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PGmatrix-Spheroid™ Using Guide

The **PepGel PGmatrix-Spheroid™ (PG-S)** is a powerful tool for in vitro 3D cell spheroid culture with more accurate in vivo predictions for life science research and development. The kit consists of a vial of ***PGmatrix-spheroid*** 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-spheroid™ Research Kit
CONTENT:	PGmatrix-spheroid solution and PGworks solution
QUANTITY:	20 mL of PGmatrix-spheroid and 3 mL of PGworks
	10 mL of PGmatrix-spheroid and 2 mL of PGworks
	6 mL of PGmatrix-spheroid and 1 mL of PGworks
	2 mL of PGmatrix-spheroid and 0.5 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-spheroid (PG-S) solution contains 2% W/V standard peptides. Most cells from soft tissue grow well in the peptide concentration from 0.5% to 1%. If you are first time user, we recommend using a few mixing ratios in the range of 0.3%-1.5% W/V final peptide concentration for 3D cell encapsulation to identify the best mixing ratio for your cells. The following **Table 1** presents four mixing ratios at 0.3%, 0.5%, 1% and 1.5% concentration as example, respectively. Please use the following table as reference to mix PG-S solution and cell suspension.

Remember: add the PGworks to your cell suspension FIRST before you mix **PG-S** 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 PG-S solution, PGworks* and cell suspension and maximum plating volume per well for different plates.

Well Plate Size	0.3% W/V			0.5% W/V			*Maximum plating volume per well (µL)*
	PG-S : (cell suspension+PGworks) =1:5.7			PG-S : (cell suspension+PGworks) =1:3			
	PG-S (µL)	*PGworks (µL)*	*Cell suspension (µL)*	*PG-S (µL)*	*PGworks (µL)*	*Cell suspension (µL)*	
6	300	24	1676	500	40	1460	2000
12	150	12	838	250	20	730	1000
24	75	6	419	125	10	365	500
48	37.5	3	209.5	62.5	5	182.5	250
96	15	1.2	83.8	25	2	73	100

Well Plate Size	1% W/V			1.5% W/V			*Maximum plating volume for 1 well (µL)*
	PG-S : (cell suspension+PGworks) =1:1			PG-S : (cell suspension+PGworks) = 1:0.33			
	PG-S (µL)	*PGworks (µL)*	*Cell suspension (µL)*	*PG-S (µL)*	*PGworks (µL)*	*Cell suspension (µL)*	
6	1000	80	920	1500	120	380	2000
12	500	40	460	750	60	190	1000
24	250	20	230	375	30	95	500
48	125	10	115	187.5	15	47.5	250
96	50	4	46	75	6	19	100

NOTE*: PGworks is always 8% of the total volume of PG-S solution.

**** Nontreated culture plate is recommended for 3D cell culturing in PG-S.**

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

	6-well	12-well	24-well	48-well	96-well
Volume to add (µL)	4000	2000	1000	500	200

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PGmatrix-Spheroid™ QUICK START*

For Example: 3D spheroid Culture Procedure for a 24 well plate at the ratio of 1:1 (PG-S: PGworks + Cell suspension) that will yield 1% (w/v) PG-S hydrogel concentration.
(Preparation: Bring the PG-S kit and cell solution to room temperature or 37 °C)

- **Step 1** Prepare cell suspension (920 µL), add serum or other growth factors as needed to your cell suspension (pipet well without introducing air bubbles**)
- **Step 2** Add PGworks (80µl) to the cell suspension from Step 1 (pipet well without introducing air bubbles) (Graphic illustration Step 2)
- **Step 3** Mix PG-S 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 30 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 1% PG-S concentration. **Most** cells can form spheroids in the range of 50-200 um in gel concentration of 0.5-1.5%, PGworks is always 8% of the total volume of PG-S. 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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Cell Encapsulation illustration of the Example given in the QUICK START

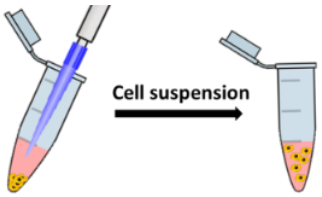
(Pipet without introducing air bubble)

1% gel concentration for a 2000 µl volume: 920 µL Cell suspension +80 µL PGworks + 1000 µL PG-S solution

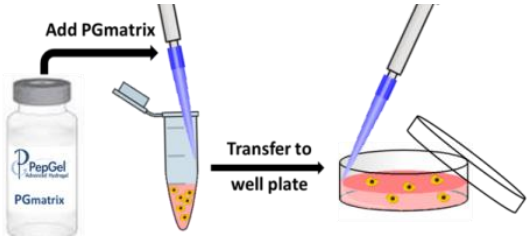
A. Protocols for 3D Cell Spheroid Culture

I ENCAPSULATE CELLS FROM SUBCULTURE

1. Bring the PG-S 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 PG-S 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 (For 6 well-plate with larger bottom area, gently hand-shaking the plate front to rear and side to side is necessary for gel to uniformly cover the entire bottom). For cell seeding density and gel concentration, please see examples in **Table 5** on page 15.

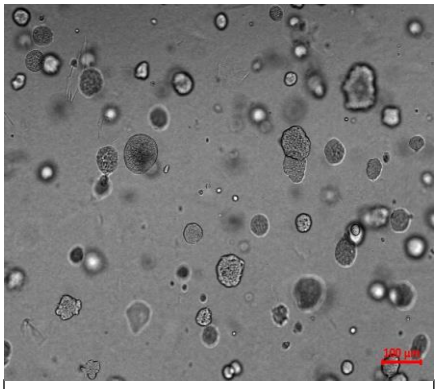


4. Incubate the plate at 37°C (5% CO_2) for 30-60 min to complete the gelation.
5. After gelation, place cell medium on top of the gel of each well to prevent the matrix from drying (recommended volume of each well is listed in **Table 2** on page 4). (**Tips**: carefully add cell culture medium along the wall of each well on the top surface of the hydrogel without disturbing the hydrogel).

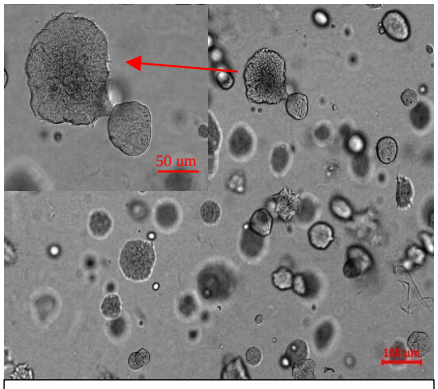
6. To feed the cells, change the medium very gently by replacing 1/3 - 2/3 of medium above the gel with fresh medium every day or every other day, depending on how often the cells need to be fed or how soon the cell medium color change to yellowish.
7. PG-S supports naturally formed spheroids directly from the single cell in 3D environment. Cells develop their own spheroids as they desire. Size and tightness of the spheroids are also regulated by PG-S gel strength, initial cell seeding density and duration of cell growth phase (i.e., Head and Neck cancer cell, **Figure 1** and **Table 3**).

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).

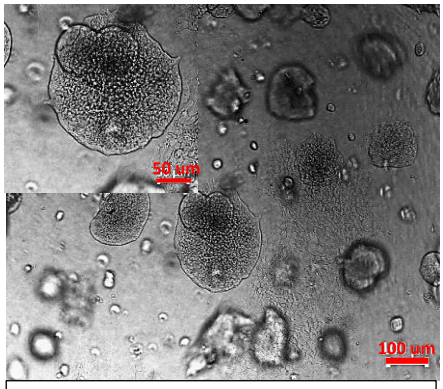
Figure 1 Examples of Spheroid culturing in 3D PG-S – Head and Neck Cancer cells



Head and Neck cancer cells, 10000 cell /well in 24well-plate, cultured in 0.5% PG-S, Day 5.



Head and Neck cancer cells, 10000 cell /well in 24well-plate, cultured in 0.5% PG-S, Day 7.



Head and Neck cancer cells, 10000 cell /well in 24well-plate, cultured in 1% PG-S, Day 10.

Table 3. Spheroid Size of Head and Neck Cancer Cell culturing in 3D PG-S (Gel strength, seeding density, duration of cell growth phase)

PG-S Gel Concentration (w/v)	Seeding density (cell/mL)	Duration of cell growth phase		
		Day 4	Day 8	Day 10
0.5% PG-S	4×10^4	20-30	40-80	40-80
	1×10^4 -2×10^4	20-30	40-100	50-150
	4×10^3	20-30	30-80	50-150
1% PG-S	1×10^5	20-30	40-80	40-100
	4×10^4	20-30	50-100	50-200

II THAWING CELLS DIRECTLY IN 3D PG-S*

1. Bring the PG-S 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 PG-S.

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

III SPHEROID 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 spheroid 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 is listed in **Table 4** on Page 11).
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 µL for 96-well plate or 1000 µL for 24-well plate).
3. **Gel dilution:** Pipette the mixture **THOROUGHLY** and add additional DPBS to further dilute the mixture by 20-25 folds of the original plating volume (See examples in **Table 4** on Page 11) and mix well.
4. **Centrifuge:** Centrifuge at 500 g-800 g for 5-6 min, depending on cell. Discard supernatant and collect the spheroid pellet. (*** Most spheroid precipitate at very bottom of centrifuge tube, you may also observe a visible thick layer of cloudy gel at bottom but above the spheroids. In this case you need to dilute the cloudy part 20-25 folds of original plating volume by adding DPBS, then centrifuge to remove gel and collect spheroids)
5. **Spheroids size distribution and separation:** Cell strainer with porosities of 40 µm, 70 µm and 100 µm or flow cytometry can be used to sort cells based on various size for downstream applications.

Note:
1. Centrifuge force for spheroid harvesting from 3D PG-S should be higher than conventional 2D cell harvesting. 500 g-800 g is the typical centrifuge force range to harvest spheroids from PG-S.
2. Always check if there are still spheroids remaining in the supernatant after FIRST centrifuge by using microscope, then you may increase the centrifuge force or time accordingly for further cell isolation.

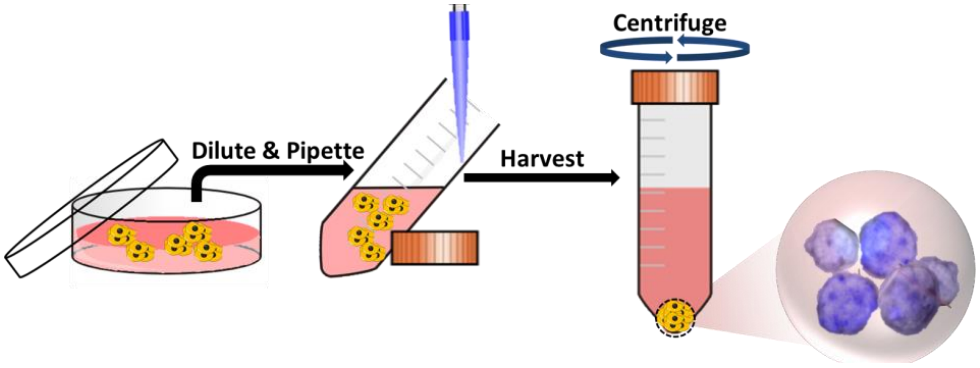


Table 4*: The recommended conical centrifuge tube size vs well plate size for gel dilution

	6-well	12-well	24-well	48-well	96-well
Gel plating volume	2000 µL	1000 µL	500 µL	250 µL	100 µL
Final volume of diluted gel and cell mixture from one well (20-25 folds)	40-50 mL	20-25 mL	10-15 mL	5-7.5mL	2-3 mL
Suggested conical tube size	50 mL	50 mL	15 mL	15 mL	2 or 15 mL

*** The conical tube size suggested here is only good for one well cultured cell 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 15 mL).**

6. **Spheroid breakup:** To break the spheroids, follow your conventional culture protocol, add enzyme solution or other dissociation agents, or follow procedures described below.

   – To break the spheroids, 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 µL for 96-well plate or 500 µL for 24-well plate).
   – Mix gently to disrupt the spheroid pellet, then incubate the conical tubes at 37°C (5% CO_2) for 5 min.
   – After incubation, pipet the cell solution up and down to mechanically break up the spheroids. If there are still cell clusters observed, continue to incubate the cell solution at 37°C (5% CO_2) for another 2-3 min and pipet, until no cell clusters 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 spheroids disruption depend on the cell types, spheroid 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 spheroids breakup.

IV CELL CRYOPRESERVATION

1. Resuspend cell pellet in freezing medium or complete growth medium with 5%-10% DMSO to a concentration of 1 x 10^6 to 1 x 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 °C/min. This can be achieved by using a programmable cooler or using Mr. Frosty Freezing container with isopropanol placed in a -80 °C freezer for at least 24 hours.
3. Quickly transfer the vial to liquid nitrogen or -130 °C freezer for long term storage.

V IMMUNO-STAINING

Spheroids can be harvested from PG-S gel for staining or can be stained directly in the gel. For staining cells in the PG-S gel, the procedure below is recommended (i.e., hiPSC cells)

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 µg/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 lgG (H+L) 2^{nd} antibody

alexa fluor 488 with final concentration of 5 µg/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. Table 5. Reference Information of 3D Spheroid Culture in PG-S

Cells	PGmatrix and Gel concentration (%)	Gelation time (min)	Cell seeding density (Cell/ml)	Spheroid size	Proliferation (times)	Cell viability (%)
MCF-7 (ATCC)	0.2-0.3% PG-S	60	2.8 x10^5	30-80	4	90
Hela cell	0.5-1% PG-S	60	8 x10^4	50-100	5-6	90-95
Head Neck cell	0.5-1% PG-S,	30	4 x10^4	50-200	15-20	90-95
hiPSC derived from fibroblast (Applied Stem Cell)	0.5% PGD-hiPSC (with PGgrow-hiPSC**)	30	1.8 x10^5 - 2 x10^5	50-100	10	80-90
Episomal hiPSC (Thermofisher)	0.5% PGD-hiPSC (with PGgrow-hiPSC**)	30	2 x10^5 - 3 x10^5	50-100	7-9	75-80
AD MSC (ATCC)* **	0.5% PGD-S	30	7x10^5	40-80	--	90

(**PGmatrix-spheroid with various basal medium would be also available upon request, i.e., PG-S = PGmatrix-spheroid peptide nanofiber solution without medium; PGD-S = PGmatrix DMEM-spheroid; PGR-S = PGmatrix RPMI-spheroid)**

* ***Note:*** Results reported here are from 48-12 well plate under culturing condition at 37°C and 5% CO_2, and can only be used as reference. It is users' responsibility to use appropriate cell medium and growth supplement or consult with PepGel.

** **PGD-hiPSC=PGmatrix DMEM-hiPSC; 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).

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