TheWell BIOSCIENCE

3D Cell Culture and Beyond

VitroGel® STEM

Ready-to-use, synthetic, chemically defined hydrogel system for hPSCs 3D static suspension culture and scale-up

Protocol
Catalog #: VHM02, VHM02-S

Revision No: v2.0
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VitroGel® STEM

Catalog #: VHM02, VHM02S

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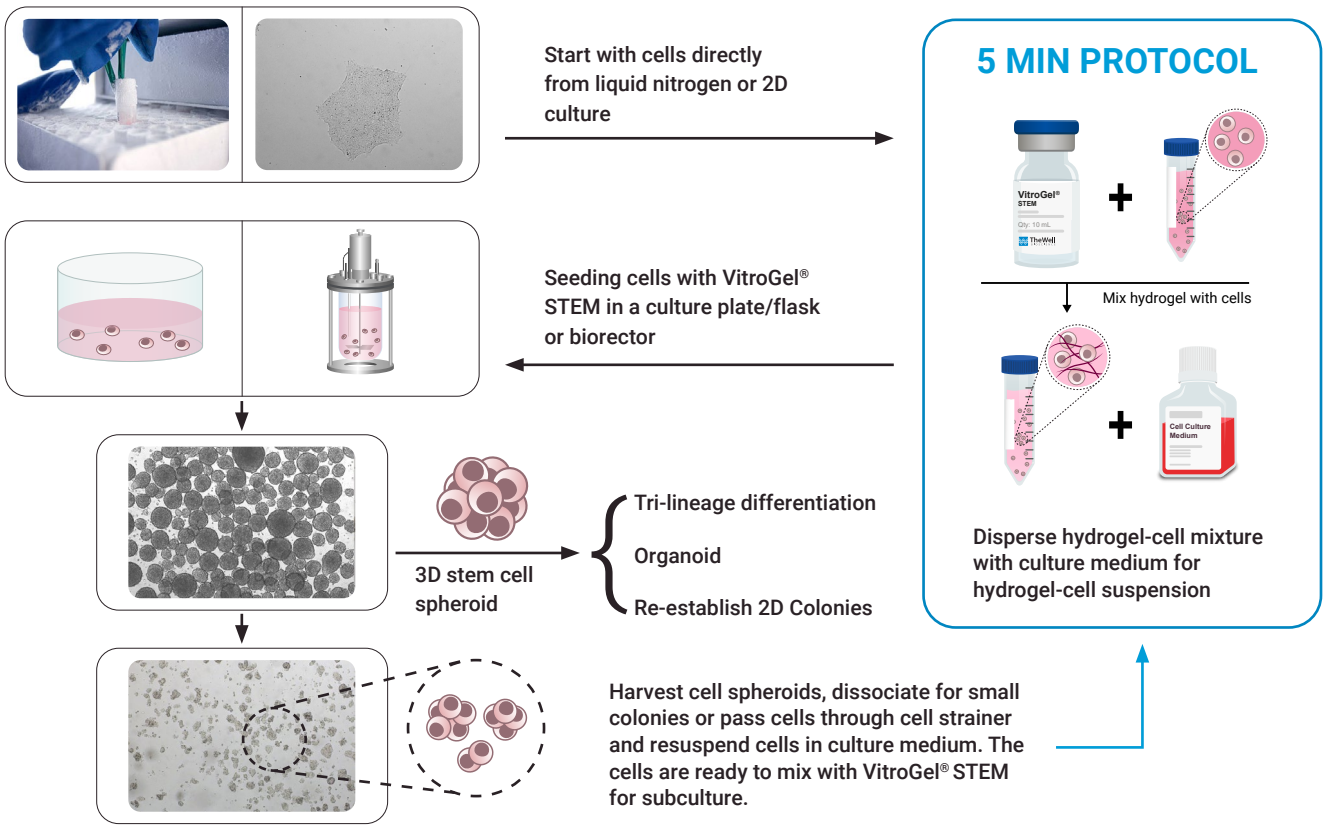
Stem Cell Static Suspension Culture Protocol

A. Introduction

VitroGel® STEM is a synthetic, chemically defined hydrogel system for improving the performance of three-dimensional (3D) cultures and scale-up of hPSCs populations to create a high-throughput system to model various issue and disease states. VitroGel® STEM is ready-to-use with an optimized formulation that fully supports the rapid expansion of high-quality 3D stem cell spheroids with pluripotent properties. The hydrogel system can support 3D cell growth through different culture methods such as 3D hydrogel encapsulation, 2D thick hydrogel coating, hydrogel-cell bead, and static suspension culture.

In this protocol, we will focus on the static suspension culture for stem cell spheroid expansion and scale-up using VitroGel® STEM. The hPSCs directly thawed from liquid nitrogen or passaged from 2D matrix coated culture vessels can immediately be mixed with the hydrogel solution for static suspension cultures. Moreover, the optimization protocol is ideal for time-sensitive experiments, as it does not require excessive medium exchanges, which can ultimately save on time and materials. VitroGel® STEM is compatible with most hPSC culture media and issue culture vessels. Due to the unique static suspension culture protocol method, microcarriers is not required when used in large-scale bioreactors thus making the cell harvesting much simpler and efficient. The 3D stem cell spheroids that are produced using VitroGel® STEM can be used for further sub-culturing, patterned differentiating, or re-establishing 2D culture morphologies.

WORKFLOW OVERVIEW



B. Initial Static Suspension Culture Protocol

MATERIALS/ REAGENTS

- **VitroGel® STEM**
- Stem cell culture medium (mTeSR-PLUS, StemFlex, mTeSR-1, Essential 8, NutriStem hPSC, etc.)
- Y-27632 (10 mM/mL)
- VitroGel® Cell Recovery Solution (Catalog #: MS03-100) (optional)
- 40, 70, or 100 µm reversible strainer (optional)
- Cell dissociation solution (optional)
- Conical tubes (15 mL or 50 mL)
- Serological pipettes
- Cell culture vessels (well plate, T-flask, Erlenmeyer Flask)

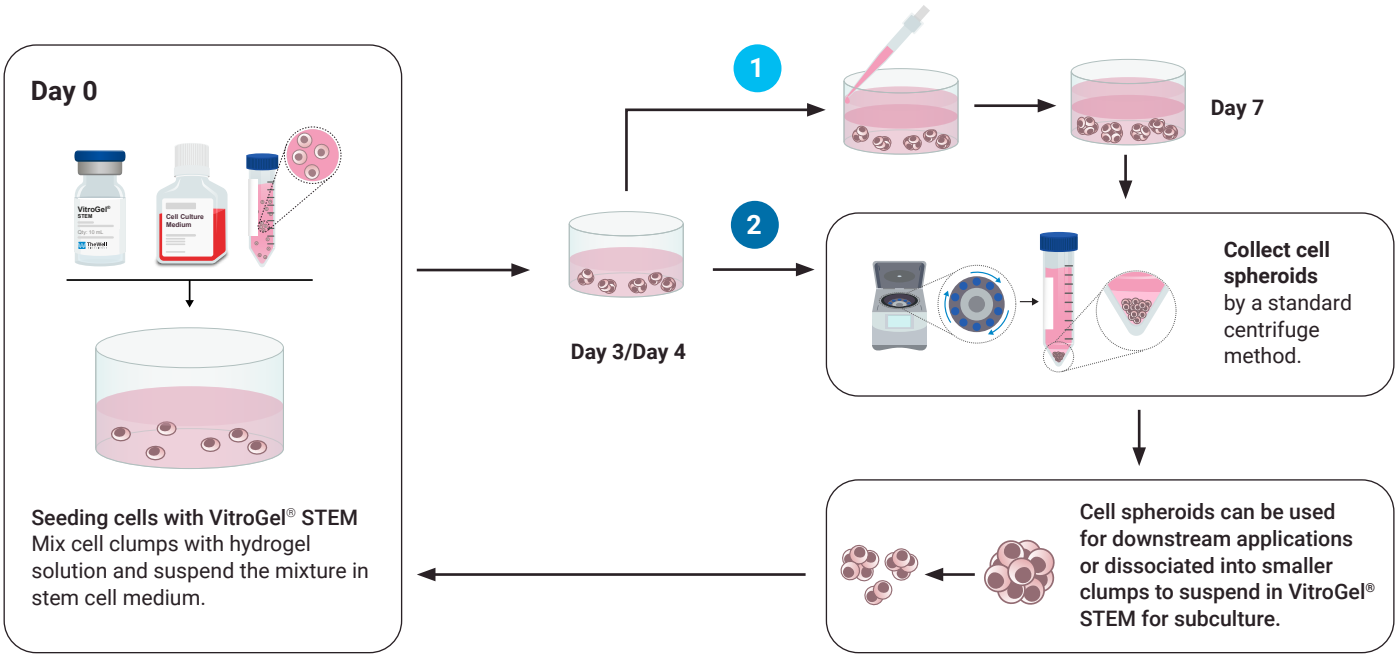
Note: VitroGel® STEM is compatible with a variety of culture vessels to grow stem cells in static suspension culture. Depending on the desired culture scale and the available systems in each laboratory, the culture volume may need to be optimized for individual cell types.

EQUIPMENT

- Biosafety cabinet (class II)
- CO_2 Incubator (37°C, 5% CO_2 and 95% humidity)
- Centrifuge
- Pipettors
- Orbital shaker, spinner flask or bioreactor at low speed 10-40 rpm (optional)

CULTURE CYCLE OF VITROGEL® STEM SYSTEM

1. 7-day culture cycle with additional medium on day 3/4
2. 3-day/4-day culture cycle without additonal medium



PROTOCOL

1. Harvest hPSC from the 2D matrix coating surface; or use cells directly from liquid nitrogen. (Centrifuge to get the cell pellet and remove the cell dissociation reagent or cell freezing solution).
2. Prepare cell clump suspension in the stem cell medium with 10 μm/mL Y-27632 Recommend cell density at 0.2-5 X 10^6 cells/mL for the final cell seeding density in the hydrogel suspension culture around 0.1-5 X 10^5 cells/mL.
    - If needed, break the clumps to 30-70 μm in size by carefully pipetting the clump suspension up and down. Single-cell suspension is not recommended.
    - Depending on the desired culture conditions and the final sizes of stem cell spheroid, the cell seeding density should be optimized for individual cell types.
3. Gently mix VitroGel® STEM with cell clump suspension at 2:1 v/v ratio (e.g., mix 2 mL VitroGel® STEM with 1 mL of cell clump suspension. Check Table 1 or Table 2 of the recommended volume of different culture vessels for different culture cycles.

Table 1. Recommend volume of for 7-day culture cycle

	Well Plate (Volume per well)			T-Flask			Erlenmayer Flask			
	96-well	**24-well**	**6-well**	**T-25**	**T-75**	**T-175**	**125 mL**	**250 mL**	**500 mL**	**1000 mL**
VitroGel® STEM	12 μL	60 μL	200 μL	400 μL	1.2 mL	4 mL	6 mL	12 mL	24 mL	50 mL
Cell clump suspension	6 μL	30 μL	100 μL	200 μL	600 μL	2 mL	3 mL	6 mL	12 mL	25 mL
Stem cell medium	90 μL	450 μL	1.5 mL	3 mL	9 mL	30 mL	45 mL	90 mL	180 mL	375 mL
Initial Culture Volume	108 μL	540 μL	1.8 mL	3.6 mL	10.8 mL	30 mL	54 mL	108 mL	216 mL	450 mL
Aditional medium	108 μL	540 μL	1.8 mL	3.6 mL	10.8 mL	36 mL	54 mL	108 mL	216 mL	450 mL

Table 2. Recommend volume of for 3-day or 4-day culture cycle

	Well Plate (Volume per well)			T-Flask			Erlenmayer Flask			
	96-well	**24-well**	**6-well**	**T-25**	**T-75**	**T-175**	**125 mL**	**250 mL**	**500 mL**	**1000 mL**
VitroGel® STEM	20 μL	100 μL	400 μL	600 μL	1.8 mL	6 mL	12 mL	24 mL	48 mL	100 mL
Cell clump suspension	10 μL	50 μL	200 μL	300 μL	900 μL	3 mL	6 mL	12 mL	24 mL	50 mL
Stem cell medium	150 μL	750 μL	3 mL	4.5 mL	13.5 mL	45 mL	45 mL	180 mL	360 mL	750 mL
Initial Culture Volume	180 μL	900 μL	3.6 mL	5.4 mL	16.2 mL	54 mL	108 mL	216 mL	432 mL	900 mL

4. Add stem cell medium (optional: with 10 µm/mL Y-27632) to the cell-hydrogel mixture at 5:1 v/v ratio (e.g. mix 15 mL stem cell medium with 3 mL of cell-hydrogel mixture). Carefully pipette up and down to mix the medium and mixture homogeneously.
    - The mixing ratios of stem cell medium and cell-hydrogel mixture can be adjusted between 1:1 to 20:1 v/v ratio, depending on the desired viscosity of the final mixture and culture conditions. If the mixing ratio is higher than 5:1 v/v, an orbital shaker or spin flask may be required and set at a speed of 10-40 rpm to maintain the cell suspension.
5. Add the desired volume of the mixture to the culture vessel and incubate at 37°C with 5% CO_2.
    - Add additional medium for 7-day culture cycle: On day 3 or day 4, add the desired volume of stem cell medium (without Y-27632) directly to the culture vessel. Check table 2 for the recommended volume of additional medium for different culture vessels.

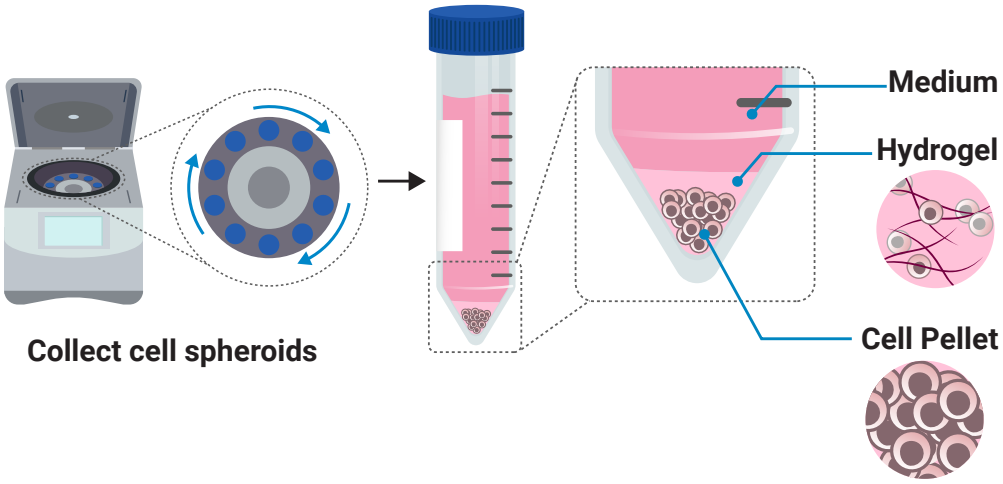
Note:

- The selection between 3-day /4-day culture cycle or 7-day culture cycle is depended on the cell seeding density and the desired conditions of stem cell spheroids. For high cell seeding density, 3-day/4-day cycle can make cell spheroids at sizes of 100-200 µm.
- Adding additional medium with a culture cycle is required whenever the culture medium color starts to turn yellow. (Add additional cell culture medium for 3-day or 4-day culture cycle may be required when the initial cell seeding density in hydrogel suspension is higher than 1 X 10^5 cells/mL).
- If additional culture medium is added more then one time within a culture cycle, an orbital shaker may be required at a speed of 10-40 rpm to maintain the cell suspension.

C. Harvesting Stem Cell Spheroids from VitroGel® STEM

1. Transfer hPSC spheroids from the culture vessel to a conical tube by using a serological pipette.
2. Centrifuge the tube for 3 minutes at 100 x g to collect the cell pellet.
    - Optimize the speed and time of centrifuge according to the experiment needs.
3. Carefully remove the supernatant to collect the cells.

Note: After centrifuge, the big cell spheroids will pellet at the bottom of the tube. There could be a layer of hydrogel on the top of the cell pellet, which contains some small cell spheroids or single cells (dead cells). See image below.



If only big cell spheroids will be harvested for downstream applications, carefully remove the supernatnat and hydrogel layer to collect the cell pellet.

To harvest cells from hydrogel layer with cell pellet together, please follow the steps below:

- Carefully remove the supernatant.
- Add VitroGel® Cell Recovery Solution (Catalog #: MS03-100) to the tube (warm the solution at 37°C before using; Keep the volumes of cell recovery solution and cell pellet/hydrogel mixture at 5:1 v/v ratio; e.g., 5 mL of cell recovery solutoon for 1 mL hydrogel-cell mixture,).
- Gently mix with a serological pipette and incubate at 37°C for 3-5 minutes.
- Centrifuge the tube for 3 minutes at 100 x g to collect the additional cell pellet.

D. Passaging: Stem Cell Spheroids Subcultured in VitroGel®

METHOD 1: Using cell dissociation solution

1. Collect cell pellet by centrifuge (check "Harvesting Stem Cell Spheroids from VitroGel® STEM" section for details).
2. Add cell dissociation solution to the tube to resuspens cells; incubate the mixture at 37°C for 3-5 min).
    - Please follow the instruction of manufacture of the cell dissociation solution to optimize the volume and time of incubation.
3. Centrifuge the tube for 3 minutes at 100 x g to collect the cell pellet.
4. Add the desired volume of stem cell medium (with 10 µm/mL Y-27632) to resuspend the cells. Use a pipettor with a 1 mL pipette tip, carefully pipette up and down to break up the clumps to 30-70 µm in size.
5. The cells are ready to mix with VitroGel® STEM for subculture. (Follow the protocol in the section of "Initial Static Suspension Culture").

METHOD 2: Using a cell strainer

1. Collect cell pellet by centrifuge (check "Harvesting stem cell Spheroids from VitroGel® STEM" section for details).
2. Add stem cell medium to the tube to resuspend cells.
3. Prepare a 40 or 70 µm strainer on a conical tube to dissociate hPSC spheroids into small clumps.
4. Transfer the cell spheroids from step 2 to a serological pipette and place the tip of the pipette directly contacting the sieve surface of the strainer without a gap. Force the cell spheroids to pass through the strainer at a low flow rate (0.5 mL/second) to generate small clumps for subsequent passage.
    - If the strainer appears clogged, increase the flow rate slightly or slide the pipette laterally on the strainer while maintaining direct contact.
5. To increase yield, rinse the strainer with an additional 1 - 5 mL stem cell medium.
6. Centrifuge the tube for 3 minutes at 100 x g to collect the cell pellet.
7. Gently aspirate the medium, leaving 0.5 mL to avoid removing any clumps.
8. Add the desired volume of stem cell medium (with 10 µm/mL Y-27632) to resuspend the cells.
9. The cells are ready to mix with VitroGel® STEM for subculture. (Follow the protocol in the section of "Intial Static Suspension Culture").

Appendix A: Other products for culture of stem cells

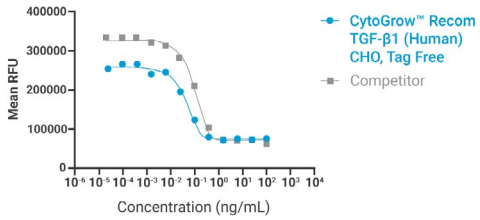
- **RocketCell™ 3D iPSC Complete Growth Kit, AOF** (Catalog #: RC02-CGK)
- **RocketCell™ iPSC Growth Medium, AOF** (Catalog #: RC02-GM)
- **CytoGrow™ Growth Factors** - over 100+ types across multiple species available - See Appendix B
- **RocketCell™ Supplements**
  - » RocketCell™ S1 Supplements (50X), AOF, (B27-like supplements)
  - » RocketCell™ S2 Supplements (100X), AOF, (N2-like supplement)
  - » RocketCell™ S3 Supplements (50X), AOF, (N2B27 like premixed supplement)
- **RocketCell™ Cell Viability Enhancer (1000X)**, (RC02-CV50)
- **VitroGel® Organoid Recovery Solution**
  - » 100 mL (Catalog # MS04-100)
  - » 500 mL (Catalog # MS04-500)
- **VitroGel® Cell Recovery Solution** (Catalog #: MS03-100)
- **VitroPrime™ Spread-Attach Plate**
  - » 6-Well (Catalog #: VP-SA6W)
  - » 12-Well (Catalog #: VP-SA12W)
  - » 24-Well (Catalog #: VP-SA24W)
  - » 48-Well (Catalog #: VP-SA48W)
  - » 96-Well (Catalog #: VP-SA96W)
- **VitroPrime™ 3D Culture and Imaging Plate**
  - » 6-Well (Catalog #: VP-3D6W5)
  - » 24-Well (Catalog #: VP-3D24W5)
  - » 96-Well (Catalog #: VP-3D96W5)
- **Cyto3D® Live-Dead Assay Kit** (Catalog #: BM01)

Appendix B: CytoGrow™ Growth Factors for Stem Cell Culture

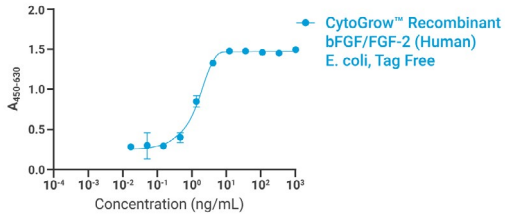
Growth Factor	Cat. No.	Species	Host cell
EGFs (Epidermal Growth Factors)			
EGF	CG014	Human	CHO
FGFs (Fibroblast Growth Factors)			
FGF-1/aFGF	CG090	Human	E. coli
FGF-2/bFGF	CG003	Human	E. coli
FGF-4	CG018	Human	CHO
Hematopoetic Stem Cell-Related			
EPO	CG015	Human	CHO
FLT-3L	CG026	Human	CHO
G-CSF	CG029	Human	HEK293
GM-CSF	CG027	Human	CHO
SCF	CG068	Human	CHO
TPO	CG079	Human	CHO
IGFs (Insulin-like Growth Factors)			
IGF-II	CG100	Human	HEK293
LR3 IGF-1	CG054	Human	CHO
NTs (Neurotrophins)			
CNTF	CG088	Human	E. coli
GDNF	CG092	Human	E. coli
Neurturin	CG103	Human	CHO
NT-3	CG105	Human	HEK293
Persephin	CG108	Human	E. coli
β-NGF	CG104	Human	HEK293
PDGFs (Platelet-Derived Growth Factors)			
PDGF-AA	CG063	Human	CHO
PDGF-AB	CG065	Human	CHO
PDGF-BB	CG0107	Human	CHO
TGFs (Transforming Growth Factors)			
Activin A	CG001	Human	CHO
BMP-2	CG006	Human	E. coli
BMP-7	CG007	Human	CHO
GDF-8	CG094	Human	CHO
TGF-β1	CG069	Human	CHO
TGF-β2	CG071	Human	CHO
TGF-β3	CG072	Human	CHO
VEGFs (Vascular Endothelial Growth Factors)			
VEGF121	CG0110	Human	CHO
VEGF165	CG080	Human	CHO
Wnt-Related Growth Factors			
DKK-1	CG013	Human	CHO
Other Growth Factors			
GH	CG073	Human	CHO
HGF	CG032	Human	CHO
OSM	CG061	Human	CHO

Growth Factor	Cat. No.	Species	Host cell
Interleukins			
IL-3	CG096	Human	E. coli
IL-6	CG041	Human	CHO
IL-10	CG098	Human	CHO
IL-11	CG099	Human	CHO
IL-34(His Tag)	CG014	Human	HEK293
ECM			
Fibronectin	CG024	Human	CHO
Fibronectin Fragment	CG130	Human	HEK293
Vitronectin	CG082	Human	CHO
Accessory Proteins (Supplements and Coagulants)			
Fetuin A	CG016	Human	CHO
Transferrin	CG075	Human	CHO

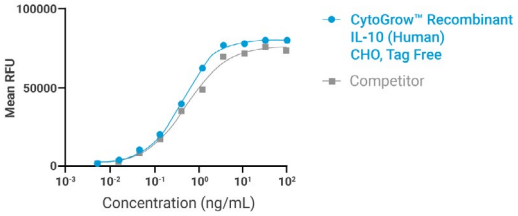
TGF-β1 (Human), Recombinant Protein, CHO, Tag Free



bFGF/FGF-2 (Human), Recombinant Protein, E. coli, Tag Free



IL-10 (Human), Recombinant Protein, CHO, Tag Free



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