VitroGel™
ready-to-use tunable hydrogel
for 3D cell culture and beyond

Cat No:   TWG001   VitroGel™ 3D
Cat No:   TWG002   VitroGel™ 3D-RGD
Cat No:   TWG003   VitroGel™ RGD-PLUS
Cat No:   MS01-100   VitroGel™ Cell Dilution Solution, TYPE 1
Cat No:   MS02-100   VitroGel™ Cell Dilution Solution, TYPE 2
Cat No:   MS03-100   VitroGel™ Cell Recovery Solution

Handbook
Rev. 2.69 Feb 2019
Check our website for latest protocol guideline revision

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Rev 2.69 updates
• Introduce 6 new products:
  VitroGel™ 3D-PLUS (TWG003)
  VitroGel™ Cell Dilution Solution, TYPE 1 (MS01-100)
  VitroGel™ Cell Dilution Solution, TYPE 2 (MS02-100)
  VitroGel™ Cell Recovery Solution (MS03-100)
• Updated protocol on diluting the hydrogel
• Updated protocols to recommend option to use of ready-to-use
  VitroGel™ Cell Dilution Solutions
• Optimized 3D Cell Culture protocol
• Optimized 2D Coating protocol
• Optimized Preparing an Injectable Hydrogel protocol
• Optimized Cell Recovery from hydrogel protocol
PRODUCT DESCRIPTION AND SPECIFICATIONS

VitroGel™ is a ready-to-use, xeno-free tunable hydrogel system which closely mimics the natural extracellular matrix (ECM) environment. VitroGel creates a functional and optimized environment to make cells feel like at home. The hydrogel system is room temperature stable, has a neutral pH, transparent, permeable and compatible with different imaging systems. The solution transforms into a tunable hydrogel matrix by simply mixing with cell culture medium. Cells cultured in this system can be easily harvested out with our VitroGel Cell Recovery Solution. The hydrogel can also be tuned to be injectable for in vivo studies. From 2D coating, 3D culture to animal injection, VitroGel makes it possible to bridge the in vitro and in vivo studies with the same platform system.

VitroGel hydrogel versions:

<table>
<thead>
<tr>
<th>Catalog No.</th>
<th>Name</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>TWG001</td>
<td>VitroGel™ 3D</td>
<td>Unmodified synthetic hydrogel</td>
</tr>
<tr>
<td>TWG002</td>
<td>VitroGel™ 3D-RGD</td>
<td>Modified with RGD peptide (1X)</td>
</tr>
<tr>
<td>TWG003</td>
<td>VitroGel™ RGD-PLUS</td>
<td>Modified with high concentration of RGD peptide (3X)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sizes</th>
<th>2 mL, 10 mL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(VitroGel are manufactured in high concentration)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Number of uses</th>
<th>2-6 of 24-well plate at 250 µL/well</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expiration</td>
<td>15 months from manufacture</td>
</tr>
<tr>
<td>Storage conditions</td>
<td>Store 2°C to 8°C. DO NOT FREEZE.</td>
</tr>
</tbody>
</table>

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Note that VitroGel comes in high concentration. The hydrogel solution can be diluted with VitroGel™ Dilution Solution before mixing with cell culture medium (with or without cells) for the desired final hydrogel strength. Please read the first-time user note to learn how to tune the hydrogel condition for different cell culture medium.
<table>
<thead>
<tr>
<th>Catalog No</th>
<th>Name</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS01-100</td>
<td>VitroGel™ Dilution Solution TYPE 1</td>
<td>For dilution of VitroGel hydrogel. Contains sucrose to maintain best osmolarity.</td>
</tr>
<tr>
<td>MS02-100</td>
<td>VitroGel™ Dilution Solution TYPE 2</td>
<td>For dilution of VitroGel hydrogel. No Sucrose formulation.</td>
</tr>
<tr>
<td>MS03-100</td>
<td>VitroGel™ Cell Recovery Solution</td>
<td>Fast and safe recovery of cells from VitroGel.</td>
</tr>
</tbody>
</table>

Sizes

<table>
<thead>
<tr>
<th>Sizes</th>
<th>100 mL</th>
</tr>
</thead>
</table>

Storage conditions

<table>
<thead>
<tr>
<th>Storage conditions</th>
<th>Store 15°C to 30°C</th>
</tr>
</thead>
</table>

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A TUNABLE HYDROGEL SYSTEM

VitroGel is a ready-to-use tunable hydrogel system designed to mimic the endogenous physiological microenvironment for in vitro culture of various cell types. Mechanical properties of the hydrogel can be adjusted via 1) concentration of the hydrogel solution and 2) the ionic concentration of the cell culture medium, giving a broad-range of elastic modulus of hydrogel strength. Rate of hydrogel formation can also be adjusted; for the same cell culture medium, the different mixing ratios of hydrogel solution and cell culture medium can be used to adjust the rate at which the hydrogel forms.

How does it work?
A two-stage tunable hydrogel forming process:

1. **STAGE ONE (forming a soft hydrogel)**

   The hydrogel formation starts when mixing the VitroGel solution with cell culture medium. Hydrogel molecules interact with ions (such as Ca$^{2+}$ or Na$^+$) from the cell culture medium to become a matrix structure (hydrogel).

   The process of hydrogel formation is slow when the ion concentration is low. At this stage, the hydrogel is soft and possesses a shear-thinning and rapid recovering mechanical property, which makes the hydrogel injectable for in vivo applications.

   The slow hydrogel forming process and the injectable property of the soft hydrogel create a time window for easy hydrogel transfer from the mixing tube to the cell culture plate.
**STAGE TWO  (forming a solid hydrogel)**

After transferring the soft hydrogel formation to a cell culture plate, adding additional cell culture medium on top of the hydrogel would increase the ion concentration, allowing more ions to penetrate into the hydrogel matrix to facilitate further hydrogel cross linking forming a solid hydrogel.

The solid hydrogel possesses higher mechanical strength than the soft hydrogel and can be broken into smaller gel pieces.

After the hydrogel solidifies, there is a clear gel-liquid phase separation, which allows medium change by removing and adding medium on top of the hydrogel.

In summary, the concentration of the VitroGel solution + the concentration of the cell culture medium tunes the mechanical properties of the hydrogel and rate of hydrogel formation.
FOR FIRST TIME USERS OF THE VITROGEL SYSTEM, PLEASE READ THE FOLLOWING NOTES BEFORE USING THE PRODUCTS

Since different cell types prefer different tissue-specific microenvironments, hydrogel conditions need to be optimized for different cell types and culture media in order to get the best results out of the VitroGel system. For first-time users, an initial test of cell growth in a gradient of hydrogel concentrations is highly recommended. Please use the following steps to setup a gradient of hydrogel concentrations.

**Dilute the hydrogel solution:**

The ready-to-use VitroGel Dilution Solution is recommend for preparing different hydrogel concentration.

*You can prepare your own hydrogel dilution solution. Mix 500 mL 1X PBS (without calcium or magnesium) with 500 mL DI water for 0.5X PBS (optional: add 5 wt% sucrose to supplement the osmolarity of the dilution solution).*

1. Directly mix VitroGel with the VitroGel Dilution Solution in ratios of 1:0, 1:1, 1:2, 1:3 (VitroGel: VitroGel Dilution Solution, v/v) at room temperature.
2. Mix 4 mL diluted VitroGel from step 1 with 1 mL cell culture medium (with or without cells, keep the mixing ratio at 4:1 (v/v)).
3. Transfer the hydrogel mixture to a well plate and wait 10-20 min at room temperature for a soft gel formation.
4. After soft gel formation, carefully add cell culture medium to cover the hydrogel.

**Note 1:**

- The VitroGel Dilution Solution will slowly initialize the hydrogel formation, therefore prepare **FRESH** diluted VitroGel and use immediately to mix with cell culture medium.
- Mixing VitroGel with 1X PBS would form a soft hydrogel, which can be use for 2D coating or preparing an injectable hydrogel. Using 1X PBS for dilution at 1:2 to 1:4 ratio, might cause the non-uniform hydrogel formation.

**TABLE 1. Volumes of solution/medium for different hydrogel dilutions**

<table>
<thead>
<tr>
<th>Dilution Ratio</th>
<th>VitroGel</th>
<th>VitroGel Dilution Solution</th>
<th>Cell Medium for Mixing</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:0</td>
<td>4 mL</td>
<td>0 mL</td>
<td>1 mL</td>
</tr>
<tr>
<td>1:1</td>
<td>2 mL</td>
<td>2 mL</td>
<td>1 mL</td>
</tr>
<tr>
<td>1:2</td>
<td>2 mL</td>
<td>4 mL</td>
<td>1.5 mL</td>
</tr>
<tr>
<td>1:3</td>
<td>1 mL</td>
<td>3 mL</td>
<td>1 mL</td>
</tr>
</tbody>
</table>
Note 2: Adjusting The Hydrogel Formation Time

- If VitroGel needs to be diluted more than 1:3 ratio, a longer waiting time (20-30 min) may be needed for soft gel formation. Using a higher volume of cell culture medium for mixing would help to accelerate the process of hydrogel formation.

- If the hydrogel solidifies too fast after mixing with culture medium (showing as small solid gel chunk), adjust the mixing ratio by using less cell culture medium. For example, if mixing 4 mL diluted hydrogel solution with 1 mL cell culture medium lead to the solid gel chunk (particles), then mixing 4 mL diluted hydrogel solution with 0.5-0.8 mL cell culture medium would help to solve the issue.

- On the other hand, if the hydrogel formation is too slow, which may happen when using low hydrogel concentration at 1:3 or 1:4 dilution or using cell culture medium with very low ionic concentration, adjust the mixing ratio by using more cell culture medium. For example, if mixing 4 mL diluted hydrogel solution with 1 mL cell culture medium lead to a slow hydrogel formation, then mixing 4 mL diluted hydrogel solution with 1.5-4 mL cell culture medium would help to solve the issue.
3D CELL CULTURE

Materials:
• VitroGel
• Cells
• Cell culture medium
• VitroGel Dilution Solution (CAT# MS01-100 or MS02-100)
• Micropipette
• Tissue culture treated plate

⚠️ Note: Non-tissue culture treated well plate may cause hydrogel detachment. If using a glass bottom plate, please use tissue culture treated glass bottom plate or Poly-D-Lysine coated glass bottom plate for a better hydrogel attachment.

PROTOCOL

Read 3D CELL CULTURE AND 2D COATING HELP GUIDE on page 11 for protocol tips carefully.

1. Bring VitroGel to room temperature and warm cell culture medium to 37° C if needed.

2. Adjust the concentration of VitroGel for different cell types by diluting the VitroGel with VitroGel Dilution Solution. After dilution, gently mix the diluted VitroGel with a cell suspension (in the desired media) without introducing bubbles. Please check the Table 2 below for solution/medium volume of different dilutions

Table 2: Volumes of solution/medium for different hydrogel dilutions for 3D cell culture

<table>
<thead>
<tr>
<th>Dilution Ratio</th>
<th>VitroGel</th>
<th>VitroGel Dilution Solution</th>
<th>Cell Medium w / Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:0</td>
<td>4 mL</td>
<td>0 mL</td>
<td>1 mL</td>
</tr>
<tr>
<td>1:1</td>
<td>2 mL</td>
<td>2 mL</td>
<td>1 mL</td>
</tr>
<tr>
<td>1:2</td>
<td>2 mL</td>
<td>4 mL</td>
<td>1.5 mL</td>
</tr>
<tr>
<td>1:3</td>
<td>1 mL</td>
<td>3 mL</td>
<td>1 mL</td>
</tr>
</tbody>
</table>

3. Transfer the hydrogel mixture to a well plate. Gently tilt/swirl the well plate to ensure there is an even coating on the bottom of each well.

⚠️ Note: Adding the hydrogel as a dome instead of covering the whole bottom of the well plate might cause hydrogel detachment.
The recommend volume of each well/insert is listed in the table 3 and table 4 below:

**Table 3: Recommended hydrogel volume for WELL PLATES**

<table>
<thead>
<tr>
<th>WELL PLATE</th>
<th>Volume of hydrogel (µL)</th>
<th>Volume of Cover Medium (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 -well plate</td>
<td>1200</td>
<td>1200</td>
</tr>
<tr>
<td>12-well plate</td>
<td>600</td>
<td>600</td>
</tr>
<tr>
<td>24 well plate</td>
<td>300</td>
<td>300</td>
</tr>
<tr>
<td>48-well plate</td>
<td>150</td>
<td>150</td>
</tr>
<tr>
<td>96-well plate</td>
<td>75</td>
<td>75</td>
</tr>
</tbody>
</table>

**Table 4: Recommended hydrogel volume for PLATE INSERTS**

<table>
<thead>
<tr>
<th>PLATE INSERTS</th>
<th>Volume of hydrogel (µL)</th>
<th>Volume of Cover Medium (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 -well plate</td>
<td>800</td>
<td>800</td>
</tr>
<tr>
<td>12-well plate</td>
<td>400</td>
<td>400</td>
</tr>
<tr>
<td>24 well plate</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>48-well plate</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>96-well plate</td>
<td>50</td>
<td>50</td>
</tr>
</tbody>
</table>

4. Wait 10-20 min at room temperature for a soft gel formation.

**Note:** During the hydrogel forming process, do not disrupt the hydrogel by tilting or shaking the well plate.

5. After soft gel formation, GENTLY tilt the well plate to check if hydrogel has formed and attached firmly to the bottom of the well plate.

6. Carefully cover hydrogel with additional medium to further stabilize the hydrogel. See Table 3 or Table 4 for recommended volume of cover medium.

7. Place the well plate in an incubator and change the cover medium every 48 hours.

**Note:** We recommend to only change 60-80% of the top medium without disturbing the hydrogel.
The final cell concentration can be optimized based on different cell types. We recommend to prepare cell suspension at concentration of $2 \times 10^5$ cells/mL for the final cell concentration around $0.4 \times 10^5$ cells/mL after mixing.

Prepare FRESH diluted VitroGel for each use. Immediately mix with cell culture medium after diluting with dilution solution.

Adding the hydrogel as a dome instead of covering the whole bottom of the well plate might cause hydrogel detachment. Please make sure the hydrogel covers the whole bottom of the well plate.

During the waiting time (step 4), please do not disrupt the hydrogel by tilting or shaking the well plate.

Do not over-pipette the hydrogel-medium mixture during the mixing and transferring steps. It might disrupt the hydrogel formation. Pipetting up and down for 5-10 times is sufficient.

After the initial soft hydrogel formation (step 5), it is important to make sure the hydrogel is stable and attached to the bottom of the well plate before adding the cover medium (if the hydrogel is not stable, it might detached from the bottom of the well after adding the cover medium). The gel might be soft, do not shake the plate or keep the plate vertically.

If the speed of hydrogel formation needs to be adjusted, please read Note 2 (adjust hydrogel formation time) of First Time User Note on page 5.

PARTIALLY CHANGING THE COVER MEDIUM

Changing 100% of the cover medium might cause the disruption of the hydrogel by accident. We recommend adding additional fresh medium without removing the top medium at first-time medium change and then only change 60-80% of the cover medium afterward. (Please check the recommend volume of the additional cover medium in the table 5 below.)

Table 5: Recommend volume for partially cover medium change

<table>
<thead>
<tr>
<th>Volume of the cover medium at Day 0</th>
<th>Additional medium volume to add without removing the cover medium at Day 2</th>
<th>Volume of partial medium to change afterwards (Day3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 well plate</td>
<td>1200 µL</td>
<td>600 µL</td>
</tr>
<tr>
<td>12 well plate</td>
<td>600 µL</td>
<td>300 µL</td>
</tr>
<tr>
<td>24 well plate</td>
<td>300 µL</td>
<td>150 µL</td>
</tr>
<tr>
<td>48 well plate</td>
<td>150 µL</td>
<td>50 µL</td>
</tr>
<tr>
<td>96 well plate</td>
<td>75 µL</td>
<td>25 µL</td>
</tr>
</tbody>
</table>
INCREASE HYDROGEL ATTACHMENT BY PRE-COATING THE WELL PLATE

• Using a low VitroGel concentration would make a hydrogel with low elastic modulus. Such hydrogel might detach from the well plate. Pre-coat the well plate with 1X PBS or 10-100 mM CaCl$_2$ can improve the hydrogel attachment at the bottom of the well plate:
  I. Add the PBS or CaCl$_2$ solution to the well plate for 30 min.
  II. Remove the PBS or CaCl$_2$ solution, open the lid under the biosafety hood for 10-20 min before adding the hydrogel. The recommend volumes of PBS or CaCl$_2$ for different sizes of well plate are list in Table 6 below.

Table 6: Recommend volume of PBS or CaCl$_2$ solution for pre-coating well plate

<table>
<thead>
<tr>
<th>Volume of PBS or CaCl$_2$ solution for each well</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-well plate</td>
</tr>
<tr>
<td>12-well plate</td>
</tr>
<tr>
<td>24-well plate</td>
</tr>
<tr>
<td>48-well plate</td>
</tr>
<tr>
<td>96-well plate</td>
</tr>
</tbody>
</table>
2D COATING

Materials:

- VitroGel
- Cells
- Cell Culture medium
- VitroGel Dilution Solution (CAT# MS01-100 or MS02-100)
- Micropipette
- Tissue culture treated plate

Note: Non-tissue culture treated well plate may cause hydrogel detachment. If using a glass bottom plate, please use tissue culture treated glass bottom plate or Poly-D-Lysine coated glass bottom plate for a better hydrogel attachment.

PROTOCOL

Read 3D CELL CULTURE AND 2D COATING HELP GUIDE on page 11 for protocol tips carefully.

1. Bring VitroGel to room temperature and warm cell culture medium to 37° C if needed.

2. Gently mix VitroGel with cell culture medium without introducing bubbles. If needed, adjust the concentration of VitroGel by diluting with VitroGel Dilution Solution. Please check the Table 7 below for solution/medium volume of different dilutions.

<table>
<thead>
<tr>
<th>Dilution Ratio</th>
<th>VitroGel</th>
<th>VitroGel Dilution Solution</th>
<th>Cell Medium for Mixing</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:0</td>
<td>4 mL</td>
<td>0 mL</td>
<td>1 mL</td>
</tr>
<tr>
<td>1:1</td>
<td>2 mL</td>
<td>2 mL</td>
<td>1 mL</td>
</tr>
<tr>
<td>1:2</td>
<td>2 mL</td>
<td>4 mL</td>
<td>1.5 mL</td>
</tr>
<tr>
<td>1:3</td>
<td>1 mL</td>
<td>3 mL</td>
<td>1 mL</td>
</tr>
</tbody>
</table>

(The diluted VitroGel coating can also be prepared by simply mixing VitroGel with VitroGel Dilution Solution (without cell medium) and transferring the hydrogel mixture to a well plate and leave overnight.)

3. Transfer the hydrogel mixture to a well plate. Gently tilt/swirl the well plate to ensure there is an even coating on the bottom of each well.

Note: Adding the hydrogel as a dome instead of covering the whole bottom of the well plate might cause hydrogel detachment.
The recommend volume of each well is listed in the tables below:

### Table 8: Recommend 2D coating hydrogel volume for WELL PLATE

<table>
<thead>
<tr>
<th>WELL PLATE</th>
<th>Volume of hydrogel (μL)</th>
<th>Volume of cover medium (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-well plate</td>
<td>1200</td>
<td>1200</td>
</tr>
<tr>
<td>12-well plate</td>
<td>600</td>
<td>600</td>
</tr>
<tr>
<td>24-well plate</td>
<td>300</td>
<td>300</td>
</tr>
<tr>
<td>48-well plate</td>
<td>150</td>
<td>150</td>
</tr>
<tr>
<td>96-well plate</td>
<td>75</td>
<td>75</td>
</tr>
</tbody>
</table>

### Table 9: Recommend 2D coating hydrogel volume for PLATE INSERT

<table>
<thead>
<tr>
<th>PLATE INSERT</th>
<th>Volume of hydrogel (μL)</th>
<th>Volume of cover medium (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-well plate</td>
<td>800</td>
<td>800</td>
</tr>
<tr>
<td>12-well plate</td>
<td>400</td>
<td>400</td>
</tr>
<tr>
<td>24-well plate</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>48-well plate</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>96-well plate</td>
<td>50</td>
<td>50</td>
</tr>
</tbody>
</table>

4. **Wait 10-20 min at room temperature for a soft gel formation.**

   **Note:** During the hydrogel forming process, do not disrupt the hydrogel by tilting or shaking the well plate.

5. **After soft gel formation, GENTLY tilt the well plate to ensure the hydrogel is formed and attached firmly to the bottom of the well plate. Carefully add cell culture media to cover hydrogel.**

6. **Place the well plate in an incubator and change the cover medium every 48 hours.**

   **Note:** We recommend to only change 60-80% of the top medium without disturbing the hydrogel.
CELL RECOVERY FROM HYDROGEL

Materials:

- Cells cultured in VitroGel system
- VitroGel Cell Recovery Solution (Cat# MS03-100)
- Centrifuge tubes
- Micropipette
- 37°C water bath or dry bath
- Centrifuge
- Lab spoon/spatula

**PROTOCOL** (using 24 well-plate, 300 µL gel/well as an example)

1. Add 6 mL VitroGel Cell Recovery Solution to a 15 mL centrifuge tube. Warm the solution to 37 °C.

2. Take the cells out of the incubator and remove the medium covering the top of the hydrogel.

3. Add 1 mL warm VitroGel Cell Recovery Solution to the well and use a spatula to detach the hydrogel from well plate.

4. Transfer the hydrogel to the 15 mL centrifuge tube containing the warm VitroGel Cell Recovery Solution from step 1.

5. Rinse the well with 1 mL warm VitroGel Cell Recovery Solution and combine the solution in the centrifuge tube.

6. Rock the centrifuge tube for 20 times and then put back to the water bath for 2-3 min. Repeat this step for 3-5 times.

7. Centrifuge at 1,200 rpm for 5 minutes at room temperature and discard supernatant and collect the cell pellet.

8. **OPTIONAL:**
   Warm additional 6 mL VitroGel Cell Recovery Solution to 37°C. Resuspend the cells and repeat steps 6 and 7 one more time if necessary.

**Note:**

- It is very important to keep the solution and tube warm during the whole process.
- Centrifuge should be done at room temperature.
• For step 3, if the cell colonies is small (< 80 µm), you can cut the pipette tip (1 mL) and using the pipette to break the hydrogel into small piece and transfer the mixture to the centrifuge tube rather than using a spatula to transfer the whole hydrogel. This process to accelerate the cell recovery in step 6.

• For step 6, the time of rocking and repeat can be optimized for different gel strengths and cell types.

• Optimize the speed and time of centrifuge according to different cell types.
PREPARING AN INJECTABLE HYDROGEL

Materials:

- VitroGel
- Cells
- Cell culture medium
- VitroGel Dilution Solution (Cat# MS01-100 or MS02-100) or 1X DPBS (no calcium, no magnesium)
- Micropipette
- Syringe

PROTOCOL

1. Bring VitroGel to room temperature and warm cell culture medium to 37° C if needed.

2. Prepare cell suspension or drug solution in cell culture medium (can also use 1X PBS or VitroGel Dilution Solution to substitute the cell culture medium).

3. Gently mix VitroGel with cell suspension (drug solution) without introducing bubbles. The recommended mixing ratio is listed in Table 10 below.

<table>
<thead>
<tr>
<th>Medium used to prepare cell suspension/drug solution</th>
<th>VitroGel</th>
<th>Cell suspension/drug solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell culture medium</td>
<td>4 mL</td>
<td>1 mL</td>
</tr>
<tr>
<td>1X PBX</td>
<td>1 mL</td>
<td>0.5 - 1 mL</td>
</tr>
<tr>
<td>VitroGel Dilution Solution</td>
<td>1 mL</td>
<td>1 mL</td>
</tr>
</tbody>
</table>

4. The mixture can be transferred to a syringe. The hydrogel is ready for injection after stabilization for 10-20 min.

Note:

- The cell concentration needs to be optimize for different application. We recommend preparing cell suspension at high concentration (10⁶ cells/mL) for the final cell concentration above 2 x 10⁵ cells/ml after mixing with hydrogel solution.

- For different hydrogel concentration need, please check the table 11 below to prepare injectable hydrogel. We recommend using higher hydrogel concentration (1:0 to 1:2 (v/v) dilution) for injection application.
Table 11: Volumes of solution/medium for injectable hydrogel preparation

<table>
<thead>
<tr>
<th>Type of medium to prepare cell suspension/drug solution</th>
<th>Dilution Ratio</th>
<th>Cell suspension/drug solution</th>
<th>Dilution Solution suspension/drug solution</th>
<th>Cell Suspension/Drug Solution</th>
<th>Waiting time for hydrogel stabilization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell culture medium</td>
<td>1:0</td>
<td>4 mL</td>
<td>0 mL</td>
<td>1 mL</td>
<td>10-20 min</td>
</tr>
<tr>
<td></td>
<td>1:1</td>
<td>2 mL</td>
<td>2 mL</td>
<td>1 mL</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1:2</td>
<td>2 mL</td>
<td>4 mL</td>
<td>1.5 mL</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1:3</td>
<td>1 mL</td>
<td>3 mL</td>
<td>1 mL</td>
<td></td>
</tr>
<tr>
<td>1X PBS</td>
<td>1:0</td>
<td>4 mL</td>
<td>0 mL</td>
<td>2-4 mL</td>
<td>15-30 min</td>
</tr>
<tr>
<td></td>
<td>1:1</td>
<td>2 mL</td>
<td>2 mL</td>
<td>2-4 mL</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1:2</td>
<td>2 mL</td>
<td>4 mL</td>
<td>2-4 mL</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1:3</td>
<td>1 mL</td>
<td>3 mL</td>
<td>2-4 mL</td>
<td></td>
</tr>
</tbody>
</table>
LIVE DEAD ASSAY

Materials:

- Cells cultured in VitroGel system
- Live/Dead staining solution
- DPBS (no calcium, no magnesium)
- Micropipette
- Fluorescent microscopy

PROTOCOL

1. Remove the cover media on the hydrogel and wash the hydrogel with PBS 2-3 times.

2. Prepare live/dead staining solution with PBS.

3. Add live/dead staining solution directly to the hydrogel.
   The volume of the live/dead staining solution is 1-3 times of the volume of hydrogel.

4. Incubate in the dark for 5 min and observe under fluorescent microscopy.

Note:

- The optimal concentration needs to be adjusted based on the cell line and the imaging system. The concentration of the staining solution might need to be adjusted to 1-5X of the recommend concentration for 2D culture.

- Analyze the cells quickly after staining.
FLUORESCENT STAINING

Materials:

- Cells cultured in VitroGel system
- PBS
- Micropipette
- Fixation solution (4% formaldehyde solution in PBS)
- Permeabilization solution (0.1% Triton X-100 in PBS)
- F-actin filament staining solution
- Nucleus staining solution
- Micropipette
- Fluorescent microscopy

PROTOCOL

1. Remove the cover media on the hydrogel and wash the hydrogel with PBS 3 times.
2. Add fixation solution to hydrogel and incubate at room temperature for 10-30 min.
3. Remove the fixation solution and wash hydrogel with PBS 3 times.
4. Add permeabilization solution to submerge the hydrogel for 5 min at room temperature.
5. Remove the permeabilization solution and wash with PBS 3 times.
6. Add F-actin staining solution to the hydrogel and incubate in the dark for 30-60 minutes at room temperature.
7. Remove the F-actin staining solution and wash with PBS 3 times.
8. Add nucleus staining solution to the hydrogel and incubate in the dark for 5 min at room temperature.
9. Remove the nucleus staining solution and wash with PBS 3 times.
10. Observe under a fluorescent microscopy.

Note:

- Prepare the staining solution according to product manual. The final concentration might need to be optimized depending on the different cell types and hydrogel sizes.
- During the washing steps, carefully add or remove the solution to avoid possible lost of hydrogel/cells.
- The incubation time needs to be adjusted accordingly to the different cell types.
• Protect the hydrogel from light after adding the staining solution.

• Make sure the PBS wash steps are thorough and the hydrogel is submerged within the different solutions during the whole process.
HISTOLOGICAL ANALYSIS

Materials:

- Cells cultured in VitroGel system
- PBS
- Cassettes
- Paraffin, xylene, fixation solution (10% buffered formalin)
- Ethanol (50-100%)
- Microscopy

PROTOCOL

1. Remove the cover media on the hydrogel and wash the hydrogel with PBS 3 times.

2. Add fixation solution to the hydrogel and incubate at room temperature for 30 min.

3. Remove the fixation solution and wash with PBS 3 times.

4. Place the hydrogel in cassette, dehydrate the sample using ethanol in the following sequence: 50% (10 min) - 70% (10 min) - 80% (10 min) - 95% (10 min) - 100% (10 min) - 100% (10 min) - 100% (10 min).

5. Exchange ethanol with xylene in the following sequence: 2:1 ethanol : xylene (10-15 min) - 1:1 ethanol : xylene (10-15 min) - 1:2 ethanol : xylene (10-15 min) - 100% xylene (10-15 min) - 100% xylene (10-15 min) - 100% xylene (10min).

6. Exchange xylene with paraffin in the following sequence: 2:1 xylene : paraffin (30 min) - 1:1 xylene : paraffin (30 min) - 1:2 xylene : paraffin (30 min) - 100% paraffin (1-2 hrs) - 100% paraffin (1-2 hrs or overnight).

7. After embed in fresh new paraffin, section onto microscope slides.

8. Stain and mount according to different applications.

Note:

- Optimize the incubation time according to different cell types and hydrogel sizes. Make sure the hydrogel is submerged within the different solutions during the whole process.