PepGel

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 solution***. 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, PGworks solution, PGgrow-hiPSC solution.
QUANTITY:	PGmatrix-hiPSC (2, 6, 10, or 20ml), PGworks (0.3, 0.3, 0.5, or 1ml), PGgrow-hiPSC (50,150,250, or 500 µL).
STORAGE:	PGmatrix-hiPSC solution and PGworks solution should be stored at 4°C PGgrow-hiPSC 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.

CONTACT: PepGel LLC

101 BIVAP Bldg.
1980 Kimball Ave
Manhattan, KS 66506
E-mail: info@pepgel.com
Phone: 785-226-6917
Fax: 785-532-7193
Web: www.pepgel.com

To Order: customerservice@pepgel.com, or online www.pepgel.com

PepGel

Home for Cells and Beyond

Table of Contents

1. 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	40.8	1.2	18	28.8	1.2	30	60

NOTE: *PGworks is always 2% of the total volume of PGmatrix + cell suspension + PGworks 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. **Table 2** presents the recommend initial volume of medium added on top of gel for different sizes of well plate.

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

	6-well*	12-well	24-well	48-well	96-well**
Plating hydrogel volume (µL)	2000	1000	500	250	60
Initial medium added on top of gel (µL)	6000	3000	1500	750	180

*6-well is not recommended for hiPSC 3D embedded culture; PGmatrix3D-Suspension kit is a better option for large scale hiPSC production with 6-well plate
**96-well is not usually recommended for hiPSC 3D culture

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-hiPSC	0.5	30	(2-3) x10^5	mTeSR™1 complete medium + PGgrow
Episomal hiPSC (Thermofisher)	PGmatrix-hiPSC	0.5	30	(2.5 -3.5) x10^5	mTeSR™1 complete medium + PGgrow

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

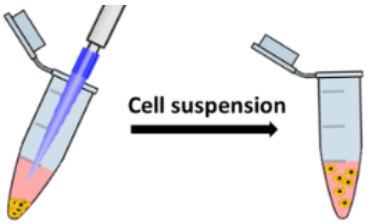
2. Protocols for 3D hiPSC Culture

A) CELL ENCAPSULATION AND CULTURE (cell performance given in section 2C.1)

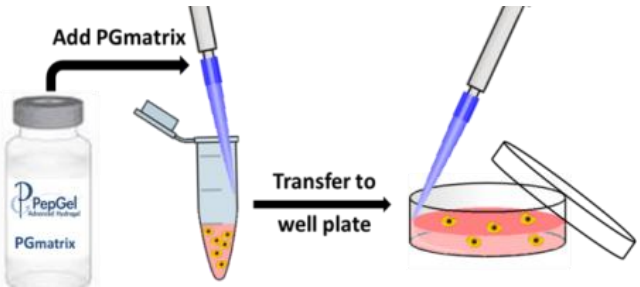
I. Encapsulate cells from subculture (2D or 3D) or cryopreserved hiPSC.

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-hiPSC (PGgrow)* and add it into mTeSR1 complete medium at ratio 1:1000 v/v (PGgrow : mTeSR1 complete medium)

   * **PGgrow** is used as a cell culture supplement, PGgrow should be diluted into mTeSR1 complete medium immediately before use and use within two weeks after dilution. Medium used for cell culture in this protocol are all supplemented with PGgrow.
   ** **ROCK Inhibitor** is not necessary for 3D hiPSC culture using PGmatrix-hiPSC kit.
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 (***Tips: Fully expel air from pipet before immersing pipet tip in cell solution, and keep 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.



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 feed cells and prevent the matrix from drying (recommended initial medium volume of each well vs plating volume 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. Feeding cells, medium feeding volume vs well plate size can follow **Table 4** as below:
   - **On day 0:** see step 6 above.
   - **On day 1 and day 2:** no medium change is needed because initial medium feeding is enough to support cell growth.
   - **On day 3 and day 4:** Gently remove about 2/3 medium above gel, then feed fresh medium along the wall of well plate slowly following the recommended feeding volume in **Table 4** below.
   - **hiPSCs are usually harvested on day 5.**

Table 4: The recommend medium feeding volume for different size of well plate

Well plate	Day 0	Day 1, 2	Day 3		Day 4	
	Initial medium added on top of gel (µL)		Medium removal above gel (µL)	Fresh medium (µL)	Medium removal above gel (µL)	Fresh medium (µL)
6-well	6000	No medium change	4000	4000	4000	6000
12-well	3000		2000	2000	2000	3000
24-well	1500		1000	1000	1000	1500
48-well	750		600	600	600	700
96-well	180		100	100	100	150

Notes: * hiPSCs are usually harvested on day 5 with 10-15 growth rate, if customer prefers to harvest on day 4 (96 hr or few hours over of cell culture), it recommends feeding cells with more medium on day 3. For day 6 harvesting, cells can be fed twice on day 5 due to the fast cell growth.

** The feeding volume from Table 4 can be used as reference, there are several factors including cell lines, cell seeding density, culture passages and culture duration which can affect hiPSC growth, therefore, it is user's responsibility to feed cells as needed; culture medium color can be used as indicator to determine how often to feed cells.

II. Thawing hiPSC *

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, follow the same procedure in Section 2. A-I #2.
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 above water surface.
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 2. A-I "Encapsulate cells from subculture (2D or 3D) or cryopreserved hiPSC"** for the hiPSC encapsulation and culture 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 SPHEROID RECOVERY FOR PASSAGING, COUNTING AND DOWNSTREAM APPLICATIONS (a Quick Example is given in section 2.C.2 for new users)

1. **Gel disruption:** First transfer about 1/3 of the culture medium to a conical centrifuge tube A, then mechanically disrupt the gel **GENTLY** and **THOROUGHLY** by pipetting the mixture of gel and the remaining medium a few times (**6-8 times**), then transfer the mixture to conical centrifuge tube A (recommended conical centrifuge tube size vs well-plate size are listed in **Table 5**).

   Notes: * Gel disruption **thoroughly** is very important for cell isolation from the gel. Follow the steps below:

   * 1). Slightly tilt the well plate; 2). Be sure to expel air from the pipette; 3). Immerse pipette tip into the gel but not touch the well plate bottom; 4). Aspirate the mixture of gel and medium slowly and then dispense along the top wall of the well plate for better gel disruption results; 5). Repeat steps 3 and 4 for **6-8 times**. * Avoid air bubbles during

pipetting. More tips can be also found in Section 4 for FAQs.

2. **Rinse the well:** Use PBS or DPBS (without Mg^{2+} / Ca^{2+}) to rinse the well and combine the solution to the centrifuge tube A, mix thoroughly (**3-4 times** pipetting). Recommend using PBS/DPBS volume as double the maximum plating volume for each well listed in Table 1 (i.e., 200 µL PBS for 96-well plate or 1000 µL PBS for 24-well plate to rinse the well).
3. **Gel dilution:** Then add additional PBS or DPBS to further dilute the mixture by about 20 folds of the original plating volume (see examples in **Table 5**) and mix well (**3-4 times** pipetting).
4. **Centrifuge:** Centrifuge at **200-400 g for 5 min** using **swing bucket centrifuge**. 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 spheroid pellet).

Table 5*: 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	60 µL
Final volume of diluted gel and cell mixture from one well (20 folds)	40 mL	20 mL	10 mL	5 mL	1.2 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 10 mL=30 mL).**

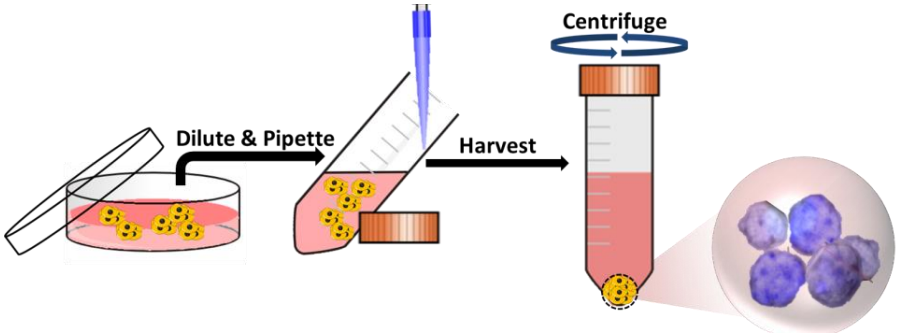
**** 50 mL tube is recommended for better pipetting result, then the mixture will be transferred to one 15 ml conical tube for centrifuge in order to easily collect hiPSC pellet.**

5. **hiPSC spheroid colony breakup (dissociation)**
   - **To** break the hiPSC spheroid colony, add TrypLE™ Express Enzyme (1X) to the spheroids pellet, and the TrypLE™ volume depends on the spheroids amount and size. ***For example,*** hiPSC cell pellet harvested from 1 wells of 24-well plate on Day 5 with seeding density 2 $x10^5$ cell/mL, and about 10 proliferation folds (final single hiPSC around 1-1.5 $x10^6$ cells), needs 1 mL TrypLE Express Enzyme (1X) solution to break up the spheroids,
   - **Mix** gently to disrupt the cell spheroids pellet, then incubate at 37°C 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), then centrifuge at 200 g for 5 min.
- **Discard** supernatant and re-suspend the pellet in mTeSR1 complete medium supplemented with PGgrow for further cell counting or cell passage. (i.e., one well of 24-well plate harvested by 5 days culture usually produces about 1-1.5 x10^6 cells, which can be re-suspended into 400-500 uL medium).

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.



C) GROWTH PERFORMANCE and RECOVERY OF HIPSC in PGmatrix-hiPSC

C.1. Culture condition (24-well plate for example)

- **Culture medium**: completed mTeSR medium supplemented with PGgrow
- **Seeding density:** 2 x 10^5 cells/mL (1 x 10^5 cells per well for 24-well plate)
- **Gel concentration:** 0.5% PGmatrix-hiPSC
- **Gel plating amount:** 500 uL per well for 24-well plate (2 mm gel thickness)
- **Cell feeding:** add 1.5mL medium above gel at day 0;

  replacing 1 mL of medium above gel with 1 mL of fresh medium at day 3;

  replacing 1 mL of medium above gel with 1.5 mL of fresh medium at day 4.

  [***Tips: DO NOT disturb the gel, therefore, slightly tilt the culture plate and aspirate the conditioned medium along the wall, keep the tip close to the medium surface; then add fresh medium drop by drop along the well wall***]

Figure 1. hiPSC presents physiological spheroid morphologies

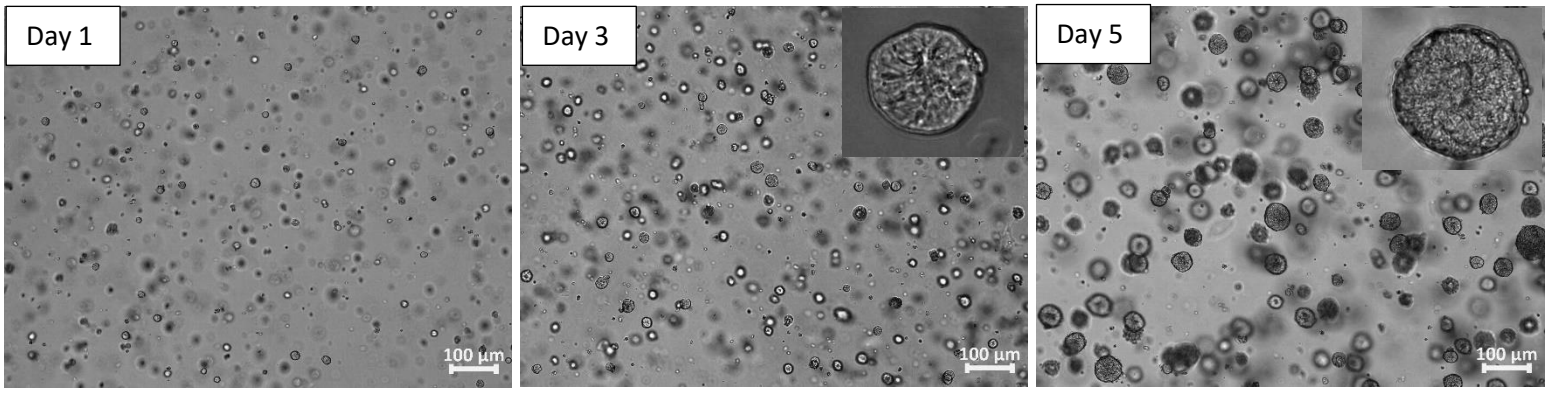


Table 6. hiPSC growth performance within 0.5%PGmatrix-hiPSC-24 well plate

Cell source	Viability	Cell amount harvested per well at day 5	Proliferation rate
hiPSC derived from fibroblast	95%-98%	1.0×10^6 - 1.5×10^6	10-15 folds

Note: Results reported here are under culturing condition of 37°C and 5% CO_2 for 24 well plate and 5 days culture duration, which can be used as reference. It is users' responsibility to select cell lines, culture medium and seeding density for 3D hiPSC growth or consult with PepGel.

C.2. Spheroid recovery using 24-well for example

hiPSC seeding density is 2×10^5 cell/mL (seeding number is 1×10^5 cell/well), hydrogel (encapsulated with cells in it) plating volume is 500 µL/well with 2000 µL culture medium on top, and cultured for 5 days.

Gel disruption: Transfer 1/3 (500-700 µL) of the old medium from the culture well to a 50 mL conical centrifuge tube A, then mechanically disrupt the gel **GENTLY** and **THOROUGHLY** by pipetting the mixture of gel and the remaining medium a few times (**6-8 times**), then transfer the mixture to conical centrifuge tube A (recommended conical centrifuge tube size vs well-plate size are listed in **Table 5**).

Notes: * Gel disruption **thoroughly** is very important for cell isolation from the gel. Follow the steps below:

* 1). Slightly tilt the well plate; 2). Be sure to expel air from the pipette; 3). Immerse pipette tip into the gel but not touch the well plate bottom; 4). Aspirate the mixture of gel and

medium slowly and then dispense along the top wall of the well plate for better gel disruption results; 5). Repeat steps 3 and 4 for **6-8 times**. * Avoid air bubbles during pipetting. More tips can be also found in Section 4 for FAQs.

Rinse the well: Use 1000 µL PBS/DPBS (without Mg^{2+} / Ca^{2+}) to rinse the well, and then combine the solution to the 50 ml centrifuge tube A, and mix thoroughly (**3-4 times** pipetting).

Gel dilution: add additional 7-8 mL PBS/DPBS to further dilute the mixture, and mix well (**3-4 times** pipetting).

Centrifuge: Transfer the mixture into a 15 mL conical centrifuge tube and centrifuge at **200 – 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, 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 Section 2B **#5** above.

D) hiPSC CRYOPRESERVATION

1. Resuspend hiPSC pellet (single cell or small clusters) 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.

E) 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

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 8]. (i.e., Rabbit anti-goat IgG (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

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.

3. Appendix

Table 7: 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
Phosphate buffered saline (PBS)	806552	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

4. FAQ

1. Poor cell performance

Poor cell performance or cell yield decrease can be caused by several reasons:

a. Cell lines – usually cell performance will be improved in 3D by passage 2 or 3.

b. Lack of nutrients – cell feeding frequency and amount can be varied depending on cell growth rate or days. Cells grow slow on day 1 and 2, but will go to exponential phase on day 3 and day 4. Please read protocol Section 2.A #7, Section 2.C-I, and Table 4 for cell feeding tips.

Notes: The feeding volume from Table 4 can be used as reference, there are several factors including cell lin*es*, cell seeding density, culture passages and culture duration which can affect hiPSC growth, therefore, it is user's responsibility to feed cells as needed; culture medium color can be used as indicator to determine how often to feed cells.

c. Lack of nutrients – can also be caused by plating volume per well for 3D embedded culture. Please follow the recommended plating volume in Tables 1 to ensure the gel thickness is within 2 mm for all types of well plate. For 3D large scale production, please use PGmatrix3D-Suspension kit.

d. Extra ROCK inhibitor (ROCKi) – ROCKi was formulated in the PGmatrix-hiPSC kit, therefore, no extra ROCKi is needed for 3D hiPSC culture.

e. Air bubbles generated in the gel – Depress the plunger to expel the air from pipette before immersing tip to gel; Tilt the culture plate at a small angle to comfortably view samples and pipetting; Depress and release the plunger slowly and smoothly to avoid uneven piston movement during pipetting.

f. Culturing condition – Regularly maintain incubator to make sure 5% CO_2 and 37 °C culturing condition for cell growth.

2. Cell harvesting

Several possible reasons contributing to the low viability for hiPSC and can be certainly improved.

a. Viability ~70% is expected for the first few passages (1-3) for 3D cultured hiPSC directly from 2D subculture, then viability should be in the range of 85%-96% depending on hiPSC sources, recovering process, skillfulness, etc.

b. hiPSC sources could be a major reason, for example hiPSCs derived from cord blood (i.e., hiPSC from Thermo Fisher) always has a few % (~5%) lower viability than the hiPSC from fibroblast (i.e., from Applied Stemcell), which should be improved by optimizing the 3D conditions.

c. Recovering process effects on cell viability: gentle vs surly pipetting, longer vs shorter recovering process, low vs high centrifuge force, using medium instead of PBS for recovering process should improve viability particularly for the first few passages in the transition from 2D to 3D cultures.

d. In hiPSC spheroid pellet breakdown for passaging, using EDTA always has lower viability (~10%) than using 1X TrypLE, enzyme concentration and trypsinization time could be another factor, it can be fine-tuned (i.e., lower enzyme concentration or shorter treatment) for hiPSCs from different sources, for example, blood sources to maximize cell viability.

e. **cell harvesting tips** -- 1). Tilt the well plate a little; 2). Be sure to expel air from the pipette; 3). Immerse pipette tip into the gel but not touch the well plate bottom; 4). Aspirate slowly the mixture of gel and medium and then dispense along the top wall of the well plate for better gel disruption results; 5). Repeat steps 3 and 4 for 6-8 times. * Avoid air bubbles during pipetting.

f. **Cell harvesting either earlier or later than day 5** – hiPSCs are usually harvested on day 5 with 10-15 growth rate, if customer prefers to harvest on day 4 (96 hr or few hours over of cell culture), it recommends feeding cells with more medium on day 3. For day 6 harvesting, cells can be fed twice on day 5 due to the fast cell growth.

If all above are tried, and still low cell viability, please contact info@pepgel.com

5. REFERENCE

1. Soltantabar P, Calubaquib EL, Mostafavi E, Ghazavi A, Stefan MC. Heart/liver-on-a-chip as a model for the evaluation of cardiotoxicity induced by chemotherapies. Organs-on-a-Chip. 2021 Nov 1;3:100008.
2. Li Q, Qi G, Liu X, Bai J, Zhao J, Tang G, Zhang YS, Chen-Tsai R, Zhang M, Wang D, Zhang Y. Universal Peptide Hydrogel for Scalable Physiological Formation and Bioprinting of 3D Spheroids from Human Induced Pluripotent Stem Cells. Advanced Functional Materials. 2021:2104046.
3. Carter T, Qi G, Wang W, Nguyen A, Cheng N, Ju YM, Lee SJ, Yoo J, Atala A, Sun XS. Self-Assembling Peptide Solution Accelerates Hemostasis. Advances in Wound Care. 2020. (https://www.liebertpub.com/doi/abs/10.1089/wound.2019.1109)
4. Harrison R, Luckett J, Marsh S, Lugo Leija HA, Salih S, Alkharji R, Sottile V. Magnetically Assisted Control of Stem Cells Applied in 2D, 3D and In Situ Models of Cell Migration. Molecules. 2019 Jan;24(8):1563.
5. Zhu Q, Hamilton M, Vasquez B, He M. 3D-printing enabled micro-assembly of a microfluidic electroporation system for 3D tissue engineering. Lab on a Chip.

2019;19(14):2362-72.
6. Thippabhotla S, Zhong C, He M. 3D cell culture stimulates the secretion of in vivo like extracellular vesicles. Scientific reports. 2019 Sep 10;9(1):1-4.
7. Xu J, Qi G, Sui C, Wang W, Sun X. 3D h9e peptide hydrogel: An advanced three-dimensional cell culture system for anticancer prescreening of chemopreventive phenolic agents. Toxicology in Vitro. 2019 Dec 1;61:104599.
8. Li L, Liu G, Timashev P, Sun XS, Criswell T, Atala A, Zhang Y. Biofabrication of tissue-specific extracellular matrix proteins to enhance the expansion and differentiation of skeletal muscle progenitor cells. Applied Physics Reviews. 2019 Jun 26;6(2):021309.
9. Nethercott HE, Brick DJ, Schwartz PH. Immunocytochemical analysis of human pluripotent stem cells. Human Pluripotent Stem Cells: Methods and Protocols. 2011:201-20.
10. Liang J, Liu G, Wang J, Sun XS. Controlled release of BSA-linked cisplatin through a PepGel self-assembling peptide nanofiber hydrogel scaffold. Amino acids. 2017 Dec 1;49(12):2015-21.
11. Liang J, Sun XS, Yang Z, Cao S. Anticancer drug camptothecin test in 3D hydrogel networks with HeLa cells. Scientific reports. 2017 Feb 1;7(1):1-9.
12. Miller PG, Shuler ML. Design and demonstration of a pumpless 14 compartment microphysiological system. Biotechnology and bioengineering. 2016 Oct;113(10):2213-27.
13. Kumar D, Kandl C, Hamilton CD, Shnayder Y, Tsue TT, Kakarala K, Ledgerwood L, Sun X, Huang H, Girod D, Thomas SM. Anti-HGF antibody ficlatuzumab mitigates tumor-associated fibroblast-facilitated head and neck cancer progression. JAMA Otolaryngology. 2015;141(12):1133-9.
14. Kumar D, Kandl C, Hamilton CD, Shnayder Y, Tsue TT, Kakarala K, Ledgerwood L, Sun XS, Huang HJ, Girod D, Thomas SM. Mitigation of tumor-associated fibroblast-facilitated head and neck cancer progression with anti–hepatocyte growth factor antibody ficlatuzumab. JAMA Otolaryngology–Head & Neck Surgery. 2015 Dec 1;141(12):1133-9.
15. Li X, Galliher-Beckley A, Huang H, Sun X, Shi J. Peptide nanofiber hydrogel adjuvanted live virus vaccine enhances cross-protective immunity to porcine reproductive and respiratory syndrome virus. Vaccine. 2013 Sep 23;31(41):4508-15.
16. Huang H, Ding Y, Sun XS, Nguyen TA. Peptide hydrogelation and cell encapsulation for 3D culture of MCF-7 breast cancer cells. PloS one. 2013 Mar 20;8(3):e59482.
17. Huang H, Shi J, Laskin J, Liu Z, McVey DS, Sun XS. Design of a shear-thinning recoverable peptide hydrogel from native sequences and application for influenza H1N1 vaccine adjuvant. Soft Matter. 2011;7(19):8905-12.