VitroGel™

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

Cat No: TWG001 VItroGel™ 3D (10 mL high concentration)
Cat No: TWG002 VitroGel™ 3D-RGD (10 mL high concentration)

Handbook

Rev. June 2018 v2.6

Check our website for latest protocol guideline revision

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3D cell culture and beyond

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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 to 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. The hydrogel can also be tuned to be injectable for in vivo study. 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 currently comes in two versions:

VitroGel™ 3D
Better for suspension cells

VitroGel™ 3D-RGD
(RGD peptide modified version)
Better for adhesion cells

	VitroGel™ 3D	VitroGel™ 3D-RGD
Catalog number	TWG001	TWG002
Description	ready-to-use, tunable xeno-free hydrogel solution	ready-to-use, tunable xeno-free hydrogel solution RGD peptide modified
Content	10 mL/vial high concentration	10 mL/vial high concentration
Number of uses	2-6 of 24-well plate at 250 µL/well	2-6 of 24-well plate at 250 µL/well
Storage conditions	Store 4-8 ˚C. **DO NOT FREEZE.** Keep away from strong acids, strong bases and strong oxidizers.	

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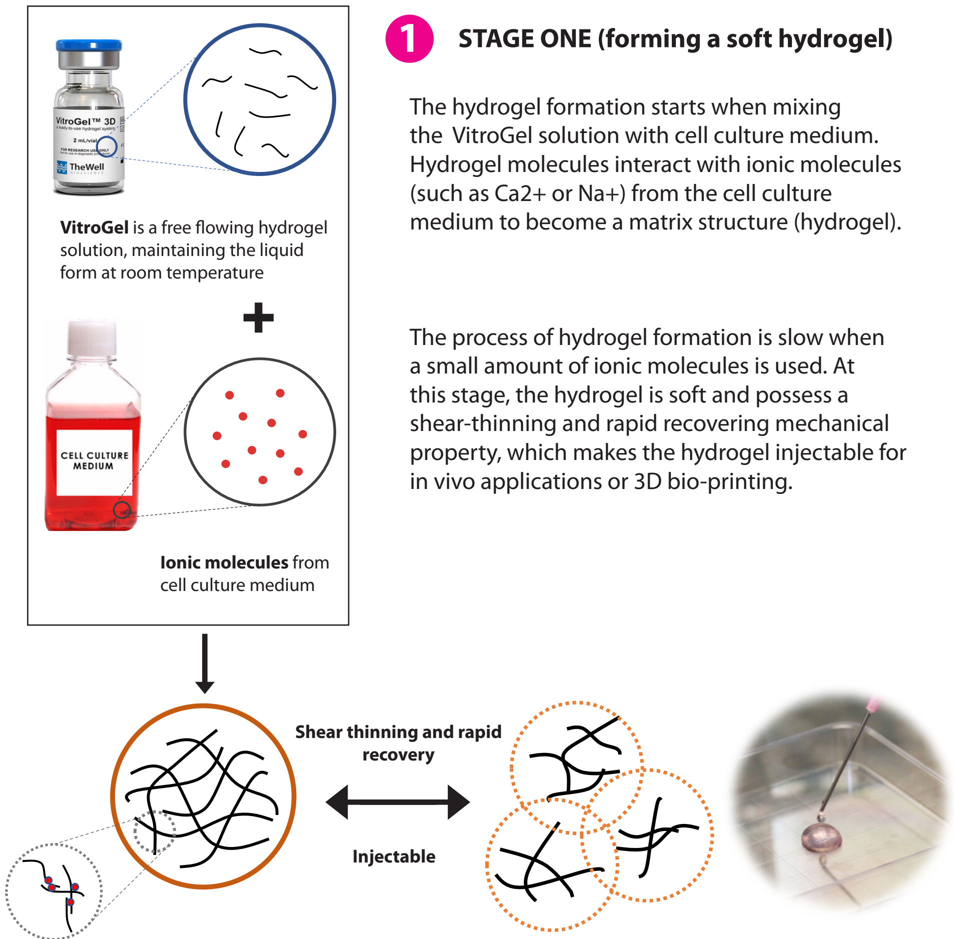
⚠ Note that VitroGel solution comes in high concentration. The hydrogel solution can be diluted with PBS or DI water before mixing with cell culture medium (w/ or w/o cells) for the desired final hydrogel strength. Please read the first-time user note to learn how to tune the hydrogel condition for different cell culture medium.

A TUNABLE HYDROGEL SYSTEM

VitroGel is a ready-to-use tunable hydrogel system to create the physiological mimicking micro-environment for in vitro culture of various cell types. Mechanical properties of the hydrogel can be adjusted by 1) concentration of the hydrogel solution and 2) the ionic concentration of the cell culture medium; to reach a wide range of elastic modulus of hydrogel strength and different speeds of hydrogel formation (for the same cell culture medium, the different mixing ratios of hydrogel solution and cell culture medium can be used to adjust the speed of hydrogel formation).

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 ionic molecules (such as Ca2+ or Na+) from the cell culture medium to become a matrix structure (hydrogel).

The process of hydrogel formation is slow when a small amount of ionic molecules is used. At this stage, the hydrogel is soft and possess a shear-thinning and rapid recovering mechanical property, which makes the hydrogel injectable for in vivo applications or 3D bio-printing.

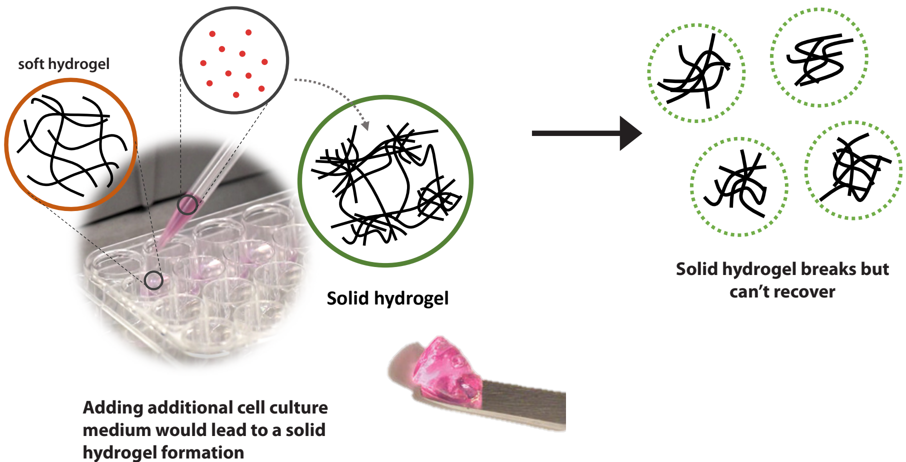
The slow hydrogel forming process and the injectable property of the soft hydrogel create a time window for easy hydrogel transfer from the mixing tube to the cell culture plate.

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 allow more ionic molecules to penetrate into the hydrogel matrix to further saturate the hydrogel cross linking. A solid hydrogel would form during this process.

The solid hydrogel possesses higher mechanical strength than the soft hydrogel. The hydrogel can be broken into smaller pieces, which is irreversible.

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



In summary, the concentration of the VitroGel solution + the concentration of the cell culture medium tunes the mechanical properties of the hydrogel and speed of hydrogel formation.

FOR FIRST TIME USER OF THE VITROGEL SYSTEM, PLEASE READ THE FOLLOWING NOTES BEFORE USING THE PRODUCTS

As different cell types prefer different tissue-appropriate microenvironment, to get the best results out of VitroGel system, the hydrogel conditions need to be optimized for different cell types and culture media. For first time users, the 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. You can choose to use PBS or DI water to make the hydrogel dilution.

OPTION 1. Use PBS to dilute the hydrogel solution:
(We recommend using 0.5X PBS)

1. Directly mix the hydrogel solution with PBS at 1:0, 1:1, 1:2, 1:3, 1:4 (hydrogel solution : PBS, v/v) ratio at room temperature.
2. Mix the diluted hydrogel solution with cell culture medium (w/ or w/o cells) according the Table 1 below. (The mixing ratio is adjusted according to the concentration of inorganic salts of the cell culture medium, especially [Ca2+]).

TABLE 1. Mixing ratios of dilution hydrogel solution with cell culture medium with different Ca2+ concentration (using 0.5X PBS dilution)

[Ca2+] in culture medium (mg/L)	Volume of diluted hydrogel solution (μL)	Volume of cell culture medium (μL)	Examples
200	400	100	DMEM, EMEM, MEM-Alpha
150	200	100	Leibovitz L-15, MEM (Hanks'), DMEM/F-12, McCoy`s 5A
100	100	100	RPMI 1640

Notes:

- PBS will initialize the hydrogel formation, so prepare **FRESH** the diluted hydrogel solution and use immediately to mix with cell culture medium after diluting with PBS.
- Mixing hydrogel solution with 1X PBS would form a soft hydrogel, which can be use for 2D coating or prepare injectable hydrogel. Using 1X PBS for dilution at 1:2 to 1:4 ratio, might cause the non-uniform hydrogel formation.
- For medium having [Ca2+] lower than 150 mg/mL, we suggest the concentration of the hydrogel solution to be not lower than 1:2 dilution.
- For more diluted hydrogel solution (e.g. 1:3 or 1:4 dilution), use a higher volume of cell culture medium 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), please adjust the mixing ratio by using less cell culture medium. For

example, if mixing 1 mL diluted hydrogel solution with 1 mL cell culture medium lead to the solid gel chuck (particles), then mixing 1 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 (normally happens when using low hydrogel concentration at 1:3 or 1:4 dilution). 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 1.5-3 mL cell culture medium would help to solve the issue.

OPTION 2. Use DI water to dilute the hydrogel solution:

1. Directly mix the hydrogel solution with DI at 1:0, 1:1, 1:2, 1:3, 1:4 (hydrogel solution: DI water, v/v) ratio at room temperature.
2. Mix the diluted hydrogel solution with cell culture medium (w/ or w/o cells) according the Table 2 below. (The mixing ratio is adjusted according to the concentration of inorganic salts of the cell culture medium, especially [Ca2+]. For the same culture medium, the mixing ratio need to be adjusted for different hydrogel dilution to ensure the hydrogel formation within a reasonable time)

TABLE 2. Mixing ratios of dilution hydrogel solution with cell culture medium with different Ca2+ concentration (using DI water dilution)

[Ca2+] in culture medium (mg/L)	Dilution of hydrogel solution with DI water	Volume of diluted hydrogel solution (µL)	Volume of cell culture medium (µL)	Examples
200	1:0, 1:1	400	100	DMEM, EMEM, MEM-Alpha
	1:2	300	100	
	1:3	300 or 200	100	
	1:4	100	100 to 300	
150	1:0, 1:1	200	100	Leibovitz L-15, MEM (Hanks'), DMEM/F-12, McCoy`s 5A
	1:2	100	100	
100	1:0, 1:1, 1:2	100	100	RPMI 1640

Note:

- Prepare **FRESH** the diluted hydrogel solution for each use.
- By using the DI water to dilute the hydrogel solution, the mixing ratio of the diluted hydrogel solution and cell culture medium need to adjust according to different dilution.
- For medium has [Ca2+] lower than 150 mg/mL, we suggest the concentration of the hydrogel solution not to be lower than 1:2 dilution.

- For more diluted hydrogel solution (e.g. 1:3 or 1:4 dilution), the volume of cell culture medium can be adjusted to higher than the recommend volume of Table 2 (page 6) to accelerate the process of hydrogel formation.
- If the hydrogel solidifies too fast after mixing with culture medium (showing as small solid gel chunk), please adjust the mixing ratio by using less cell culture medium. For example, if mixing 1 mL diluted hydrogel solution with 1 mL cell culture medium lead to the solid gel chuck (particles), then mixing 1 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 (normally happens when use low hydrogel concentration at 1:3 or 1:4 dilution). 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 1.5-3 mL cell culture medium would help to solve the issue.

3D CELL CULTURE

Materials:

- **VitroGel**
- **Cells**
- **Cell Culture medium**
- **DPBS (no calcium, no magnesium) or DI water**
- **Micropipette**
- **Tissue culture treated plate**
  ⚠ Note: Non-tissue culture treated well plate may cause hydrogel detachment.
  If use glass bottom, please use tissue culture treated glass bottom plate or Poly-D-Lysine coated glass bottom plate for a better hydrogel attachment.
- **Water bath 37°C (optional)**

PROTOCOL

Before starting, read 3D CELL CULTURE HELP GUIDE on page 9 for protocol tips

1. **Bring VitroGel solution and cell culture medium to room temperature. Optional: Warm at 37° C**

2. **Adjust the concentration of hydrogel solution for different cell types by diluting the VitroGel solution with 0.5X PBS or DI water. After dilution, gently mix the diluted VitroGel solution with a cell suspension (in the desired media) without introducing bubbles. Fot the volume of dilution and gel/cell mixing ratio, please check the Table 3 (0.5X PBS) and Table 4 (DI water) in Appendix A (page 11).**

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 table below:

WELL PLATE	Volume of hydrogel (μL)	Volume of cover medium (μL)
6 well plate	1000	1000
12 well plate	500	500
24 well plate	250	250
48 well plate	125	125
96 well plate	50	50

PLATE INSERT	Volume of hydrogel (μL)	Volume of cover medium (μL)
6 well plate	700	700

12 well plate	250	250
24 well plate	125	125
48 well plate	50	50
96 well plate	20	20

4. **Wait 15-30 min at room temperature for hydrogel stabilization.**
   Note: During the hydrogel forming process, do not disrupt the hydrogel by tilting or shaking the well plate.

5. A**fter hydrogel stabilization, tilt the well plate to check that hydrogel is formed and attached well to the bottom of the well plate. Carefully add cell culture media to cover hydrogel.**

6. **Plate the well plate in an incubator and change the cover medium every 48 hrs.**
   Note: We recommend to only change 60% of the top medium without disturbing the hydrogel.

3D CELL CULTURE HELP GUIDE

- The final cell concentration can be optimized based on different cell types. We recommend 2×10^5 to 1×10^6/mL.
- Using 0.5X PBS to dilute the hydrogel would help to accelerate the hydrogel formation when compare to DI water for hydrogel dilution.
- Prepare FRESH the diluted hydrogel solution for each use. Immediately mix with cell culture medium after diluting with PBS.
- The concentrations of PBS can be adjusted from 0.1X to 1X. When using PBS concentration lower than 0.5X, please refer the mixing ratio of DI water (table 4 of Appendix A). 1X PBS can be used for 1:1 mixing ratio.
- Adding the hydrogel as a dome instead of covering the whole bottom of the well plate might cause hydrogel detachment.
- 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. It might disrupt the hydrogel formation. Pipetting up and down for 5-10 times should be good.
- 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 media.
- At step 6, if the hydrogel is still not stable after 30 min at room temperature, the hydrogel might be too soft and may detach from the well plate after adding the cover medium or medium change. You need to consider adjusting the mixing ratio by adding more cell culture medium to mix with diluted hydrogel solution at step 3 to form a more stable hydrogel.

- If the hydrogel solidifies too fast after mixing with culture medium (showing as small solid gel chuck), please adjust the mixing ratio by using less cell culture medium. For example, if mixing 1 mL diluted hydrogel solution with 1 mL cell culture medium lead to the solid gel chuck (particles), then mixing 1 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 (normally happen when use low hydrogel concentration at 1:3 or 1:4 dilution). 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 1.5-3 mL cell culture medium would help to solve the issue.

- 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 60-70% of the cover medium afterward. (Please check the suggested volume of the additional cover medium in the tables below)

	Volume of the cover medium at Day 0	Additional volume adding at first-time medium change without removing the cover medium (Day 2)	Volume of partial to medium change afterward (Day 4)
6 well plate	1200 µL	1000 µL	1500 µL
12 well plate	500 µL	400 µL	600 µL
24 well plate	250 µL	200 µL	300 µL
48 well plate	125 µL	100 µL	150 µL
96 well plate	50 µL	50 µL	60 µL

- Using a low hydrogel concentration would make a hydrogel with low elastic modulus. Such hydrogel might detach from the well plate. Simply using more cell culture medium to mix with diluted hydrogel solution in step 2 might solve this issue. Otherwise, pre-coat the well plate with 1X PBS or 100 mM CaCl2 would help the hydrogel to attach at the bottom of the well plate:

  i. Add the PBS or CaCl2 solution to the well plate for 30 min
  ii. Remove the PBS or CaCl2 solution, open the lid under the biosafety hood for 10-20 min before adding the hydrogel. The suggested volumes of PBS or CaCl2 for different sizes of well plate are list below.

	Volume of 1X PBS or 100 mM CaCl2 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

APPENDIX A: 3D CELL CULTURE DILUTION & MIXING VOLUME

TABLE 3. Using 0.5X PBS to dilute the hydrogel solution

1. **DMEM, EMEM, MEM-Alpha**

Dilution	VitroGel solution	0.5X PBS	Culture 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
1:4	1 mL	4 mL	1.25 mL

2. **Leibovitz L-15, MEM (Hanks'), DMEM/F-12, McCoy`s 5A**

Dilution	VitroGel solution	0.5X PBS	Culture medium w/ cells
1:0	4 mL	0 mL	2 mL
1:1	2 mL	2 mL	2 mL
1:2	1 mL	2 mL	1.5 mL

3. **RPMI 1640**

Dilution	VitroGel solution	0.5X PBS	Culture medium w/ cells
1:0	2 mL	0 mL	2 mL
1:1	1 mL	1 mL	2 mL
1:2	1 mL	2 mL	3 mL

TABLE 4. Using DI water to dilute the hydrogel solution

1. **DMEM, EMEM, MEM-Alpha**

Dilution	VitroGel solution	DI water	Culture 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	2 mL
1:3	1 mL	3 mL	2 mL
1:4	1 mL	4 mL	5 mL

2. **Leibovitz L-15, MEM (Hanks'), DMEM/F-12, McCoy`s 5A**

Dilution	VitroGel solution	DI water	Culture medium w/ cells
1:0	4 mL	0 mL	2 mL
1:1	2 mL	2 mL	2 mL
1:2	1 mL	2 mL	3 mL

3. **RPMI 1640**

Dilution	VitroGel solution	DI water	Culture medium w/ cells
1:0	2 mL	0 mL	2 mL
1:1	1 mL	1 mL	2 mL
1:2	1 mL	2 mL	3 mL

2D COATING

Materials:

- **VitroGel**
- **Cells**
- **Cell Culture medium**
- **DPBS (no calcium, no magnesium) or DI water**
- **Micropipette**
- **Tissue culture treated plate**
  ⚠ Note: Non-tissue culture treated well plate may cause hydrogel detachment.
  If use glass bottom, please use tissue culture treated glass bottom plate or Poly-D-Lysine coated glass bottom plate for a better hydrogel attachment.
- **Water bath 37°C (optional)**

PROTOCOL

Before starting, read 2D COATING HELP GUIDE on page 14 for protocol tips

1. **Bring VitroGel solution and cell culture medium to room temperature.**
   Optional: Warm at 37°C

2. **Gently mix the VitroGel solution with cell culture medium without introducing bubbles. The recommended mixing ratio for the different media is listed below (without dilution of hydrogel solution). If you need to adjust the concentrations of hydrogel solution with PBS or DI water, please check the Table 5 and Table 6 in Appendix B (page 16) for the volume of dilution and gel/media mixing ratio.**

Media	DMEM, EMEM, MEM-Alpha	Leibovitz L-15, MEM (Hanks'), DMEM/F-12, McCoy`s 5A	RPMI 1640, 1X PBS
Mixing ratio (hydrogel solution : medium, V/V)	4:1	2:1	1:1

   (Can also use 1X PBS to prepare the hydrogel coating by simply mixing the hydrogel with 1X PBS at 1:1 (v/v) ratio)

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 table below:

WELL PLATE	Volume of hydrogel (μL)	Volume of cover medium (μL)
6 well plate	1000	1000
12 well plate	500	500
24 well plate	250	250
48 well plate	125	125
96 well plate	50	50

PLATE INSERT	Volume of hydrogel (μL)	Volume of cover medium (μL)
6 well plate	700	700
12 well plate	250	250
24 well plate	125	125
48 well plate	50	50
96 well plate	20	20

4. **Wait 15-30 min at room temperature for hydrogel stabilization.**
   Note: During the hydrogel forming process, do not disrupt the hydrogel by tilting or shaking the well plate.

5. **After hydrogel stabilization, tilt the well plate to ensure that hydrogel is formed and attached well 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 hrs**
   Note: We recommend to only change 60% of the top medium without disturbing the hydrogel.

2D COATING HELP GUIDE

- The final cell concentration can be optimized based on different cell types. We recommend 2×10^5 to 1×10^6/mL.
- Using PBS to dilute the hydrogel would help to accelerate the hydrogel formation when compare to using DI water.
- Prepare FRESH the diluted hydrogel solution for each use. Immediately mix with cell culture medium after diluting with PBS.
- Adding the hydrogel as a dome instead of covering the whole bottom of the well plate might cause hydrogel detachment.
- 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. It might disrupt the hydrogel formation. Pipetting up and down for 5-10 times should be good.

- 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 media.

- At step 5, if the hydrogel is still not stable after 30 min at room temperature, the hydrogel might be too soft and may detach from the well plate after adding the cover medium or medium change. You need to consider adjusting the mixing ratio by adding more cell culture medium to mix with diluted hydrogel solution at step 3 to form a more stable hydrogel.

- If the hydrogel solidifies too fast after mixing with culture medium (showing as small solid gel chuck), please adjust the mixing ratio by using less cell culture medium. For example, if mixing 1 mL diluted hydrogel solution with 1 mL cell culture medium lead to the solid gel chuck (particles), then mixing 1 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 (normally happen when use low hydrogel concentration at 1:3 or 1:4 dilution). 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 1.5-3 mL cell culture medium would help to solve the issue.

- 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 60-70% of the cover medium afterward. (Please check the suggested volume of the additional cover medium in the tables below)

	Volume of the cover medium at Day 0	Additional volume adding at first-time medium change without removing the cover medium (Day 2)	Volume of partial to medium change afterward (Day 4)
6 well plate	1200 µL	1000 µL	1500 µL
12 well plate	500 µL	400 µL	600 µL
24 well plate	250 µL	200 µL	300 µL
48 well plate	125 µL	100 µL	150 µL
96 well plate	50 µL	50 µL	60 µL

- Using a low hydrogel concentration would make a hydrogel with low elastic modulus. Such hydrogel might detach from the well plate. Simply using more cell culture medium to mix with diluted hydrogel solution in step 2 might solve this issue. Otherwise, pre-coat the well plate with 1X PBS or 100 mMCaCl2 would help the hydrogel to attach at the bottom of the well plate:

i. Add the PBS or CaCl2 solution to the well plate for 30 min
ii. Remove the PBS or CaCl2 solution, open the lid under the biosafety hood for 10-20 min before adding the hydrogel. The suggested volumes of PBS or CaCl2 for different sizes of well plate are list below.

	Volume of 1X PBS or 100 mM CaCl2 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

APPENDIX B: 2D COATING DILUTION & MIXING VOLUME

TABLE 5. Using 0.5X PBS to dilute the hydrogel solution

1. **DMEM, EMEM, MEM-Alpha**

Dilution	VitroGel solution	0.5X PBS	Culture 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
1:4	1 mL	4 mL	1.25 mL

2. **Leibovitz L-15, MEM (Hanks'), DMEM/F-12, McCoy`s 5A**

Dilution	VitroGel solution	0.5X PBS	Culture medium w/ cells
1:0	4 mL	0 mL	2 mL
1:1	2 mL	2 mL	2 mL
1:2	1 mL	2 mL	1.5 mL

3. **RPMI 1640**

Dilution	VitroGel solution	0.5X PBS	Culture medium w/ cells
1:0	2 mL	0 mL	2 mL
1:1	1 mL	1 mL	2 mL
1:2	1 mL	2 mL	3 mL

TABLE 6. Using DI water to dilute the hydrogel solution

1. **DMEM, EMEM, MEM-Alpha**

Dilution	VitroGel solution	DI water	Culture 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	2 mL
1:3	1 mL	3 mL	2 mL
1:4	1 mL	4 mL	5 mL

2. **Leibovitz L-15, MEM (Hanks'), DMEM/F-12, McCoy`s 5A**

Dilution	VitroGel solution	DI water	Culture medium w/ cells
1:0	4 mL	0 mL	2 mL
1:1	2 mL	2 mL	2 mL
1:2	1 mL	2 mL	3 mL

3. **RPMI 1640**

Dilution	VitroGel solution	DI water	Culture medium w/ cells
1:0	2 mL	0 mL	2 mL
1:1	1 mL	1 mL	2 mL
1:2	1 mL	2 mL	3 mL

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PREPARING AN INJECTABLE HYDROGEL

Materials:

- VitroGel
- Cells
- Cell Culture medium
- DPBS (no calcium, no magnesium)
- Micropipette
- Syringe
- Water bath 37°C (optional)

PROTOCOL

1. Bring VitroGel solution and cell culture medium to room temperature. Optional: Warm at 37°C)

2. Gently mix the VitroGel solution with cell suspension without introducing bubbles. The recommended mixing ratio for the different media is listed below.

Media	DMEM, EMEM, MEM-Alpha	Leibovitz L-15, MEM (Hanks'), DMEM/F-12, McCoy`s 5A	RPMI 1640, 1X PBS
Mixing ratio (hydrogel solution : medium, V/V)	4:1	2:1	1:1

(Can also use 1X PBS to prepare the injectable by simply mixing the hydrogel with 1X PBS at 1:1 (v/v) ratio)

3. The mixture can be transferred to a syringe. The hydrogel is ready for injection after stabilization for 15-30 min.

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 3 times.**

2. **Prepare live/dead staining solution with PBS for 5X solution.**

3. **Add live/dead staining solution directly to the hydrogel to submerge the whole gel.**

   The recommend volume is listed in the table below:

Volume of hydrogel (μL)	Volume of staining solution (μL)
500	1500
250	750
50	150

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

Note:

- Using 5X staining solution is a recommended starting concentration. The optimal concentration needs to be adjusted based on the cell lines and the imaging system.
- Analyze the cells quickly after staining.

CELL HARVESTING

Materials:

- **Cells cultured in VitroGel system**
- **0.1X PBS or DI water**
- **Centrifuge tubes**
- **Micropipette**
- **Water bath 37˚C, vortex**

PROTOCOL (using 24 well-plate, 250 µL gel/well as example)

1. **Warm up 0.1X PBS (or DI water) and empty centrifuge tubes at 37˚C water bath.**

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

3. **Add 1 mL warm 0.1X PBS to each well and pipette up and down to mix thoroughly.**
4. **Transfer the mixture to a warm centrifuge tube.**

5. **Rinse each well with 1 mL warm 0.1X PBS and combine the solution in the centrifuge tube.**

6. **Pipette the mixture thoroughly and add additional warm 0.1X PBS to dilute the mixture to 10 mL.**

7. **Vortex the centrifuge tube for 5-10 seconds and incubate in 37°C water bath for 1-5 minutes (optional).**

8. **Centrifuge at 1,000 rpm for 2-5 minutes and discard supernatant and collect the cell pellet.**

9. **Re-suspend the cells with warm 0.1X PBS and repeat steps 6-8 one more time if necessary (optional).**

Note:
- Use warm 0.1X PBS or DI water instead of using cold solutions or 1X PBS (or cell culture media with high ionic concentrations).
- Optimize the speed and time of centrifuge according to different cell types.

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 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 1 hr 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.