROUND

Self-assembling peptide hydrogels can be used in a variety of cell culture applications, providing a 3D environment in which cells can be encapsulated. PeptiMatrix™ hydrogels are formed from short peptide sequences of between 8-20 amino acids. At the correct pH and temperature, they self-assemble to form a stable entangled network of nanofibers. The non-covalent nature of the peptide interactions enables the hydrogel to undergo gel-liquid-gel transitions upon shear stress, allowing for uniform cell encapsulation.

2. RISK ASSESSMENT

Please refer to PM-RA0 and PM-RA2 for a full risk assessment of the following protocol. Ensure that you fully understand the potential risks and have received the appropriate training before starting any work.

3. MATERIALS

- Class II Microbiology Safety Cabinet (or appropriate class for your cells/materials)
- Centrifuge
- Centrifuge tubes (e.g., 15 mL falcon tube)
- 80 °C oven
- P1000, P200, P20, P10 filter tips
- P1000, P200, P20, P10 pipette
- 96-well plate **OR** 24-well plate with 24-well cell culture inserts
- Cells to be encapsulated
- Soluble matrix additions (optional)

4. METHODS

Video Demonstration

To see a video demonstration of the below methods, visit: [How to create 3D cell culture models with PeptiMatrix hydrogel (youtube.com)]

Final Gelation / Cell encapsulation

1. Transfer 1 mL of hydrogel to a 15 mL centrifuge tube and centrifuge at 600 x g for 1 min to remove any air bubbles.
    1. **OPTIONAL:** If gels have been stored for longer periods (>1 month), it can be beneficial to heat gels in an 80 °C oven for at least 20 min before prior to cell encapsulation to ensure a homogeneous end gel. Gels of lower concentration will be free-flowing at this temperature. Gels of higher concentration may remain self-supporting but will be less viscous.
    2. This can be done either before or after transfering the 1 mL of hydrogel to a 15 mL centrifuge tube (the vials and caps are heat resistant). The advantage of doing this before transfer is that it can make it make the transfer easier, as the gels will be less viscous at higher temperatures.
    3. **CAUTION:** Ensure proper temperature control during this step, as temperatures above 90 °C can damage the gels.
2. Transfer the gel to the 37 °C water bath while preparing cell suspension for final gelation. The gels should be self-supporting again when cooled to this temperature.
3. Follow separate protocols (as appropriate for the cell line) for detaching and counting cells to be encapsulated.
4. For each tube, prepare 250 µL of cell suspension at the desired concentration. A good initial seeding density for these hydrogels is 5x10^5 cells/mL. However, depending on your cell type, densities between 1x10^4 and 1x10^6 cells/mL may be more appropriate.
    1. **OPTIONAL:** If you would like to add soluble matrix components to the gel environment, a cell pellet containing the desired cell quantity can either be resuspended in 250 µL of the pure matrix component, or a mixture of matrix and media. It may be beneficial to keep the cell suspension on ice, as many matrix components will rapidly gel at room temperature.
5. Using a P1000 pipette, add the cell suspension dropwise onto the top of the gel in the 15 mL tube.
6. Using a P1000 pipette again, change pipette tips, and then slowly mix by a combined action of pipetting and stirring. It is helpful to reverse pipette* (on a setting of 200 µL) to avoid introducing air bubbles.
7. Using a P200 pipette, when the gel and media have been thoroughly mixed, the gel can be plated out. We recommend either:
    1. Adding 100 µL to the center of each well of a 96-well plate, or
    2. 200 µL to each 24-well cell culture insert in a 24-well plate.
8. Incubate gels at 37 °C for 10 min.
9. Add media to each gel.
    1. If using a 96-well plate, add up to 200 µL of media, depending on the remaining working volume, dropwise onto each gel.
    2. If using cell culture inserts, add 1 mL of media divided between the cell culture insert and the well.
    3. **OPTIONAL:** If your cell type is particularly sensitive to alkaline pH, change the media on the gels twice in the first hour after plating (see **Notes of Gel pH** section below for further infomation).
    4. **TIP:** When performing these media changes, it is helpful to exchange only 75% of the media each time (e.g., exchanging 150 µL of media, instead of 200 µL, when using a 96-well plate), to avoid disturbing the gel.
10. Change media the next day and then as needed during culture, depending on cell type and number seeded.

1. **TIP:** When performing media changes during culture, it is also helpful to exchange only 75% of the media each time (e.g., exchanging 150 µL of media instead of 200 µL when using a 96-well plate), to avoid disturbing the gel.

* see section below for instructions on how to reverse pipette

Preparing Hydrogels with Indirect Co-culture

1. Where the hydrogels are seeded into 24-well plate inserts, a feeder layer of cells in 2D can be plated on the bottom of the well plate itself.
2. To do this, plate the feeder layer at a density of 1-5x10^4 cells/well and leave to attach overnight.
3. The hydrogel seeding itself should take place in a separate well plate. The inserts can be transferred to the wells containing the feeder layer on the following day.
4. Fresh feeder layers should be prepared every 3-5 days to avoid over-confluence, following the same method.

Notes on Gel pH

1. The precursor hydrogels (prior to cell encapsulation) have a slightly alkaline pH, such that complete gelation does not occur until after cell encapsulation where the precursor gel is combined with cell culture media.
2. After combining with cell culture media, the gel is rapidly neutralised to pH 9 and continues to drop to 7 over the next 12 hours.
3. If your cells are particularly sensitive to alkaline pH, you can perform two media changes on the gels within the first hour after seeding, which will more rapidly neutralise the gels to pH 7.
    1. Typically, we do this with more sensitive stem cell and primary lines, but is not necessary with continuous cancer cell lines.

5. DISPOSAL

Hydrogels seeded with cells and/or matrix components should be treated in the same way as suspensions of the cell type and matrix components in use.

6. REVERSE PIPETTING

Forward pipetting is considered a standard technique and is ideal for most aqueous solutions. In forward pipetting, the target volume is aspirated and dispensed, and a separate blowout step is used to completely empty the tip by pressing the plunger to the second stop.

In reverse pipetting technique, the pipette aspirates the selected volume plus an excess volume. After dispensing, the excess volume remains in the tip and is then discarded. This technique is recommended when handling more viscous samples (such as hydrogels).

How to reverse pipette:

1. Adjust volume of pipette.
2. Fit the tip into the pipette tip cone.
3. Press the operating button all the way to the second stop.
4. Insert the tip just under the surface of the liquid.
5. Slowly release the operating button, allowing it to return to the starting position. For particularly viscous liquids, you may need to wait a few seconds for the full desired volume to be drawn into the tip.
6. Carefully withdraw the tip from the liquid.
7. Press the operating button smoothly to the first stop to deliver the desired volume. Wait one second.
8. Discard the remaining liquid by pressing the operating button to the second stop.

To see a video of this protocol, see: Tutorial: How to Reverse Pipette? (youtube.com)

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