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. 2.72 Aug. 2019

Check our website for latest protocol guideline revision

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Information in this document is subject to change without notice.

TheWell BIOSCIENCE

3D cell culture and beyond

Table of Contents

REVISIONS

Rev 2.72 updates
- Correct table 11 header on page 18.

Rev 2.71 updates
- Added "How to Prepare Cell Suspesion" on page 7.

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:

VitroGel® 3D

VitroGel® 3D-RGD

VitroGel® RGD-PLUS

Catalog No.	Name	Description
TWG001	VitroGel® 3D	Unmodified synthetic hydrogel
TWG002	VitroGel® 3D-RGD	Modified with RGD peptide (1X)
TWG003	VitroGel® RGD-PLUS	Modified with high concentration of RGD peptide (3X)

Sizes	2 mL, 10 mL (VitroGel are manufactured in high concentration)
Number of uses	2-6 of 24-well plate at 250 µL/well
Expiration	15 months from manufacture
Storage conditions	Store 2°C to 8°C. **DO NOT FREEZE.**
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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.

SUPPORT MEDIUMS

Catalog No	Name	Description
MS01-100	VitroGel® Dilution Solution TYPE 1	For dilution of VitroGel hydrogel. Contains sucrose to maintain best osmolarity.
MS02-100	VitroGel® Dilution Solution TYPE 2	For dilution of VitroGel hydrogel. No Sucrose formulation.
MS03-100	VitroGel® Cell Recovery Solution	Fast and safe recovery of cells from VitroGel. Enzyme-free.

Sizes	**100 mL**
Number of uses	Dilution Solutions: Good for 3-4 vials of 10 mL VitroGel system at 1:3 dilution ratio
Storage conditions	Store 15°C to 30˚C

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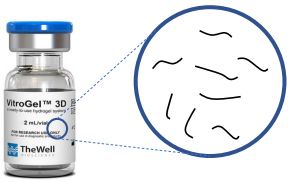
A TUNABLE HYDROGEL SYSTEM

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.

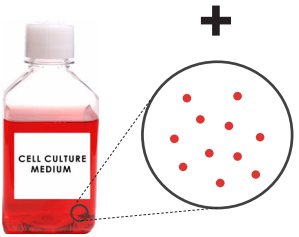
How does it work?
A two-stage tunable hydrogel forming process:



VitroGel is a free flowing hydrogel solution, maintaining its liquid form at room temperature

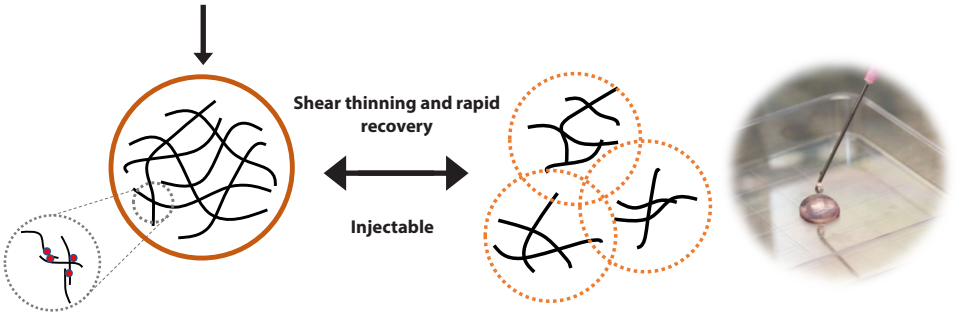
1 **STAGE ONE (forming a soft hydrogel)**

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).



Ions from cell culture medium

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.



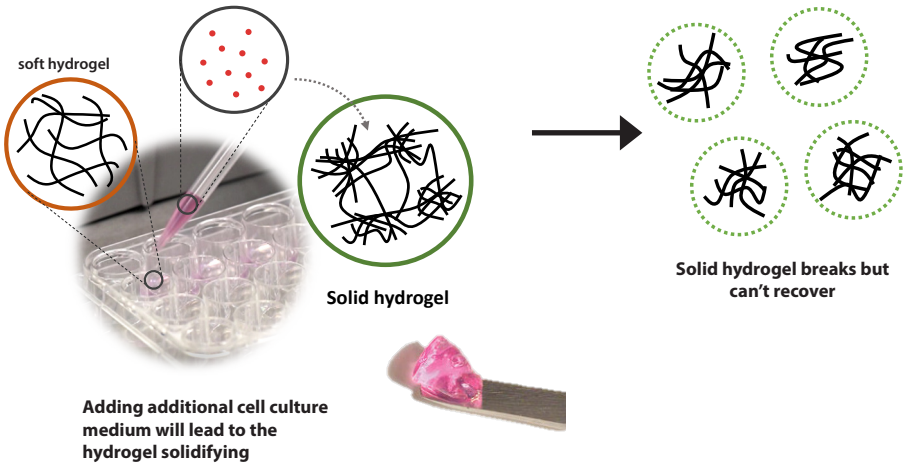
The slow hydrogel forming process and the injectable property of the soft hydrogel create a time window for easy hydrogel transfer from the mixing tube to the cell culture plate.

2 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.

*Users can prepare their 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)).
   IMPORTANT NOTE: Please read the "How To Prepare The Cell Suspension" below.
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.

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

Dilution Ratio	VitroGel	VitroGel Dilution Solution	Cell Medium for Mixing
1:0	4 mL	0 mL	1 mL
1:1	2 mL	2 mL	1 mL
1:2	2 mL	4 mL	1.5 mL
1:3	1 mL	3 mL	1 mL

How To Prepare Cell Suspension

If cells cultured in complete cell culture medium, which is supplement with 10% FBS or other critical supplement, please prepare the cell suspension using the following methods before mixing it with hydrogel solution.

1. Prepare the cell suspension with 2X concentration (e.g. 100K), and mix with 100% FBS at 1:1 (v/v) ratio to get 1X cell suspension (50K) with 50% FBS.

2. Mix the diluted hydrogel solution with the cell suspension from above at 4:1 (v/v) ratio to get the final cells in the hydrogel at 10K with 10% FBS supplement.

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.
- After mixing with cell culture medium, immediately transfer the mixture to the tissue culture plate.
  Note: If you have multiple samples with different hydrogel conditions or cell types to prepare, transferring mixture of sample 1 to the tissue culture plate before mixing the hydrogel with cell culture medium for sample 2.
- 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.

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 chuck (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

Video Protocol Online

Materials:

- **VitroGel**
- **Cells**
- **Cell culture medium**
- **VitroGel Dilution Solution (CAT# MS01-100 or MS02-100)**
- **Micropipette; Low rentention pipette tips**
- **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

Dilution Ratio	VitroGel	VitroGel Dilution Solution	Cell Medium w / Cells
1:0	4 mL	0 mL	1 mL
1:1	2 mL	2 mL	1 mL
1:2	2 mL	4 mL	1.5 mL
1:3	1 mL	3 mL	1 mL

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

WELL PLATE	Volume of hydrogel (µL)	Volume of Cover Medium (µL)
6 -well plate	1200	1200
12-well plate	600	600
24 well plate	300	300
48-well plate	150	150
96-well plate	75	75

Table 4: Recommended hydrogel volume for PLATE INSERTS

PLATE INSERTS	Volume of hydrogel (µL)	Volume of Cover Medium (µL)
6 -well plate	800	800
12-well plate	400	400
24 well plate	200	200
48-well plate	100	100
96-well plate	50	50

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 2-10 x 10^5 cells/mL for the final cell concentration around 0.4-2 x 10^5 cells/mL after mixing.

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

- After mixing with cell culture medium, immediately transfer the mixture to the tissue culture plate.
  Note: If you have multiple samples with different hydrogel conditions or cell types to prepare, transferring mixture of sample 1 to the tissue culture plate before mixing the hydrogel with cell culture medium for sample 2.

- 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

	Volume of the cover medium at Day 0	Addtional medium volume to add without removing the cover medium at Day 2	Volume of partial medium to change afterwards (Day3)
6 well plate	1200 µL	600 µL	1200 µL
12 well plate	600 µL	300 µL	600 µL

24 well plate	300 µL	150 µL	300 µL
48 well plate	150 µL	50 µL	150 µL
96 well plate	75 µL	25 µL	75 µL

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

	Volume of PBS or $CaCl_2$ solution for each well
6-well plate	2000 µL
12-well plate	1000 µL
24-well plate	500 µL
48-well plate	250 µL
96-well plate	100 µL

2D COATING

Materials:

Video Protocol Online

- **VitroGel**
- **Cells**
- **Cell Culture medium**
- **VitroGel Dilution Solution (CAT# MS01-100 or MS02-100)**
- **Micropipette; Low rentention pipette tips**
- **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.

Dilution Ratio	VitroGel	VitroGel Dilution Solution	Cell Medium for Mixing
1:0	4 mL	0 mL	1 mL
1:1	2 mL	2 mL	1 mL
1:2	2 mL	4 mL	1.5 mL
1:3	1 mL	3 mL	1 mL

(The diluted VItroGel coating can also be prepared by simply mixing VitroGel with VitroGel Dilution Solution (without cell medium) and transfering 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

WELL PLATE	Volume of hydrogel (µL)	Volume of cover medium (µL)
6-well plate	1200	1200
12-well plate	600	600
24-well plate	300	300
48-well plate	150	150
96-well plate	75	75

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

PLATE INSERT	Volume of hydrogel (µL)	Volume of cover medium (µL)
6-well plate	800	800
12-well plate	400	400
24-well plate	200	200
48-well plate	100	100
96-well plate	50	50

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:

Video Protocol Online

- Cells cultured in VitroGel system
- VitroGel Cell Recovery Solution (Cat# MS03-100)
- Centrifuge tubes
- Micropipette; Low rentention pipette tips
- 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:

Video Protocol Online

- VitroGel
- Cells
- Cell culture medium
- VitroGel Dilution Solution (Cat# MS01-100 or MS02-100) or 1X DPBS (no calcium, no magnesium)
- Micropipette; Low rentention pipette tips
- 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 10: Volumes of solution/medium for an injectable hydrogel preparation

Medium used to prepare cell suspension/drug solution	VitroGel	Cell suspension/drug solution
Cell culture medium	4 mL	1 mL
1X PBS	1 mL	0.5 - 1 mL
VitroGel Dilution Solution	1 mL	1 mL

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 (10^6 cells/mL) for the final cell concentration above 2 x 10^5 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

Type of medium to prepare cell suspension/ drug solution	Dilution Ratio	VitroGel	Dilution Solution suspension/ drug solution	Cell Suspention/ Drug Solution	Waiting time for hydrogel stabilization
Cell culture medium	1:0	4 mL	0 mL	1 mL	10-20 min
	1:1	2 mL	2 mL	1 mL	
	1:2	2 mL	4 mL	1.5 mL	
	1:3	1 mL	3 mL	1 mL	
1X PBS	1:0	4 mL	0 mL	2-4 mL	15-30 min
	1:1	2 mL	2 mL	2-4 mL	
	1:2	2 mL	4 mL	2-4 mL	
	1:3	1 mL	3 mL	2-4 mL	

LIVE DEAD ASSAY

Materials:

- **Cells cultured in VitroGel system**
- **Live/Dead staining solution**
- **DPBS (no calcium, no magnesium)**
- **Micropipette; Low rentention pipette tips**
- **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; Low rentention pipette tips
- 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.