Home for Cells and Beyond

hiPSC-qualified PGmatrix™ Using Guides

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

PRODUCT:	PepGel hiPSC-qualified PGmatrix™ Research Kit
CONTENT:	PGmatrix-hiPSC solution, PGgrow solution and PGworks solution
QUANTITY:	20 mL of PGmatrix-hiPSC, 1 mL of PGworks and 500 µL of PGgrow
	10 mL of PGmatrix-hiPSC, 0.5 mL of PGworks and 250 µL of PGgrow
	6 mL of PGmatrix-hiPSC, 0.3 mL of PGworks and 150 µL of PGgrow
	2 mL of PGmatriax-hiPSC, 0.3 mL of PGworks and 50 µL of PGgrow
STORAGE:	PGmatrix-hiPSC solution and PGworks solution should be stored at 4°C
	PGgrow should be stored at -20 °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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Table of Contents

First Time User Messages……………………………………………………………3

Message I: Mixing Ratio Notice……………………………………………………………3

Message II: Medium Volume on Top of Gel……………………………………………4

Message III: Examples of Cell Density and Gel Concentration…………………4

Protocols for 3D hiPSC Culture…………………………………………………….5

A) Cell Encapsulation and Culture…………………………………………………………5

I. Encapsulate Cells from Subculture……………………………………………5-6

II. Thawing Cells in 3D PGmatrix-hiPSC…………………………………………6

B) Spheroid Colony Recovery……………………………………………………………...7-9

C) hiPSC Cryopreservation……………………………………………………………………9

D) Immuno-Staining …………………………………………………………………..10-11

Appendix: Reagents Used for 3D hiPSC Culture……………………………..12

References…………………………………………………………………………………..13

FOR FIRST TIME USER, PLEASE READ THE FOLLOWING THREE MESSAGES

MESSAGE I: Mixing Ratio Notice

The PGmatrix-hiPSC solution (PGmatrix) contains 1% W/V standard peptides. If you are first time user, we recommend using a few mixing ratios in the range of 0.3-0.6% W/V final peptide concentration for hiPSC encapsulation to identify the best mixing ratio for your cells. The following **Table 1** presents two mixing ratios at 0.3% 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.**

Well Plate Size	0.3% 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)*	
6	1360	40	600	960	40	1000	2000
12	680	20	300	480	20	500	1000
24	340	10	150	240	10	250	500
48	170	5	75	120	5	125	250
96	68	2	30	48	2	50	100

NOTE: *Pgworks is always 2% of the total volume of PGmatrix + cell suspension together.
**** Nontreated culture plate is recommended for 3D cell culturing in PGmatrix. For hiPSC culture, 0.5% is suitable for all sizes listed for mTeSR based medium, while 0.3% can be used for 48-well and 96-well plates or E8 medium.**

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 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 recommend volume of medium to add to each well based on different well sizes.

Table 2: **The recommend 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 - 5000	2000 – 3000	1000-1500	500 – 700	200 – 300

MESSAGE III: Examples of Cell Density and Gel Concentration

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

Cells	PGmatrix-hiPSC	Gel concentration (%)***	Gelation time (min)	Cell seeding density (Cell/mL)	Cell medium
hiPSC derived from Fibroblast (Applied Stem Cell)	PGmatrix DMEM-hiPSC	0.5	30	(2-3) x10^5	mTeSR™1 complete medium + PGgrow**
Episomal hiPSC (Thermofisher)	PGmatrix DMEM-hiPSC	0.5	30	(2.5 -3.5) x10^5	mTeSR™1 complete medium + PGgrow**

Note: Results reported here are from 48-6 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.

**** PGgrow is optional but highly recommended for hiPSC culturing in PGmatrix**. Without PGgrow, it is users' responsibility to choose appropriate growth supplement.

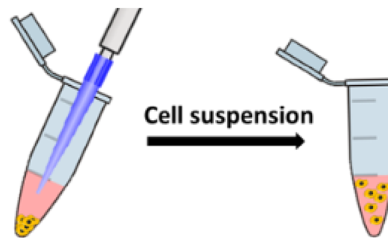
*** For E8 medium, 0.3% gel concentration is recommended.

Protocols for 3D hiPSC Culture

A) CELL ENCAPSULATION AND CULTURE

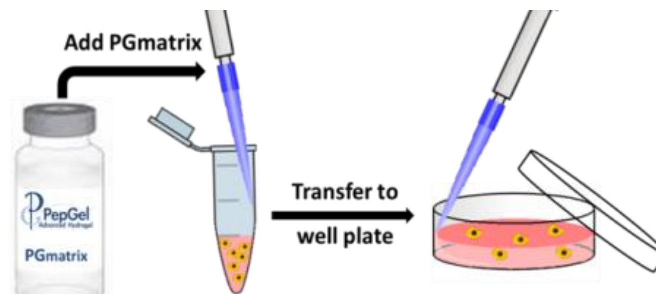
I. Encapsulate cells from subculture.

1. Bring the PGmatrix solution and PGworks solution to room temperature (15 – 25 °C) or 37 °C (37 °C water bath).
2. To prepare cell medium stock solution, thaw PGgrow* and add it into mTeSR1 complete medium at ratio 1:1000 v/v (PGgrow : mTeSR1 complete medium).
3. Suspend cells in mTeSR1 complete medium supplemented with PGgrow, 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).



4. Mix the PGmatrix solution carefully into the cell suspension of step 3 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 3** on page 4.

   Note: Please contact PepGel for special medium requirement by email to customerservice@pepgel.com).



5. Incubate the plate at 37°C (5% CO_2) for 30 min or longer as needed to complete the gelation.

6. After gelation, add mTeSR1 complete medium supplemented with PGgrow 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**: Gently add cell culture medium along the wall of each well on the top surface without disturbing the hydrogel).
7. To feed the cells, change the medium very gently by adding or replacing part (2/3) of medium above the gel with fresh medium every day start from day 2 of each passage. (Tips, usually 60% of the medium is replaced at each time to avoid disruption gel surface).

*** PGgrow is used as a cell culture supplement, PGgrow should be diluted into mTeSR1 complete medium immediately before use (see step 2), and use within two weeks after dilution. Medium used for cell culture in this protocol are all supplemented with PGgrow.**

II. Thawing hiPSC *

1. Bring the PGmatrix-hiPSC solution and PGworks solution to room temperature (15 – 25 °C) or 37 °C (37 °C water bath).
2. To prepare cell medium stock solution, thaw PGgrow and add it into mTeSR1 complete medium at ratio 1:1000 v/v (PGgrow : mTeSR1 complete medium).
3. Thaw the vial with frozen hiPSC by gently agitating in a 37 °C water bath. To reduce the possibility of contamination, keep the O-ring and cap out of the water.
4. Remove the vial from the water bath as soon as the contents start to thaw. Pre-warm mTeSR1 complete medium to room temperature and add 1 mL to the vial and pipette until the cell suspension is totally thawed.
5. Transfer the cell suspension to a 15 mL conical tube, and use another 1 mL of mTeSR1 complete medium to rinse the vial for the remaining cells, and combine the solution to the conical tube.
6. Dilute the mixture to 10 mL by using mTeSR1 complete medium. And centrifuge at 200 g for 5 min.
7. Remove the supernatant and resuspend the cell pellet gently in mTeSR1 complete medium supplemented with PGgrow (the medium stock solution from step 2).
8. Follow the steps 3-7 in section I **“Encapsulate cells from subculture”** for the hiPSC encapsulation in 3D PGmatrix-hiPSC.

* **The recommended hiPSC seeding density from thawing in hiPSC-qualified PGmatrix can be higher (i.e. 3-4 x10^5 cell/mL) than encapsulation cells from subculture in order to maintain good cell viability and proliferation.**

B) hiPSC RECOVERY FOR PASSAGING, COUNTING AND DOWNSTREAM APPLICATIONS (A Quick Example in #6 below for new users)

1. **Gel disruption:** First remove about 1/3 of the culture medium to a conical centrifuge tube A, then mechanically disrupt the gel **THOROUGHLY** by pipetting the mixture of gel and the remaining medium (**gel disruption thoroughly is very important for the cell isolation from the gel), then transfer the mixture to conical centrifuge tube A (recommended conical centrifuge tube size vs well-plate size are listed in **Table 4**).
2. **Rinse the well:** Use Dulbecco's Phosphate-Buffered Saline (DPBS, without Mg^{2+} / Ca^{2+}) to rinse the well and combine the solution to the centrifuge tube A. Recommend using DPBS volume as double the maximum plating volume for each well listed in Table 1 (i.e., 200 µL DPBS for 96-well plate or 1000 µL DPBS for 24-well plate to rinse the well).
3. **Gel dilution:** Pipette the mixture in tube A **THOROUGHLY** and add additional DPBS to further dilute the mixture by 20-25 folds of the original plating volume (see examples in **Table 4**) and mix well.
4. **Centrifuge:** Centrifuge at 200-400 g for 5-6 min using swing bucket centrifuge. Longer time and higher centrifuge force are used for large sample size such as 50 ml. Discard supernatant and collect the cell pellet (**Tips:** when approaching the bottom of tube A, use 1 ml pipette to gently remove the supernatant without disturbing the cell pellet).

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
Total plating volume per well	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	10-12.5 mL	5-6.5 mL	2-2.5 mL
Suggested conical tube size	50 mL**	50 mL	50 mL ***	15 mL	5 mL

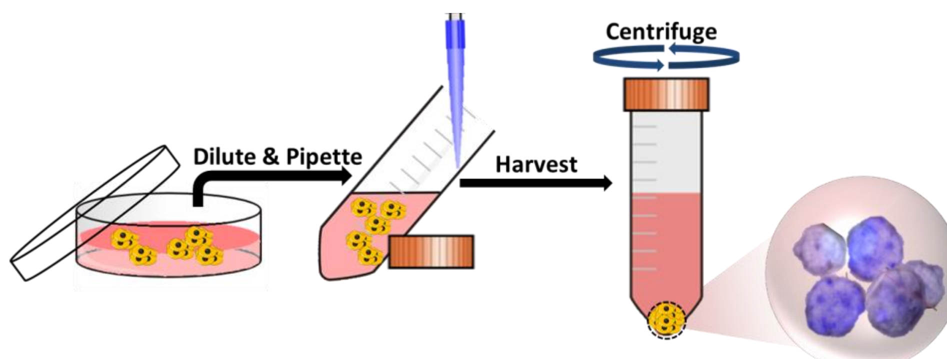
*** The conical tube size suggested here is only good for one well cultured cell harvesting. If more than one well cultured cells are harvested at the same time, the tube size need to be larger accordingly. For example, 50 mL tube can be used for harvesting no more than 3 wells of 24-well plate at the same time (3 x 12.5 mL=37.5 mL).**

**** For better pipetting result, two of 50 mL tubes are recommended for one well of 6-well plate cell harvesting, each tube can hold equally 20-25 mL of cell and gel mixture.**

*****50 mL tube is recommended for better pipetting result, then the mixture will be transferred to one 15 ml conical tube for centrifuge in order to easily collect hiPSC pellet.**

5. **hiPSC spheroid colony breakup (dissociation)**
   - To break the hiPSC spheroid colony, add TrypLE™ Express Enzyme (1X) to the spheroids pellet, and the volume depends on the spheroids amount and size.
     For example, hiPSC cell pellet harvested from 1 wells of 24-well plate at Day 5 with seeding density 2 x10^5 cell/mL needs 1 mL TrypLE Express Enzyme (1X) to break up the spheroids, and final single hiPSC amount is 1-1.5 x10^6 cells.
   - Mix gently to disrupt the cell spheroids pellet, then incubate at 37°C (5% CO_2) for 10 -20 min. The incubation time may change accordingly, depending on the hiPSC spheroids size.
     Tips: At around 10 min of incubation, pipet the spheroids and TrypLE Express Enzyme (1X) solution mixture gently to help breaking the spheroids, then observe the cell cluster size under the microscope to determine if it needs to extend incubation time. If the majority of the cells (90%) become single cells, then it is ready for next step. If you prefer larger cell cluster for passage, you can shorten incubation time as needed.
   - After incubation, add mTeSR1 complete medium equal to half volume of TrypLE Express Enzyme (1X) solution used to each tube (i.e., 1-1.5 x10^6 cells need 0.5 mL mTeSR1), centrifuge at 200g for 5 min.
   - Discard supernatant and re-suspend the pellet in mTeSR1 complete medium supplemented with PGgrow for further cell counting or cell passage.

Note: TrypLE Express Enzyme (1X) is highly recommended for hiPSC spheroids dissociation. Usually, the dissociated hiPSC (single and small clusters) has viability above 95%, compared to 80-85% if 0.05 mM EDTA is used for hiPSC spheroids dissociation.



6. **Quick Example for cell recovery using 24-well**

hiPSC seeding density is 2 x 10^5 cell/ml (seeding number is 1 x 10^5 cell/well), hydrogel (encapsulated with cells in it) plating volume is 500 µl/well with 1500 µl culture medium on top, and cultured for 5 days.

Gel disruption: First remove 500 µl of the culture medium to a 50 ml conical centrifuge tube A, then mechanically disrupt the gel **THOROUGHLY** by pipetting the mixture of gel and the remaining medium (** gel disruption thoroughly is very important for the cell isolation from the gel), then transfer the mixture to the 50 ml conical centrifuge tube A.

Rinse the well: Use 1000 µl Dulbecco's Phosphate-Buffered Saline (DPBS, without Mg^{2+} / Ca^{2+}) to rinse the well and combine the solution to the 50 ml centrifuge tube A, and mix thoroughly.

Gel dilution: add additional 10-15 ml DPBS to further dilute the mixture, and mix well.

Centrifuge: Transfer the mixture into a 15 ml conical centrifuge tube and centrifuge at 200 g – 400 g for 5 min using swing bucket centrifuge. Discard supernatant and collect the spheroid pellet for further application (**Tips:** when approaching the bottom of tube A, use 1 ml pipette to gently remove the remaining supernatant without disturbing the cell pellet), or break the spheroid into smaller cluster or single cells following the procedure in #5 above.

C) hiPSC CRYOPRESERVATION

1. Resuspend hiPSC pellet in ESC-Sure™ Human ESC Freezing Medium or complete growth medium with 5%-10% DMSO to a concentration of 1 x 10^6 to 5 x 10^6 cells/mL. 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.

D) 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 more 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 is used for 2D culture staining. Typically, dilute primary antibody with washing buffer at ratio 1:100 [reference 9]. (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)[9].

   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 that that is used for 2D culture staining. Typically, using the concentration

recommended by vendor or determined empirically [reference 9]. (i.e., Rabbit anti-goat lgG (H+L) 2nd antibody alexa fluor 488 with final concentration of 5 µg/mL was used for hiPSC staining directly in 3D PGmatrix).

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

<u>Note:</u> 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.

Appendix

Table 5: Reagents used for 3D hiPSC culture in PGmatrix-hiPSC and immune-staining for the reference data provided in this guidance

Reagents	Catalog #	Suppliers
Human induced pluripotent stem cell (hiPSC)	ASE-9203	Applied Stem Cell
	A18945	Thermofisher
mTeSR™1 / mTeSR™1 Plus medium	05850 / 05825	Stem Cell Technology
E8	A1517001	Thermofisher
Dulbecco's Phosphate-Buffered Saline (DPBS)	D8537	Sigma-Aldrich
TrypLE™ Express Enzyme (1X)	12604021	Thermal Scientific Fisher
EDTA	IB70185	MidSci
10% neutral buffered formalin	23-305510	Fisher Scientific
Triton X-100	T8787	Sigma-Aldrich
Cold water fish gelatin (Teleostean Gelatin)	G7765	Sigma-Aldrich
Normal rabbit serum control	31883	Thermo Fisher Scientific
Goat Oct 3/4 antibody (N-19) 100 µg/mL	sc-8628	Santa Cruz Biotechnology
Rabbit anti-goat lgG(H+L) 2nd antibody alexa fluor 488	A-11078	Thermo Fisher Scientific
ESC-Sure™ Human ESC Freezing Medium	ASM-5004	Applied Stem Cell
Mr. Frosty™ Freezing container	5100-0001	Thermo Fisher Scientific

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