PROTOCOL

VitroGel® STEM

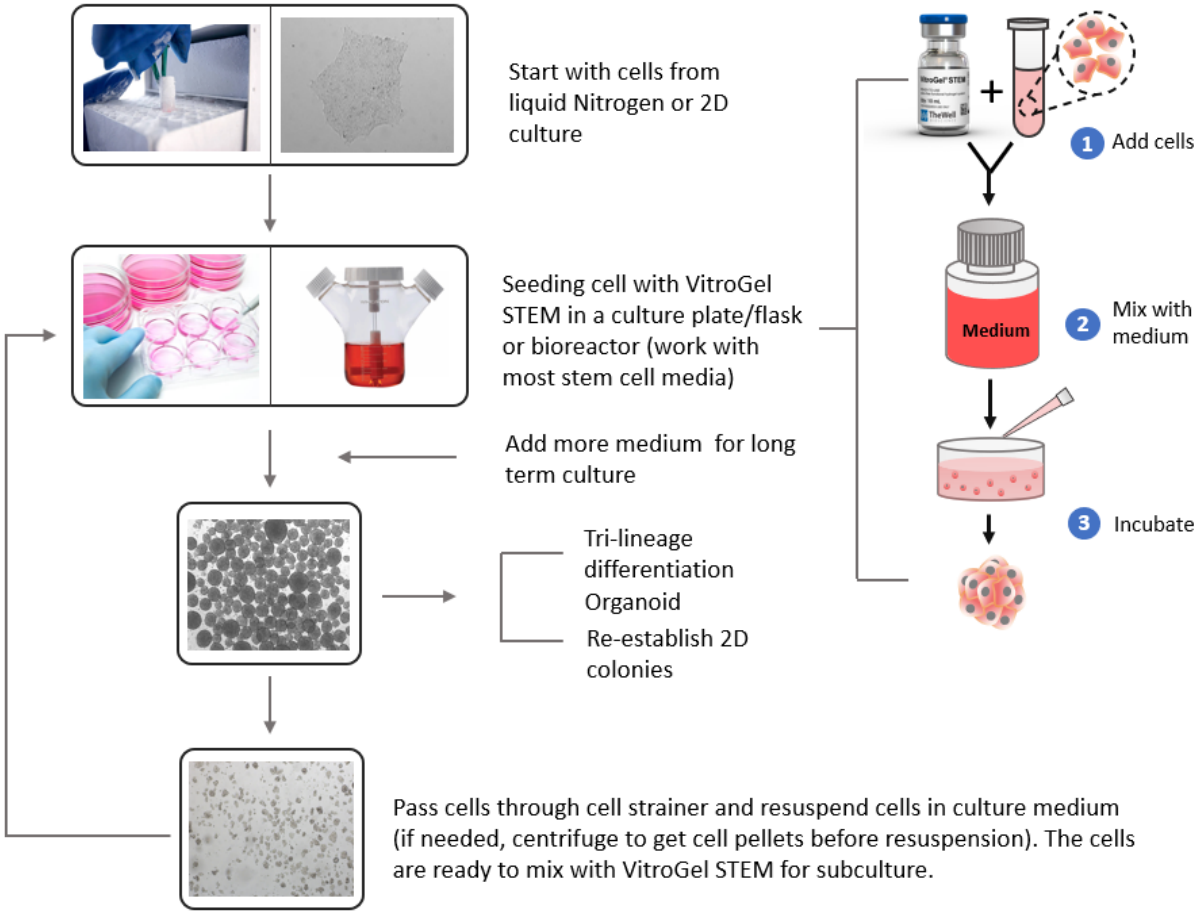
CAT NO. VHM02, VHM02S

TheWell BIOSCIENCE

Growing Cells in New Dimensions

VitroGel STEM is a xeno-free hydrogel system developed to improve the performance of three-dimensional (3D) static suspension cultures and scale-up hPSCs populations to create a high-throughput system to model various tissue 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. 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 tissue culture vessels. Furthermore, in cases where hPSC expansion is needed, this system does not require any special, expensive suspension culture vessels. Due to the unique static suspension culture procedure, the requirement for microcarriers for large-scale bioreactors is eliminated, making the cell harvesting simple and effective. The 3D stem cell spheroids that are developed using VitroGel STEM can be used for further sub-culturing, patterned differentiating, or re-establishing 2D culture morphologies.

WORKFLOW OVERVIEW



RECOMMENDED MATERIALS AND 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 (Cat No. MS03-100) (optional)
- 40, 70, or 100 µm reversible strainer
- Conical tubes (15 mL or 50 mL)
- Serological pipettes
- Non-treated 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 lines.

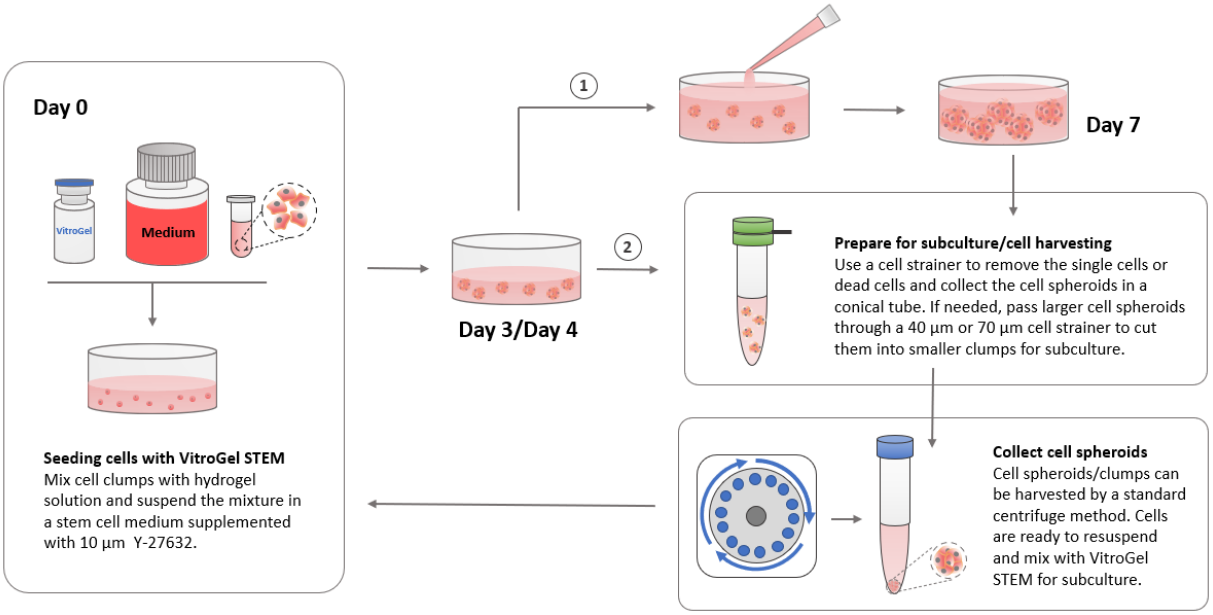
EQUIPMENT

- 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)
  Note: Orbital shaker or spinner flask is not necessary for most culture conditions using VitroGel STEM.

Culture Cycle of hPSCs with VitroGel STEM System

① **7-day culture cycle** with additional medium on day 3/4

② **3-day/4-day culture cycle** without additional medium



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Initial Static Suspension Culture of hPSC Using VitroGel STEM

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 0.5-2 X 10^6 cells/mL for the final cell seeding density in the hydrogel suspension culture around 0.3-1 X 10^5 cells/mL.

- If needed, break up the clumps for 30-70 µm in size by carefully pipetting the clump suspension up and down. Single-cell suspension is not recommended.
- The typical working range of cell density is around 0.2-5 X 10^6 cells/mL, which will make the final cell seeding density in the hydrogel suspension culture around 0.1-3 X 10^5 cells/mL. 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.
4. Add stem cell medium (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.
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:

- It is recommended to use the same type of stem cell culture medium for 2D matrix coating culture to culture cells in 3D suspension culture. If a different type of medium is used for 3D culture, the cells may take 1-3 days to adapt to the new medium (check the instruction of the medium providers for medium switch procedure).
- The selection between 3-day or 4-day culture cycle or 7-day culture cycle is depended on the cell seeding density and the desired conditions of stem cell spheroids.
- 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.

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Initial Static Suspension Culture of hPSC Using VitroGel STEM

Table 1. Recommend volume of VitroGel STEM, cell clump suspension and stem cell medium for 7-day culture cycle

	Well Plate (volume per well)			T-flak			Erlenmeyer Flask			
	96 well plate	24 well plate	6 well plate	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	36 mL	54 mL	108 mL	216 mL	450 mL
Additional 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 VitroGel STEM, cell clump suspension and stem cell medium for 3-day or 4-day culture cycle

	Well Plate (volume per well)			T-flak			Erlenmeyer Flask			
	96 well plate	24 well plate	6 well plate	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
Cell clump suspension	10 µL	50 µL	200 µL	300 µL	900 µL	3 mL	6 mL	12 mL	24 mL	50
Stem cell medium	150 µL	750 µL	3 mL	4.5 mL	13.5 mL	45 mL	90 mL	180 mL	360 mL	750
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

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Harvesting hPSC 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.

   Note: Optimize the speed and time of centrifuge according to the experiment needs

3. Carefully remove the supernatant to collect the cells.

   Optional: When removing the supernatant, leave about 1 mL of medium on top of the cell pellet. There could be a layer of hydrogel on the top of the cell pellet, which contains some small cell spheroids or single cells. To increase the yield, collect the hydrogel-cell mixture to a new conical tube. Add VitroGel Cell Recovery Solution (MS03-100) to the tube. (Keep the volumes of cell recovery solution and cell suspension at 1:1 v/v ratio; e.g., 1 mL of cell recovery solution 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.

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Passaging hPSC Spheroids Static Suspension Cultured in VitroGel STEM

Method 1

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 100x g to collect the cell pellet.

   Note: Optimize the speed and time of centrifuge according to the experiment needs

3. Carefully remove the supernatant to collect the cells. Resuspend the cells with stem cell medium.

   Optional: When removing the supernatant, leave about 1 mL of medium on top of the cell pellet. There could be a layer of hydrogel on the top of the cell pellet, which contain some small cell spheroids or single cells. To increase the yield, collect the hydrogel-cell mixture to a new conical tube. Add VitroGel Cell Recovery Solution to the tube (Keep the volumes of cell recover solution and cell suspension at 1:1 v/v ratio; e.g. 1 mL of cell recovery solution 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 100x g to collect the additional cell pellet.

4. Prepare a 40 or 70 µm strainer on a conical tube to dissociate hPSC spheroids into small clumps.
5. Transfer the cell spheroids from step 3 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.

   Note: If the strainer appears clogged, increase the flow rate slightly or slide the pipette laterally on the strainer while maintaining direct contact with it.

5. To increase yield, rinse the strainer with an additional 1 - 5 mL stem cell medium.
6. Centrifuge the tube for 3 minutes at 100X *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 to the tube and gently pipette up and down to prepare the stem cell clump suspension. The cells are ready to mix with VitroGel STEM for subculture. (Follow the protocol of "Initial Static Suspension Culture of hPSC using VitroGel STEM")

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Passaging hPSC Spheroids Static Suspension Cultured in VitroGel STEM

Method 2

1. Transfer hPSC spheroids from the culture vessel to a serological pipette and pass the entire volume through a reversible strainer into a conical tube to filter out single cells.

   Optional: Before passing through a reversible strainer, use a serological pipette to transfer hPSC spheroids from the culture vessel to a conical tube first. Add VitroGel Cell Recovery Solution to the tube (Keep the volumes of cell recover solution and cell suspension at 1:1 v/v ratio; e.g., 3.6 mL of cell recovery solution for 3.6 mL cell suspension from one well of a 6-well plate). Gently mix cell suspension and recovery solution and incubate at 37 °C for 3-5 minutes.

   Note: Choose the strainer from 40, 70, or 100 µm: the bigger the pore size of the strainer, the easier the single cells and small clumps will pass through.

2. Flip the strainer onto a new conical tube and rinse with 5 mL of stem cell medium, gently tapping the strainer to dislodge all spheroids into the new tube.
3. Prepare a 40 or 70 µm strainer on a conical tube to dissociate hPSC spheroids into small clumps. If a 40 or 70 µm strainer is used in step 1, the same strainer could be used on a new conical tube. (Please make sure the site of the strainer contacted cell spheroids is facing up to prevent any non-dissociated spheroids from being re-seeded into the subsequent passage).
4. Transfer the cell spheroids from step 2 to a serological pipette and place the tip of 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.

   Note: 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 100X *g* to collect the cell pellet.
7. Gently aspirate the medium, leaving 0.5 mL to avoid removing any clumps.
   (If use VitroGel Cell Recovery solution in step 1, carefully remove all medium.)
8. Add the desired volume of stem cell medium to the tube and gently pipette up and down to prepare the stem cell clump suspension. The cells are ready to mix with VitroGel STEM for subculture (follow the protocol of "Initial Static Suspension Culture of hPSC using VitroGel STEM").

REFERENCE DATA

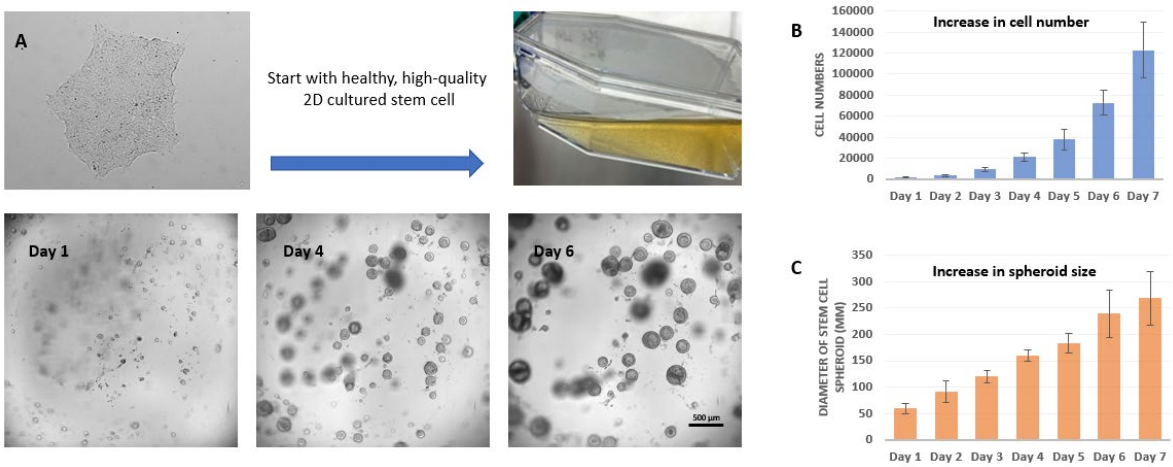

Figure 1. 3D static suspension culture of hPSC from 2D matrix culture

As shown in Figure 1, after 24 hours, small hPSC spheroids starts to form. From day 1 to 6, cells in the suspension cultures quickly grow, leading to the generation of healthy and high-quality stem cell spheroids. After day 3, cell number grow exponentially (Figure 1B) and spheroid size steadily increases (Figure 1C). The hPSC spheroids display characteristics of shallow craters or pockmarks, indicating expression of hPSC markers and successful expansion of healthy and high-quality stem cell spheroids. The resulting spheroids provide researchers with large numbers of healthy hPSCs for further experiments.

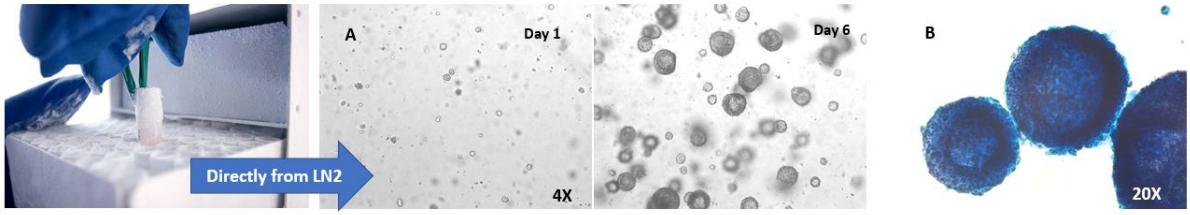

Figure 2. 3D static suspension culture of hPSC directly from Liquid Nitrogen (LN2)

Start the suspension culture by using the healthy and high-quality cells directly from LN2. hPSC-hydrogel aggregates successfully tp form healthy spheroids after 1 day in culture. The hPSC spheroids continue to expand from day 1 to 6 (Figure 2A). The resulting hPSC spheroids also show hallmark features of of healthy and high-quality stem cell spheroids, i.e., shallow craters or pockmarks. Figure 2B shows that hPSC static suspension cultures from liquid nitrogen are positive for Alkaline Phosphatase, indicating successful expansion of healthy stem cell populations.

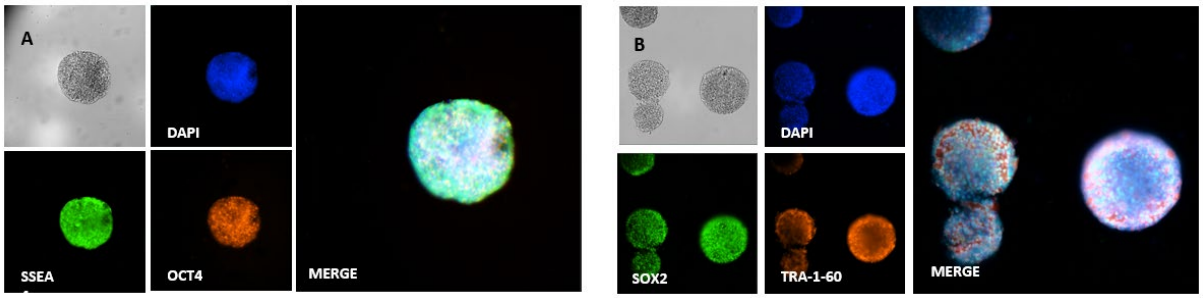

Figure 3. Immunofluorescence images of hPSC spheroids with key pluripotent stem cell markers

VitroGel STEM ensures the undifferentiated state of stem cell lines during scaling up. As shown in Figure 3, hPSC aggregates in VitroGel STEM hydrogel and retain pluripotency after 7 days, evidenced by the expression of key pluripotent stem cell markers, SSEA4, OCT4, SOX2, and TRA-1-60.