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PGmatrix™ Using Guides

The **PepGel PGmatrix™** is a powerful tool for in vitro 3D cell culture with more accurate in vivo predictions for life science research and development. PepGel PGmatrix kit consists of a vial of **PGmatrix** patented peptides nanofiber solution, a vial of **PGworks** trigger solution and a vial of **PGgrow** (optional). The PGmatrix nanofibrils are formulated into a basic or a customer desired cell culture medium in neutral pH. A 3D microenvironment can be formed accordingly for cell growth. With PGmatrix, cells no longer suffer acidic or chill conditions; cultured cells are easily harvested from the matrix; all operating and growth procedures can be completed at room temperature or 37°C in neutral pH.

PRODUCT:	PepGel PGmatrix™ Research Kit
CONTENT:	PGmatrix solution and PGworks solution
QUANTITY:	20 mL of PGmatrix and 1 mL of PGworks 10 mL of PGmatrix and 0.5 mL of PGworks 6 mL of PGmatrix and 0.3 mL of PGworks 2 mL of PGmatrix and 0.3 mL of PGworks
STORAGE:	Stored at 4°C
LOT NUMBER:	See product label

FOR IN VITRO RESEARCH USE ONLY. PLEASE READ MATERIAL USING AGREEMENT FOR MORE DETAILS. FOR IN VIVO TEST, PLEASE ASK FOR PG IN VIVO PRODUCTS.

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FOR FIRST TIME USER, PLEASE READ THE FOLLOWING THREE MESSAGES

MESSAGE I: Mixing Ratio Notice

The PGmatrix solution contains 1% W/V standard peptides. Most cells from soft tissue grow well in the peptide concentration from 0.1% to 0.5%. If you are first time user, we recommend using a few mixing ratios in the range of 0.1-0.6% W/V final peptide concentration for 3D cell encapsulation to identify the best mixing ratio for your cells. The following **Table 1** presents two mixing ratios at 0.2% and 0.5% concentration as example, respectively. Please use the following table as reference to mix PGmatrix solution and cell suspension.

Remember: add the PGworks to your cell suspension FIRST before you mix PGmatrix solution with cell suspension. If you still have questions, please contact technical support by email to customerservice@pepgel.com

Table 1: Examples of Mixing ratios of PGmatrix solution, cell suspension and PGworks* solution and maximum plating volume for 1 well for different plates. (See NOTE for PGworks concentration)

Well Plate Size	0.2% W/V			0.5% W/V			Maximum plating volume for 1 well (µL)
	Cell suspension (µL)	PGworks solution (µL)	PGmatrix solution (µL)	Cell suspension (µL)	PGworks solution (µL)	PGmatrix solution (µL)	
12	780	20	200	480	20	500	1000
24	390	10	100	240	10	250	500
48	195	5	50	120	5	125	250
96	78	2	20	48	2	50	100

NOTE*: PGworks is always 2% of the total volume of PGmatrix + cell suspension together.

MESSAGE II: Add medium on the top of hydrogel to prevent drying and to feed the cells for long term culture

After hydrogel formation (30-60 min at 37 °C after mixing), cell medium needs to be added on the top of the gel to provide fresh nutrition and prevent drying for long term culture. The following **Table 2** presents the recommended volume of medium to add to each well based on different well sizes.

Table 2: The recommended volume of medium to be added on the top of hydrogel

	12-well	24-well	48-well	96-well
Volume to add (µL)	2000	1000	500	200

MESSAGE III: Examples of Cell Density and Gel Concentration

***Table 3: Cell density and gel concentration recommendation for 3D cell cultures in PGmatrix Products**

Cells	PGmatrix [£]	Gel concentration (%)	Gelation time (min)	Cell seeding density (Cell/mL)
rESC 101	PGR2TD	0.5	60	1 x10 ⁵
Jurkat Clone E6-1 (ATCC)	PGR	0.2	60	1 x10 ⁵ - 2 x10 ⁵
Jurkat Cell (ASC)	PGR	0.2	60	1 x10 ⁵ - 2 x10 ⁵
CHO-S (ASC)	PGP/PGD	0.2-0.4	60	1 x10 ⁵ - 2 x10 ⁵
MCF-7 (ATCC)	PGD	0.2-0.3	60	2.8 x10 ⁵
Hela cell	PGD	0.2	60	8 x10 ⁴
Head Neck cell	PGD	0.5	30	4 x10 ⁴
hiPSC derived from fibroblast (Applied Stem Cell)	PGD-hiPSC (with PGgrow-hiPSC**)	0.5	30	1.8 x10 ⁵ - 2 x10 ⁵
Episomal hiPSC (Thermofisher)	PGD-hiPSC (with PGgrow-hiPSC**)	0.5	30	2 x10 ⁵ - 3 x10 ⁵
AD MSC (ATCC)***	PGD	0.3	60	1.4 x10 ⁶

£ PGR2TD= PGR2Tmatrix DMEM; PGD=PGmatrix DMEM; PGD-hiPSC=PGmatrix DMEM-hiPSC; PGR=PGmatrix RPMI; PGP=PGmatrix PURE, PGmatrix with other basal medium would be also available upon request.

***Note:** Results reported here are from 48-12 well plate under culturing condition at 37°C and 5% CO₂, and can only be used as reference. It is users' responsibility to use appropriate cell medium and growth supplement or consult with PepGel.

**PGgrow is optional but highly recommended for hiPSC culturing in PGmatrix-hiPSC. Without PGgrow, It is users' responsibility to choose appropriate growth supplement. (See hiPSC-qualified PGmatrix™ Using Guides at www.pepgel.com)

*** AD MSC grows better at O₂ level of 3% or in the presence of antioxidants at regular cell culturing condition.



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PGmatrix™ QUICK START* for 3D cell culture

For Example: 3D Cell Culture Procedure for a 24 well plate at the ratio of 1:1 (PGmatrix:Cell suspension) that will yield 0.5% (w/v) PGmatrix hydrogel concentration.

(Preparation: Bring the PGmatrix kit and cell solution to room temperature or 37 °C)

- **Step 1** Prepare cell suspension (960 µL), add serum or other growth factors as needed to your cell suspension (pipet well without introducing air bubbles**)
- **Step 2** Add PGworks (40µl) to the cell suspension from Step 1 (pipet well without introducing air bubbles) (Graphic illustration Step 2)
- **Step 3** Mix PGmatrix solution (1000µL) with the cell suspension from Step 2 at 1:1 ratio (pipet well without introducing air bubbles) (Graphic illustration Step 3)
- **Step 4** Transfer the mixture of step 3 to a 24 well plate (500 µL/well, 4 wells total)
- **Step 5** Place the 24-well plate in a 37 °C incubator for 30 min or up to 60 min for gelation
- **Step 6** After gelation, add 1000 µL of the cell medium gently on top of the gel to prevent the matrix from drying and to feed the cells, (**HINT:** add the medium along the well wall to avoid disturbing the gel, change top medium every other day to provide fresh nutrition and prevent drying for long term culture)
- **Step 7** Place the 24-well plate back to the incubator for cell growth

*The mixing ratio given above is 1:1 at 0.5% PGmatrix concentration. **Most** cells grow well in gel concentration of 0.1-0.5%, in those cases, ratio of PGmatrix vs cell suspension can be 1:2, 1:3, 1:4, 1:5 or 1:9. PGworks is always 2% of the total volume of PGmatrix + cell suspension together. For more detailed information, such as other ratios, cell feeding, cell isolation or immuno-staining, please read the complete User Guides.

**NOTE: To avoid introducing air bubbles, keep pipetting within the solution or mixture)

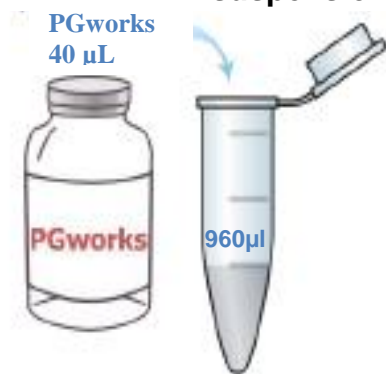
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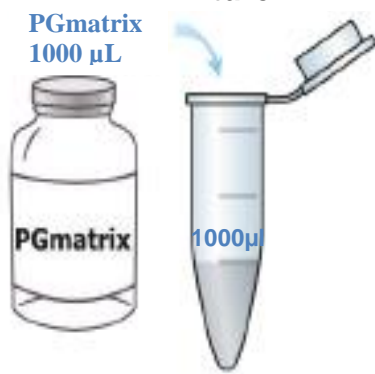
To order: customerservice@pepgel.com,

Cell Encapsulation illustration of the Example given in the QUICK START (Pipet without introducing air bubble)

STEP 2: Add PGworks to cell suspension



STEP 3: Add PGmatrix solution to mixture

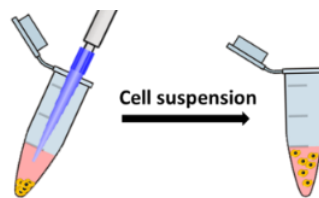


0.5% gel concentration for a 2000 µl volume: 960 µL Cell suspension +40 µL PGworks + 1000 µL PGmatrix solution

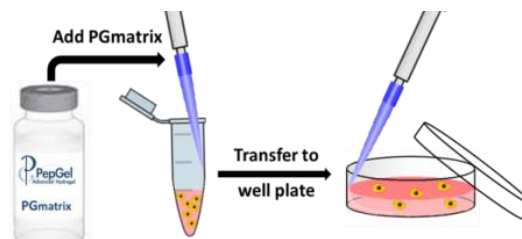
A. Protocols for 3D cell culture

I ENCAPSULATE CELLS FROM SUBCULTURE

1. Bring the PGmatrix solution and PGworks solution to room temperature (15 - 25 °C) or 37 °C (37 °C water bath).
2. Suspend cells in desired cell culture medium then add PGworks solution to the cell suspension according to the Mixing Ratio in **Table 1** on page 3, pipet well without introducing air bubbles (always immersing pipet tip in cell solution during pipetting).



3. Mix the PGmatrix solution carefully into the cell suspension of step 2 at the Mixing Ratio indicated in **Table 1** on page 3 (pipet well without introducing air bubbles). Transfer the mixture into the center of each well, then swirl the plate to uniformly cover the entire well bottom surface. (**Note:** Please select the PGmatrix solution accordingly that matches your cell culture medium or contact PepGel for special medium requirement by email to customerservice@pepgel.com). For cell seeding density and gel concentration, please see examples in **Table 3** on page 4.



4. Incubate the plate at 37°C (5% CO₂) for 30-60 min to complete the gelation.
5. After gelation, place cell medium on top of the gel of each well to prevent the matrix from drying (recommended volume of each well is listed in **Table 2** on page 3). (**Tips:** carefully add cell culture medium along the wall of each well on the top surface of the hydrogel without disturbing the hydrogel).
6. To feed the cells, change the medium very gently by replacing 1/3 - 2/3 of medium above the gel with fresh medium every day or every other day, depending on how often the cells



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need to be fed or how soon the cell medium color change to yellowish.

Note: Cells will not perform well without appropriate growth factors, it is users' preference what growth factors are needed for their cells, or contact customerservice@pepgel.com for suggestion. PGgrow kit is also available at PepGel for stem cells (i.e., hiPSC).

II THAWING CELLS DIRECTLY IN 3D PGMATRIX*

1. Bring the PGmatrix solution and PGworks solution to room temperature (15 - 25 °C) or 37 °C (37 °C water bath).
2. Thaw the vial with frozen cells by gently agitating on in a 37 °C water bath. To reduce the possibility of contamination, keep the O-ring and cap out of the water.
3. Remove the vial from the water bath as soon as the contents start to thaw. Add 1 mL of pre-warmed complete culture medium and pipet until the cell suspension is totally thawed.
4. Transfer the cell suspension to a 15 mL conical tube, and use another 1 mL of complete culture medium to rinse the vial for the remaining cells, and combine the solution to the conical tube.
5. Dilute the mixture to 10 mL by using complete culture medium. And centrifuge at 100 g 200 g for 5 min (depending on cell types).
6. Remove the supernatant and resuspend the cell pellet gently in complete culture medium.
7. Follow the steps 2-6 in Section I "ENCAPSULATE CELLS FROM SUBCULTURE" for the cells encapsulation in 3D PGmatrix.

*** The recommended cell seeding density for direct thawing cells in 3D PGmatrix is one to two times higher than encapsulation cells from subculture, in order to maintain good cell viability and proliferation.**

III CELL RECOVERY FOR PASSAGING, COUNTING AND DOWNSTREAM APPLICATIONS

1. **Gel disruption:** Without removing the upper layer cell medium, mechanically disrupt the gel **THOROUGHLY** (**gel disruption thoroughly is very important for the cell isolation from the gel) by pipetting the gel and medium mixture. Transfer the mixture to a 2 mL-50 mL conical centrifuge tube (recommended conical centrifuge tube size vs well plate size are listed in **Table 4** on Page 10).
2. **Rinse:** Use DPBS to rinse the well and combine the solution to the centrifuge tube. Recommend using the volume as double the maximum plating volume for each well listed in **Table 1** on page 3 (i.e., 200 μ L for 96-well plate or 1000 μ L for 24-well plate).
3. **Gel dilution:** Pipette the mixture **THOROUGHLY** and add additional DPBS to further dilute the mixture by 10-20 folds of the original plating volume (See examples in **Table 4** on Page 10), and mix well.
4. **Centrifuge:** Centrifuge at 200 g-600 g for 5-6 min, depending on cell. Discard supernatant and collect the pellet.

- Note:**
1. Centrifuge force for cells harvesting from 3D PGmatrix should be higher than conventional 2D cell harvesting. 200 g-600 g is the typical centrifuge force range to harvest cells or cell colonies from PGmatrix.
 2. Always check if there are many cells or cell colonies remaining in the supernatant after centrifuge, then you may increase the centrifuge force or time accordingly for further cell isolation.
 3. If you still have cell isolation issues, you may reduce your gel concentration for cell culture (i.e., if you used 0.5% PGmatrix for cell culture, you may reduce gel concentration to 0.3% or 0.2%), or consult with PepGel for technical support.

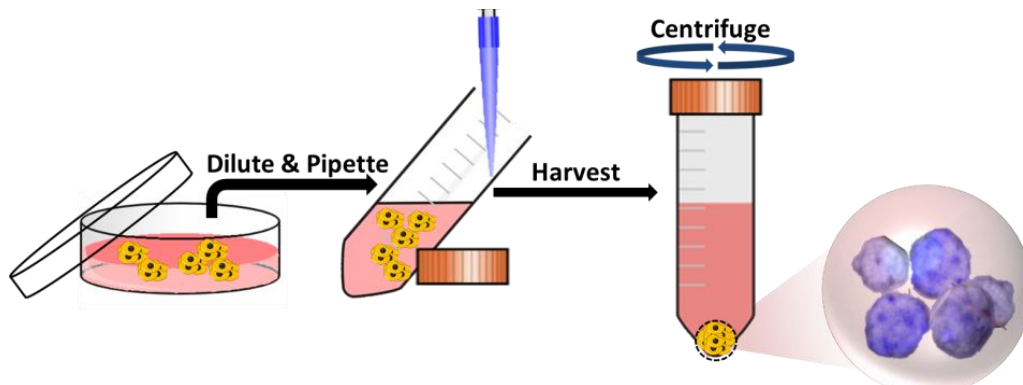


Table 4*: The recommended conical centrifuge tube size vs well plate size for gel dilution

	12-well	24-well	48-well	96-well
Gel plating volume	1000 μ L	500 μ L	250 μ L	100 μ L
Final volume of diluted gel and cell mixture from one well (10-20 folds)	10-20 mL	5-10 mL	2.5-5 mL	1-2 mL
Suggested conical tube size	50 mL	15 mL	15 mL	2 mL

* The conical tube size suggested here is only good for one well cultured cells harvesting. If more than one well cultured cells are harvested at the same time, the tube size need to be larger accordingly. For example, 50 mL tube can be used if 3 wells of 24-well plate cultured cells are harvested at the same time (3 x 10 mL).

5. **Colonies breakup:** To break the colonies, follow your conventional culture protocol, add enzyme solution or other dissociation agents, or follow procedures described below.
 - To break the colonies, add Trypsin-EDTA solution (i.e., 0.25%Trypsin+0.02%EDTA) to the cell pellet, the recommended Typsin-EDTA solution amount can be equal to the gel plating volume listed in **Table 1** on page 3 (i.e., 100 μ L for 96-well plate or 500 μ L for 24-well plate).
 - Mix gently to disrupt the cell pellet, then incubate the conical tubes at 37°C (5% CO₂) for 5 min.
 - After incubation, pipet the cell solution up and down to mechanically break up the cell colonies. If there are still some colonies left, continue to incubate the cell solution at 37°C (5% CO₂) for another 2-3 min and pipet, until no cell colonies are observed.
 - Then add culture medium or trypsin neutralization solution equal to the volume of Trypsin-EDTA solution to the conical tube, centrifuge at 100 g-200 g for 5 min (depending on cell types).
 - Discard supernatant and resuspend the pellet in complete culture medium for further cell counting or cell passage.

Note: The Enzyme concentration and incubation time for cell colonies disruption depend on the cell types, colony size and number, therefore, the condition listed above can only be used as reference. It is the users' responsibility to choose the appropriate condition for their cell colonies breakup.



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IV CELL CRYOPRESERVATION

1. Resuspend cell pellet in freezing medium or complete growth medium with 5%-10% DMSO to a concentration of 1×10^6 to 1×10^7 cells/mL depending on cell types. Allow the cells to sit at room temperature 15 min, so the cryoprotectant can diffuse into the cell.
2. Cells should be frozen slowly at $1 \text{ }^\circ\text{C}/\text{min}$. This can be achieved by using a programmable cooler or using Mr. Frosty Freezing container with isopropanol placed in a $-80 \text{ }^\circ\text{C}$ freezer for at least 24 hours.
3. Quickly transfer the vial to liquid nitrogen or $-130 \text{ }^\circ\text{C}$ freezer for long term storage.

V IMMUNO-STAINING

1. Gently remove the “free” medium on top of the gel in the culture well
2. Gently rinse the gel surface once with DPBS (without Ca^{2+} and Mg^{2+} ions)
3. Fix cells in gels with 10% neutral buffered formalin for 30-40 min
4. Wash the formalin out twice with DPBS (without Ca^{2+} and Mg^{2+} ions), 15 min for each rinsing.
5. Prepare washing buffer and blocking solution
 - A): Washing buffer: DPBS (with Ca^{2+} and Mg^{2+} ions) + 0.2% triton X-100 + 0.1% cold water fish gelatin
 - B): Blocking solution: washing buffer + 10% serum from the same source animal as the secondary antibody. Mix blocking solution thoroughly by pipetting
 - C): Filter through syringe filter (Millex GP .22 micrometer PES membrane). Part of the solution will be lost due to filter clogging. Mix at least one extra mL more than you need.

Note: Blocking solution should be prepared just before applying it to the sample.

6. Remove the DPBS on top of the gel, add washing buffer and incubate at room temperature for 10 min. Wash twice.

Note: If the sample is not blocked and stained immediately, it can be stored after adding washing buffer. To store the sample, the plate should be sealed with parafilm and stored at 4°C.

7. Blocking (Day 1):

Remove the washing buffer, add blocking solution to the fixed samples (i.e., 500-800 μL per well blocking solution, for 24-well plate). Let set overnight (or > 12 hrs).
8. Primary Antibody (1AB) (Day 2)
 - A): Prepare primary antibody solution by adding the primary antibody solution to washing buffer. The primary antibody concentration for 3D staining can be similar or higher than that used for 2D culture staining (i.e., Goat Oct 3/4 antibody (N-19) with final concentration of 3 $\mu\text{g}/\text{mL}$ was used for hiPSC staining directly in 3D PGMATRIX-hiPSC).
 - B): Add primary antibody solution to the gel, incubate at room temperature overnight

Note: Use sufficient volume of antibody solution to completely soak the gel (i.e., 1 mL for gel in 24-well plate, 500 μL for gel in 48-well plate).

- C): Process one sample without 1AB as a negative control to assess background staining
9. Rinse (Day 3): Rinse with washing buffer 4 times (2 hrs per wash)
 10. Secondary antibody (2AB) (Day 3)
 - A) Prepare secondary antibody solution by adding the secondary antibody solution to washing buffer. The secondary antibody concentration for 3D staining can be similar or higher than that used for 2D culture staining (i.e., Rabbit anti-goat IgG (H+L) 2nd antibody alexa fluor 488 with final concentration of 5 $\mu\text{g}/\text{mL}$ was used for hiPSC staining directly in 3D PGMATRIX-hiPSC).

B) Wrap the plate with aluminum foil, incubate at room temperature, overnight (or > 12 hrs).

11. Rinse and Imaging (Day 4)

A): Rinse at least 6 times (1 hr per wash) with washing buffer

B): Replace washing buffer with Glycerin for imaging

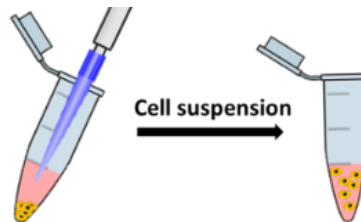
C): Proceed with imaging

Note: 1. to achieve a strong and specific signal, it is necessary to thoroughly block non-specific binding of antibodies to the gel matrix (PGmatrix) as well as allowing diffusion of antibodies through the gel matrix (PGmatrix). Therefore, extended blocking and incubation times and multiple washes are required.

2. Since it is a 3D system, the background is influenced by other cells at different planate positions. Therefore, post-processing of images may be needed to reduce background brightness.

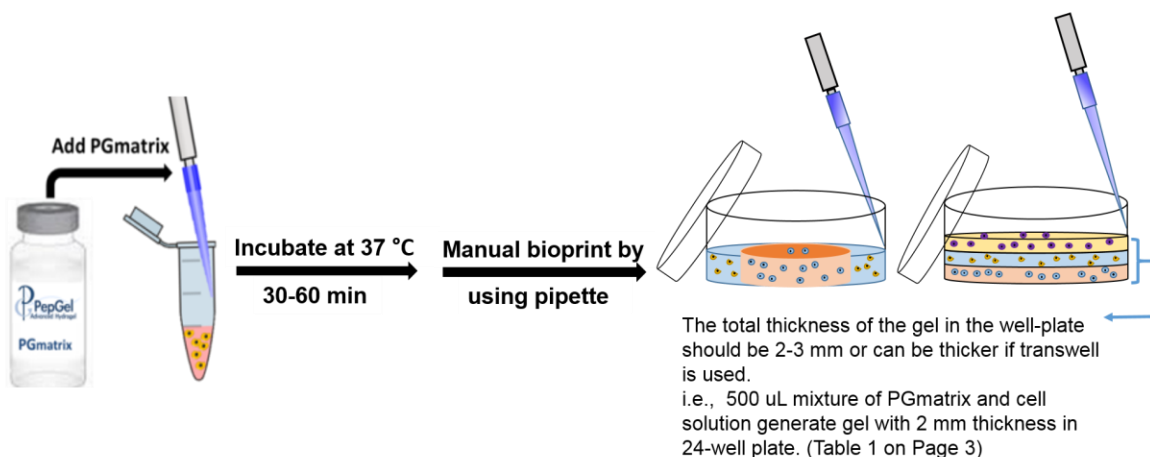
B. Protocols for 3D Manual Bioprinting

1. Bring the PGmatrix solution and PGworks solution to room temperature (15 - 25 °C) or 37 °C (37 °C water bath)
2. Suspend cells in desired cell culture medium with appropriate growth factors then add PGworks solution to the cell suspension according to the Mixing Ratio in **Table 1** on page 4, pipet well without introducing air bubbles (always immersing pipet tip in cell solution during pipetting).



3. Mix the PGmatrix solution carefully into the cell suspension of step 2 at the Mixing Ratio indicated in **Table 1** on page 3 (Please select the PGmatrix solution accordingly that matches your cell culture medium or contact PepGel for special medium requirement by email to customerservice@pepgel.com). For cell seeding density and gel concentration, please see examples in **Table 3** on page 4*.
4. Incubate the vial with mixture of cell and PGmatrix (PGmatrix-Cell) at 37°C (5% CO₂) for 30-60 min to complete the gelation. (**Note:** 30 min gelation time is recommended for 0.5% PGmatrix concentration, and 60 min gelation time for PGmatrix concentration lower than 0.5%). Then PGmatrix-Cell gel should be ready for manual bioprinting by using the pipette (i.e., the following diagram shows the potential for co-culture of two or three different cells by manual bioprinting, or users can bioprint other patterns for cell co-culture based on their needs).

* **Note:** To avoid bubbles, the PGmatrix-cell solution can be uploaded into **syringe** at Step 3, and incubate, then bioprint with the syringe with needle gage 27 or above.



C. Protocols for cell invasion (Thin gel method)

1. Bring the PGmatrix solution and PGworks solution to room temperature (15 - 25 °C) or 37 °C (37 °C water bath)
2. Mix cell medium with PGworks first, and then PGmatrix solution according to the Mixing Ratio in **Table 1** on page 3 (pipet well without introducing air bubbles)
Note: Please select the PGmatrix solution accordingly that matches your cell culture medium or contact PepGel for special medium requirement by email to customerservice@pepgel.com
3. When properly mixed, plate the mixture into the center of each well (i.e., volume per well is recommended as 250 µl - 500 µl for 24-well plate), then swirl the plate to coat the entire well bottom surface, or different amount of mixture to get a desirable gel thickness as user wishes.
4. Place plates at 37°C for 30-60 min for gelation, then plate cell suspension on top of the thin gel for cell growth.

Note: Please follow the detailed procedures described in the user guides for desirable ratio of each mixing step, or conduct preliminary experiment to find out optimum mixing ratio for your experiment.



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D. Protocols for thin gel coating for 2D cell culture

PGmatrix 2D-hiPSC kit for 2D culturing of Human Induced Pluripotent Stem Cell (hiPSC) is available upon request.

PGR2Tmatrix 2D-ESC kit for 2D culturing of Rat Embryonic Stem Cell (Rat ESC) is available upon request.

Please contact PepGel at customerservice@pepgel.com for order and culturing protocol.

E. Protocols for PGbioInk for 3D bioprinting

PGbioInk kit for 3D printing is available upon request. Please contact PepGel at customerservice@pepgel.com for order.

PGbioInk using guides are available at www.pepgel.com.

F. Protocols for 3D hiPSC culture in PGmatrix-hiPSC

hiPSC-qualified PGmatrix™ kit (PGmatrix-hiPSC) is available upon request. Please contact PepGel at customerservice@pepgel.com for order.

hiPSC-qualified PGmatrix™ Using guides is available at www.pepgel.com.



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G. Reference Information of 3D Culture in PGmatrix Products

Cells	PGmatrix	Gel concentration (%)	Gelation time (min)	Cell seeding density (Cell/ml)	Proliferation (times)	Cell viability (%)
rESC 101	PGR2TD	0.5	60	1 x10 ⁵	8-9	76-77
Jurkat Clone E6-1 (ATCC)	PGR	0.2	60	1 x10 ⁵ - 2 x10 ⁵	40-45	90-95
Jurkat Cell (ASC)	PGR	0.2	60	1 x10 ⁵ - 2 x10 ⁵	40-45	90-95
CHO-S (ASC)	PGP/PGD	0.2-0.4	60	1 x10 ⁵ - 2 x10 ⁵	40-45	90-95
MCF-7 (ATCC)	PGD	0.2-0.3	60	2.8 x10 ⁵	4	90
Hela cell	PGD	0.2	60	8 x10 ⁴	5-6	90-95
Head Neck cell	PGD	0.5	30	4 x10 ⁴	6-7	90-95
hiPSC derived from fibroblast (Applied Stem Cell)	PGD-hiPSC (with PGgrow-hiPSC**)	0.5	30	1.8 x10 ⁵ - 2 x10 ⁵	10	80-90
Episomal hiPSC (Thermofisher)	PGD-hiPSC (with PGgrow-hiPSC**)	0.5	30	2 x10 ⁵ - 3 x10 ⁵	7-9	75-80
AD MSC (ATCC)***	PGD	0.4	60	1.4 x10 ⁶	1.5-2	90

(PGR2TD= PGR2Tmatrix DMEM; PGD=PGmatrix DMEM; PGD-hiPSC=PGmatrix DMEM-hiPSC; PGR=PGmatrix RPMI; PGP=PGmatrix PURE, PGmatrix with other basal medium would be also available upon request)

* **Note:** Results reported here are from 48-12 well plate under culturing condition at 37°C and 5% CO₂, and can only be used as reference. It is users' responsibility to use appropriate cell medium and growth supplement or consult with PepGel.

** **PGgrow is optional but highly recommended for hiPSC culturing in PGmatrix-hiPSC.** Without PGgrow, It is users' responsibility to choose appropriate growth supplement (See hiPSC-qualified PGmatrix™ Using Guides at www.pepgel.com).

*** AD MSC grows better at O₂ level of 3% or in the presence of antioxidants at regular cell culturing condition.

REFERENCE

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