TheWell BIOSCIENCE

3D Cell Culture and Beyond

RocketCell™ 3D iPSC Xeno-Free Complete Growth Kit

An all-in-one optimized kit for 3D expansion of pluripotent stem cells

Protocol

Catalog #: RC02-CGK

Revision No: v1.0
Revision Date: 12/2025

www.thewellbio.com
support@thewellbio.com
US TF: +1 866.3D. CELLS
TEL: +1 973.855.4955

Scan the QR code
to access the latest product updates and resources.

thewellbio.com/product/rocketcell-3d-ipsc-xeno-free-complete-growth-kit/

TABLE OF CONTENTS

A. Introduction 2

B. Materials and Reagents 3

C. Preparation of Complete Growth Medium 3

D. Preparation of RocketCell™ 3D iPSC Xeno-Free Suspension Medium 4

E. Warming Complete Growth Medium for Feeding Existing 3D Cultures 4

F. Initiating Primary 3D Cultures using 2D source 4

G. Maintenance of iPSCs in RocketCell™ 3D iPSC Xeno-Free Complete Growth Medium 7

H. 3D Subculture from Existing 3D Cultures 7

I. Initiating 3D Cultures From Cryopreserved iPSCs 9

Tables 10

Appendix 10

RocketCell™ 3D iPSC Xeno-Free Complete Growth Kit - RC02-CGK				
Components	**Catalog No.**	**Size**	**Storage Temp**	**Shipping Temp**
VitroGel® STEM	VHM02	10 mL	4°C	Ambient
RocketCell™ 3D iPSC Xeno-Free Suspension Medium	RC02-SM	5 mL	-20°C	Dry Ice
RocketCell™ Cell Viability Enhancer (1000X)	RC02-CV	50 µL	-20°C	Dry Ice
RocketCell™ iPSC Xeno-Free Basal Medium	RC02-BM	490 mL	4°C	Ice Pack
RocketCell™ iPSC Xeno-Free Supplement (50X)	RC02-S50	10 mL	-20°C	Dry Ice

Storage/Stability:

Product	Temperature	Time
RocketCell™ iPSC Xeno-Free Basal Medium	2°C - 8°C, in the Dark	6 months
RocketCell™ 3D iPSC Xeno-Free Suspension Medium	-20°C to -80°C	6 months
RocketCell™ iPSC Xeno-Free Supplement (50X)	-20°C to -80°C	1 Year
RocketCell™ Cell Viability Enhancer (1000X)	-20°C	1 Year

Growth and Maintenance of Human Induced Pluripotent Stem Cells (iPSCs) Using RocketCell™ 3D iPSC Xeno-Free Complete Growth Kit

TheWell BIOSCIENCE

A. Introduction

RocketCell™ 3D iPSC Xeno-Free Complete Growth Kit (TheWell Bioscience, Cat. #: RC02-CGK) is an all-in-one, fully defined, animal-component-free system optimized to support the 3D expansion of pluripotent stem cells (iPSCs and hESCs) in VitroGel® STEM hydrogel. The kit includes a phenol-red free basal medium and a 50X supplement that together forms a Complete Growth Medium for expansion of IPSCs in 2D and 3D. In addition, the kit includes VitroGel® STEM, a synthetic hydrogel tuned for the growth of iPSCs in 3D; RocketCell™ 3D iPSC Xeno-Free Suspension Medium (Cat. #: RC02-SM), optimized for the resuspension of passaged cell pellets and mixing with VitroGel® STEM in a 2:1 gel:cell ratio; and RocketCell™ Cell Viability Enhancer (Cat. #: RC02-CV), required for the most robust outcomes when passaging cells in 3D.

This medium supports the growth in the undifferentiated state without the need of additional components, as demonstrated by positive immunofluorescence in 2D or 3D with a combination of the following markers: Nanog+/Lin28+/Tra-1-60+; SSEA4+/Oct4+; and demonstrates less than 15% spontaneous differentiation as indicated by the lack of SSEA1+ cells. If possible, we recommended that iPSCs be expanded in a low-oxygen environment of 5-6% O_2 to optimize pluripotency, however, this formulation will support growth in NORM-OXY conditions.

The 3D kit includes a 490 mL of basal medium with a separate frozen 10 mL vial of 50X Supplement. Once the supplement is thawed at 4°C, it can be completely mixed into the medium and is stable for up to 2 weeks. We recommend that the user warms the volume of media needed to room temperature, or briefly at 37°C prior to use. Do not warm up the entire 500 mL bottle. Alternatively, the user may mix desired amount of medium with 1/50 of supplement as needed. Repeated freeze-thaw of supplement is not recommended, however the user may aliquot the thawed supplement, and freeze in small aliquots. Similarly, once thawed, RocketCell™ 3D iPSC Xeno-Free Suspension Medium should be used within two weeks, otherwise, the user may freeze smaller aliquots. RocketCell™ Cell Viability Enhancer should be stored at -20°C, however this solution may not appear to be frozen. Prior to use, centrifuge the tube to ensure the reagent is at the bottom of the tube.

This innovative 3D culture system enables alternate-day feedings and a weekend-free schedule, providing flexibility for diverse research schedules. The workflow has been optimized so that the user can use standard 2D feeding schedules, either daily or alternate day with weekend free period. Some iPSCs accommodate well to alternate day feedings, however others may require daily feeds. This adaptation is to be expected when users switch from a 2D paradigm to 3D. This feeding schedule can also be used for thawing cells directly into 3D paradigms using validated and high viability cryopreserved iPSC lines.

Routine 7 Day Growth/Passaging cycle

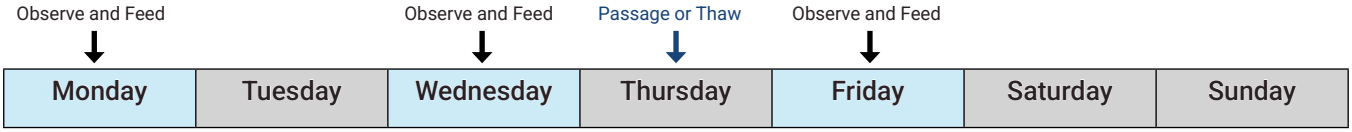
a. Passage cells from 2D paradigm to 3D on a Thursday*

b. Verify culture integrity and double volume feed on Friday

c. Feed on following Monday and Wednesday

d. Passage on Thursday

7 Day Cycle with Observation following Passaging or Thaw



*Cells can also be passaged from existing 3D cultures on Friday depending on empirical observations with the continued presence of ROCK inhibitor and effect on pluripotency. We recommend changing out or adding to the cover media on the Friday before weekend-free feeding schedule.

B. Materials and Reagents

1. RocketCell™ iPSC Xeno-Free Basal Medium, 490 mL (Catalog #: RC02-BM)
2. RocketCell™ iPSC Xeno-Free Supplement (50X), 10 mL(Catalog #: RC02-S50)
3. VitroGel® STEM, 10 mL (Catalog #: VHM02)
4. RocketCell™ 3D iPSC Xeno-Free Suspension Medium, 5 mL (Catalog #: RC02-SM)
5. RocketCell™ Cell Viability Enhancer (1000X), 50 µL (Catalog #: RC02-CV)
6. VitroGel® Cell Recovery Solution, (Catalog #: MS03)
7. VitroGel® Organoid Recovery Solution, 100 mL or 500 mL (Catalog #: MS04)
8. PBS/EDTA
9. Accutase (for 2D dissociation)
10. Accumax (for 3D dissociation)
11. 40 and 70 micron 50 mL tube top filters
12. P1000 pipette and tips
13. 5 mL glass serological pipettes
14. Refer to Appendix A for additional Material and Reagents from TheWell Bioscience (VitroPrime™ Spread-Attach Plates and VitroPrime™ 3D Culture and Imaging Plates).

C. Preparation of Complete Growth Medium

The frozen 50X supplement should be defrosted at 4°C and used to complete the Basal Medium. Once the RocketCell™ iPSC Xeno-Free Basal Medium is supplemented, the Complete Growth Medium has a validated shelf life of 2 weeks. The user may aliquot the thawed supplement and re-freeze **one** time to preserve smaller amounts of Complete Growth Medium over a longer period. The aliquot of the supplement may be re-frozen at -20°C or -80°C in small sterile o-ring screw top tubes and defrosted at 4°C as needed. We do not recommend more than **one** additional freeze-thaw cycle. The media is phenol-red free, however if so desired the user may add this pH indicator to visually monitor the acidification (growth) of the cultures to help determine optimal growth and feeding regimen. Please note that phenol-red has described estrogenic effects and will interfere with fluorescent imaging.

1. Defrost the 10 mL frozen RocketCell™ iPSC Xeno-Free Supplement (50X) at 4°C or on ice.
2. Aseptically transfer the entire contents of the RocketCell™ iPSC Xeno-Free Supplement (50X) into the basal medium. Otherwise, divide the RocketCell™ iPSC Xeno-Free Supplement (50X) into aliquots (1 mL screw top o-ring tubes) and refreeze. Use 1 mL per 49 mL of medium to achieve the correct 1X final dilution.
   - » **Do not freeze thaw supplement a second time.**
   - » The complete supplemented medium should be used within two weeks.
   - » Antibiotic/antimycotics agents may be added to the medium at the user's discretion.
3. Label the bottle with the date of preparation and the calculated expiration date (2 weeks from creation), and store at 2-8°C.

D. Preparation of RocketCell™ 3D iPSC Xeno-Free Suspension Medium

1. Thaw the frozen bottle of RocketCell™ 3D iPSC Xeno-Free Suspension Medium at 4°C and use within 2 weeks.
2. Users may aliquot the solution into tubes, refreeze and thaw only one time as described in Section C.
3. Prior to using in **all** protocols below, the RocketCell™ 3D iPSC Xeno-Free Suspension Medium **must** be supplemented with a final of 3X of the RocketCell™ Cell Viability Enhancer.
    a. For example, when resuspending a cell pellet in 1000 mL of Suspension Medium, add 3 µL of RocketCell™ Cell Viability Enhancer. Scale as needed.

E. Warming Complete Growth Medium for Feeding Existing 3D Cultures

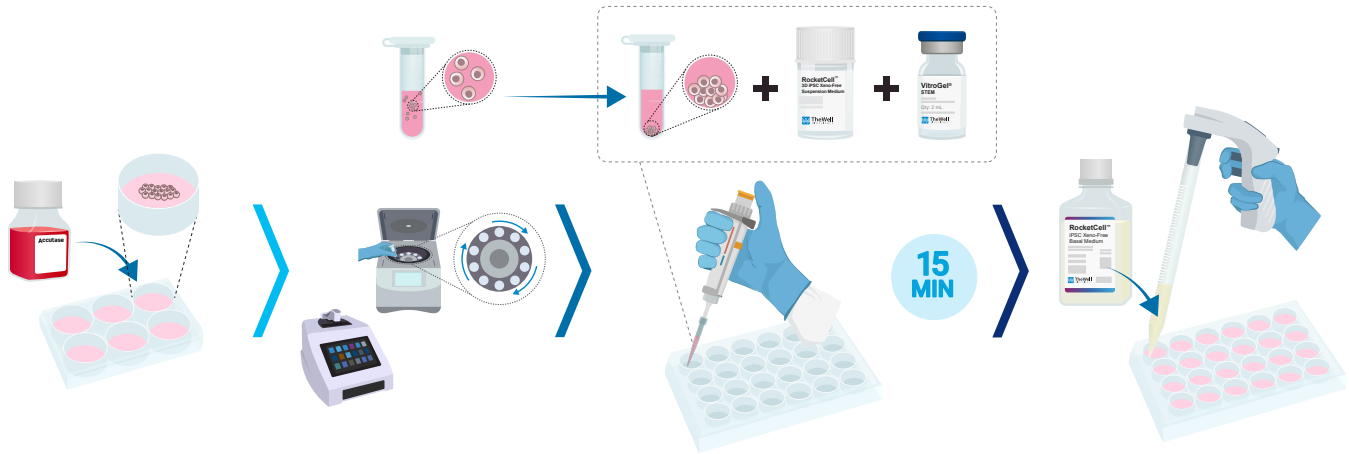
1. Prior to feeding, determine the volume of complete medium that will be required. It is generally recommended that the following volumes be used for the following standard culture plate sizes taking into consideration the range of densities of the cultures:

Table 1: Suggested volumes of Complete Growth Media for feeding 3D cultures

Plate Size	Growth Surface/Well	Volume
96-well	0.32 cm^2	0.1 - 0.2 mL
48-well	1 cm^2	0.2 - 0.4 mL
24-well	1.9 cm^2	0.5 - 1 mL
12-well	3.5 cm^2	1 - 2 mL
6-well	9.5 cm^2	2 - 4 mL

2. Remove the calculated amount from the media bottle into 50 mL conical tube and warm to room temperature. Optionally, place tube into a 37°C bath (dry bead or water bath) for 10-15 minutes for more rapid warming.

F. Initiating Primary 3D Cultures using 2D source



1. Wash out and replace medium with PBS/EDTA or Accutase.
2. Collect cell suspension, count aliquot and centrifuge main tube.
3. Resuspend cell pellets in 1 volume of RocketCell™ 3D iPSC Xeno-Free Suspension Medium and combine with 2 volumes of VitroGel® STEM. Mix 10X gently. Place mixture into culture vessel.
4. After 15 min, gently overlay RocketCell™ iPSC Xeno-Free Basal Medium. Observe and return to incubator.

Note: The end user should always be mindful that overexposure to passaging reagents or digestive enzymes has the potential to damage cells and in the case of pluripotent stem cells, alter cellular phenotypes. It is recommended that Primary Initiating 3D Culture passaging be accomplished using Accutase (or similar) or PBS-EDTA. It has been demonstrated that these agents allow for gentler passaging, greater viability, and less disruption of cellular phenotypes.

A table of volumes recommended for all commonly used multiwell plates, for RocketCell™ 3D iPSC Xeno-Free Suspension Medium with RocketCell™ 3X Cell Viability Enhancer, VitroGel® STEM, and Complete Growth Medium with 1X RocketCell™ Cell Viability Enhancer (Overlay Media) is seen below.

The example below will use exact volumes, however we recommend the user calculate **extra** volume for each component according to known level of precision of their pipetting apparatus.

A typical near confluent iPSC culture from one well of a 6-well plate may contain **2-4** million viable cells. The example below assumes the user will obtain more than the minimum of 2.4 million cells needed to seed each well of a 24-well plate with 100,000.

Table 2: Suggested Volumes for Cell Suspension, Hydrogel and Overlay Media per Well

Plate Size	Surface Area	RocketCell™ 3D iPSC Xeno-Free Suspension Medium + 3X RocketCell™ Cell Viability Enhancer	VitroGel® STEM (2:1 ratio)	Complete Growth Medium + 1X RocketCell™ Cell Viability Enhancer Overlay Media
96-well	0.32 cm^2	16.7 µL	33.3 µL	50 µL
48-well	0.95 cm^2	50 µL	100 µL	150 µL
24-well	1.9 cm^2	100 µL	200 µL	300 µL
12-well	3.8 cm^2	200 µL	400 µL	600 µL
6-well	9.5 cm^2	400 µL	800 µL	1200 µL

Example - Passaging 1 well of a 6-well plate 2D culture to 3D using a 24-well plate

1. Remove the bottle of RocketCell™ 3D iPSC Xeno-Free Suspension Medium from 4°C storage, spray with disinfectant, wipe, and place in biosafety cabinet. For this example, we will use **2.4 mL of RocketCell™ 3D iPSC Xeno-Free Suspension Medium**.
2. Remove the tube of 1000X RocketCell™ Cell Viability Enhancer from -20°C, let come to RT for 2-3 min, centrifuge, and disinfect, and transfer to Biosafety cabinet. Remove 7.2 µL of 1000X RocketCell™ Cell Viability Enhancer and add to the 2.4 mL of RocketCell™ 3D iPSC Xeno-Free Suspension Medium.
3. Remove the bottle of VitroGel® STEM from 4°C storage, spray with disinfectant, wipe, and place in biosafety cabinet. If new, remove the top blue cover, and peel off the metal retaining ring. For this example, we will use **4.8 mL of VitroGel® STEM**.
4. For each well of a 6-well plate, prepare a tube with 1 mL of Complete Growth Medium containing 1X RocketCell™ Cell Viability Enhancer (1 µL/mL). This is used to neutralize passaging reagent. In this example we will use 1 mL. This can be pooled in tube with media prepared in the step below.
5. For **each** well to be plated, i.e. 24 wells, prepare 300 µL of Complete Growth Medium containing 1x RocketCell™ Cell Viability Enhancer (Overlay Media). In this example 24 wells x 300 µL = **7,200 µL Media + 7.2 µL of 1000X RocketCell™ Cell Viability Enhancer**.
6. **Optional:** Warm PBS-EDTA to 37°C (or RT), if using an enzyme warm to recommended temperature as per manufacturer.
7. Aspirate medium from one well of the 6-well plate with near confluent iPSC cultures.
8. Add 2 mL of PBS-EDTA/well, and aspirate immediately. This will help to remove any dead cells, residual proteins and allow for quicker dissociation.

9. Add 1 mL of PBS-EDTA or Accutase and incubate at 37°C for 5 min.
10. Observe the well with an inverted microscope. If the well was confluent additional time may be required. If needed, incubate an additional 2-3 minutes.
11. Colonies will begin to dissolve into single cells or small clumps.
12. Once cells have rounded up completely and/or begun to detach, add 1 mL of growth medium with 1X RocketCell™ Cell Viability Enhancer and gently triturate cells gently 2-3 times using a 5 mL glass pipette or P1000 pipette.
13. Transfer the dislodged cells into a 15 mL conical tube. The total volume should be approximately 2 mL.

    Note: If processing more than 1 well, the user may scale up volumes and tube size.

14. Remove 10 µL from the cell suspension, combine into new tube with equal amount of Trypan Blue dye and count viable cells.
15. We recommend initiating the Primary 3D cultures with 100,000 viable cells per well of a 24-well plate, but the **initiating** density can range from 50,000 to 150,000 cells.
    a. In this example, the user counts a total of 4 million cells in 2 mL, therefore the density is 2 million cells per mL.
    b. For a 24-well plate at a density of 100,000 cells/well, 2.4 million cells are required. Therefore, the user removes 1.2 mL to a 15 mL conical new tube. As above, we recommend accommodating for pipetting error and prepare slightly more than needed. Scale up all solutions.
16. Centrifuge 15 mL conical tube in at 300 x g for 3-4 min at RT.
17. Carefully aspirate the supernatant, so as not to disturb the pellet.
18. Resuspend the pellet with **2.4 mL of RocketCell™ 3D iPSC Xeno-Free Suspension Medium with 3X RocketCell™ Cell Viability Enhancer**.
19. Add **2 Volumes** of VitroGel® STEM, i.e. **4.8 mL** and gently triturate 10 times using a glass 5 mL pipette.
    a. This is a crucial step! iPSCs are sensitive to shear force, so use gentle action to homogenize the gel with cells.
20. Once mixed, transfer 300 µL of the 2:1 gel:cell mixture per well of a 24-well VitroPrime™ Spread-Attach Plate.
    a. If using other plasticware, a quick mixing of the plate by hand may be required to ensure the hydrogel mixture uniformly covers the well.
    b. If plating multiple wells, we recommend using a repeater pipette
    c. When plating into 96-well platees, the mixture can be transferred to a standard microwell trough, and then dispensed using a multichannel (8 or 12 channel) pipette or automated liquid handling machine.
21. Set plate aside, cover and incubate for 15 min at room temperature. It is essential not to disturb the plate during the polymerization step, as this will lead to loose gel formation, subsequent heterogenous growth pattern of iPSCs, and potential cell loss of cells during routine feeding.
22. After 15 min (but no longer than 30 min), slowly overlay 300 µL/well of the Complete Growth Medium containing RocketCell™ Cell Viability Enhancer. Use a serological pipette on slow speed or a P1000 pipette, and run media down the side of well.
23. Observe, and place it in the incubator overnight.
24. Following day replace the entire medium with Complete Growth Medium without 1X RocketCell™ Cell Viability Enhancer.

    Optional: In place of changing cover medium, user may add 1 mL of Complete Growth Medium without enhancer per well of a 24-well plate for weekend-free culture technique.

G. Maintenance of iPSCs in RocketCell™ iPSC Xeno-Free Complete Growth Medium

1. Feed cells every day or every other day as with 2D culturing techniques by carefully removing all the media using P1000 or similar pipette and replacing it with fresh RocketCell™ Complete Growth Medium (without RocketCell™ Cell Viability Enhancer).
2. Observe daily and proceed with downstream protocols (like passaging, freezing, or immunostaining) as described in sections below after desired amount of time.

H. 3D Subculture from Existing 3D Cultures

Note: As with **Initiating Primary 3D Cultures**, the end user should be mindful that overexposure to passaging reagents or digestive enzymes has the potential to damage cells and in the case of pluripotent stem cells, alter cellular phenotypes. It is recommended that **3D Subculture** passaging technique be accomplished first with VitroGel® Cell Recovery Solution (more gentle) or VitroGel® Organoid Recovery Solution (more robust) followed by incubation with **Accumax** (or similar). Refer to **Table 2** in section above for volumes of RocketCell™ 3D iPSC Xeno-Free Suspension Medium with 3X RocketCell™ Cell Viability Enhancer, VitroGel® STEM, and Overlay Medium needed for desired multiwell plate configuration.

Additionally, if the user observes heterogenous sphere size after the passaging procedure, they may incorporate a physical filtration step to remove larger spheroids using a 40 or 70 micron (50 mL) tube top filter. Failure to input a well dissociated homogenous starting population will lead to heterogenous sizing of iPSC colonies, and possible polarization and differentiation. Loss of some cells may occur, so we recommend counting as best possible to accommodate single cells and small spheroids. Alternatively, the user may passage one replicate well from a 24-well plate to determine approximate cell number in parallel wells, and then use routine division such as 1:2, 1:4, 1:8 ratio to dilute and plate the cell in new wells. If the user initiate cultures as described above, it is expected to obtain roughly 1 million cells/well of 24-well plate after 7 day expansion. In the example below we will recommend dissociating cells from 4 wells of a 24-well plate to ensure the user has enough cells to initiate plating of a new complete 24-well plate. Scale up or down accordingly, and calculate in desired amount of extra volume based on precision of pipetting.

Protocol (Example: Passaging 4 wells from an existing 3D culture from a 24-well plate to a new 24-well plate.)

1. Prepare needed solutions by following Steps 1-6 in Section F above "Initiating Primary 3D Cultures using 2D source", but for Step 4 here, prepare 4 mL of Complete Growth Medium with 1X RocketCell™ Cell Viability Enhancer. Total volume together with Overlay Medium = 11.2 mL + 11.2 µL of 1000X RocketCell™ Cell Viability Enhancer.
2. Warm 10 times the volume of either VitroGel® Cell Recovery Solution or VitroGel® Organoid Recovery Solution to the ratio of hydrogel:cell mixture in 4 wells of a 24-well plate. In 1 well of 24-well plate volume = 300 µL (See Table 2 above). Use 3 mL per well = 12 mL total.
    a. Warm 3 mL x 4 wells = 12 mL of VitroGel® Cell Recovery (MS03) or VitroGel® Organoid Recovery (MS04) Solution to 37°C.
3. Warm Accumax solution (0.5 to 1 mL per well used) to recommended temperature as per manufacturer.
4. Label a new 50 mL conical tube for collection of cells and set aside.
5. Remove the medium from 4 wells of the 24-well plate after a 7 day cycle.

   Note: The 7 day cycle appears to be an optimal expansion period, however, if recovery from previous 3D passaging is robust, cells may be passaged before 7 day time point. Likewise, if growth appears slower than expected, cultures can be kept for 10 days to allow for more robust outcomes. Usually, we observe very homogenous and robust outcomes during Primary Initiating Cultures, whereas a more stringent technique is required for subsequent 3D cultures to obtain expected outcomes.

6. Add 1.5 mL of VitroGel® Cell Recovery Solution or VitroGel® Organoid Recovery Solution to the well.

   Note: The VitroGel® Cell Recovery Solution is gentler, while the VitroGel® Organoid Recovery Solution is more robust and may help initiate spheroid breakdown.

7. Using a 5 mL glass serological pipette, transfer the hydrogel/solution to the new 15 or 50 mL tube. The entire gel should get sucked into the pipette.
8. Add an additional 1.5 mL to the well, triturate once, and collect into the 15 mL tube.
9. Gently triturate the Gel:Recovery Solution in tube 2-3 times to help breakup the hydrogel. Be mindful of the total volume as not to overflow the tube.
10. Observe the tube, and place in 37°C bead or water bath for 5 min with occasional inversion.
11. Remove to biosafety cabinet, disinfect, and gently triturate 2-3 times with glass 5 mL pipette. At this point the gel should be well dissociated.
12. Centrifuge at 300 x g for 5 min at RT.
13. Remove media. If there is a small gel layer above cell pellet, carefully remove using P1000 or P200 pipette.
14. Add 4 mL of Accumax and gently triturate 1-2 times to resuspend spheroids.
15. Incubate for 5 min, and triturate 3-5 times to help dissociate cells.
16. Add 4 mL of RocketCell™ iPSC Complete Growth Medium with 1X RocketCell™ Cell Viability Enhancer.
17. **Optional:** if user observes heterogenous dissociation, the suspension may be filtered using a 50 mL tube top filter device (40 or 70 microns). If filtering in a 50 mL tube, user may centrifuge in that tube or transfer to a 15 mL tube.
18. Remove 10 µL for cell counting. If user obtains 1 million cell from each well, then total will be 4 million cells in 8 mL = 500,000 cells/mL. For a complete 24-well plate, 2.4 million cells are needed = 4.8 mL of cell suspension.
19. As before, remove 4.8 mL = 2.4 million cells into new 15 mL tube.
20. Centrifuge at 300 x g for 5 min at RT.
21. Resuspend the pellet with 2.4 mL of RocketCell™ 3D iPSC Xeno-Free Suspension Medium with 3X RocketCell™ Cell Viability Enhancer (density of 1 million cells per mL).
22. Using a 5 mL glass pipette, add 4.8 mL of VitroGel® STEM into the tube, and gently triturate 10 times.
    a. **This is a crucial step!** However, since iPSCs are sensitive to shearing, use of gentle force here is required.
23. One mixed properly, transfer 300 µL of the 2:1 gel:cell mixture to each well of the 24-well plate. When using VitroPrime™ plasticware, the hydrogel will flow evenly without mixing or agitation.
    a. If using other plasticware, a quick mixing of the plate by hand may be required to ensure the hydrogel mixture covers the well evenly.
    b. If plating multiple wells, we recommend using a repeater pipette
    c. When plating into 96-well plates, the mixture can be transferred to a standard microwell trough and then dispensed using a multichannel (8 or 12 channel) pipette or automated liquid handling machine.
24. Set plate aside, cover and incubate for 15 min at room temperature. It is **essential** not to disturb the plate during the polymerization step, as this will lead to loose gel formation, subsequent heterogenous growth pattern of iPSCs, and potential cell loss of cells during routine feeding.
25. After 15 min (but no longer than 30 min), slowly overlay 300 µL/well of the Complete Growth Medium containing 1X RocketCell™ Cell Viability Enhancer. Use a serological pipette on slow speed or a P1000 pipette, and run media down the side of well.
26. Observe, and place in the incubator overnight.
27. Following day replace the entire medium with Complete Growth Medium without 1X RocketCell™ Cell Viability Enhancer.
    a. **Optional:** In place of changing cover medium, user may add 1 mL of Complete Growth Medium without enhancer per well of a 24-well plate for weekend-free culture technique.

I. Initiating 3D Cultures From Cryopreserved iPSCs

Note: Thawing frozen cells is a rapid process wherein the entire ampoule is warmed rapidly at the same rate. This can be accomplished using a clean 37°C water bath, or with a water-free thawing device such as a ThawStar™ (BioLife). If done correctly, thawing a single vial of cells frozen as a 1 mL aliquot should take approximately 2 minutes. It is critical that once thawed the cell suspension be diluted and transferred into fresh cell culture medium (cool to room temperature). This dilutes the DMSO, a commonly used cryopreservative. We recommend freezing cell lines in 1 mL aliquots (500,000 – to 5,000,000 cells per ampoule) in 2 mL cryovials, as it leaves room for initial backfilling fresh medium into the cryovial prior to full transfer to larger volume. The example provided below is for defrosting a vial with a minimum of 2,400,000 cells (assuming 100% viability) and plating cells into all the wells of a 24-well plate (100,000 cells per well). It is vital to know if cells are viable, therefore we highly recommend pre-testing the viability of the cells from a parallel ampoule in a parallel 2D culture to determine if the lot of frozen stocks are of high quality. One may plate 20,000 cells in a matrix coated well alongside a 3D counterpart. Failure of Initiating 3D cultures is often a result of poor cryopreservation and poor recovery. For example, if the quantitated viability of cells is 50%, then adjust calculations so that 100,000 viable cells are resuspended in 100 µL of RocketCell™ 3D iPSC Xeno-Free Suspension Medium with 3X RocketCell™ Cell Viability Enhancer/well. In this example, as above, we are thawing sufficient cells to plate an entire 24-well plate (2.4 million viable cells).

Protocol:

1. Keep the vial of cells frozen in the vapor-phase nitrogen as most plastic cryovials are not rated for immersion in liquid phase. Vials can be transferred to dry ice for transport from the liquid nitrogen tank to the lab but not stored for more than 4 hours outside LN_2 temperatures.
2. Prepare needed solutions by following Steps 1-6 in Section F above "Initiating Primary 3D Cultures using 2D source", but for Step 4 here, prepare 9 mL of Complete Growth Medium with 1X RocketCell™ Cell Viability Enhancer. Total volume together with Overlay Medium = 7.2 mL + 9 mL = 16.2 mL +16.2 µL of 1000X RocketCell™ Cell Viability Enhancer.
3. Place a 9 mL of cold Complete Growth Medium with 1X RocketCell™ Cell Viability Enhancer into a fresh 15 mL conical tube labeled "Cells".
4. Remove cells from dry ice and place vial in a floater rack in a 37°C water bath or a vial thawing device such as a ThawStar™ (or similar). If using a device, first spray vial with ethanol, and place into the device till thawed. If using water bath proceed with next step, otherwise proceed to Step 8.
5. Inspect the vial and remove it from the 37°C water bath when a small piece of ice is still visible. Do not shake or mix.
6. Carefully and quickly sanitize the exterior by generously spraying it with 70% ethanol, and transfer into biosafety cabinet.
7. Using sterile technique, wipe the excess ethanol, and remove the vial cap.
8. Take 1mL of Complete Growth Medium with 1X RocketCell™ Cell Viability Enhancer from the 15 mL conical tube labeled "Cells" and slowly and gently add it to the 2 mL cryovial while stirring. This procedure is called "back-fill" and more slowly accommodates the cells suspended in 10% DMSO cryopreservation solution to lower less toxic concentrations. If this process is rushed, cells may experience osmotic shock, resulting in lower post-thaw viability.
9. Gently and slowly transfer the 2 mL into the 8 mL remaining in the 15 mL conical tube.
10. If needed, the user may count cells to help determine number of cells and viability. Remove 2.4 million cells to fresh tube, or proceed with entire vial as calculated.
11. Centrifuge tube at 100-300 x g for 5 minutes at RT. Aspirate the supernatant, taking care not to disturb the cell pellet.
12. Continue with Step 21 as described in Section H above.

Tables

Table 3: Immunophenotyping of iPSC populations via immunofluorescence microscopy

Marker	iPSC
Oct4	Positive
Lin28	Positive
Sox2	Positive
Tra-1-60/Podocalyxin	Positive
EpCAM	Positive
SSEA4	Positive
SSEA1	Negative/Weak

Appendix A: Other products for culture of iPSCs

RocketCell™ iPSC Xeno-Free Growth Medium (RC02-GM)

Cyto3D® Live-Dead Assay Kit (BM01)

VitroPrime™ Spread Attach Plate, 6-Well (VP-SA6W)

VitroPrime™ Spread Attach Plate, 12-Well (VP-SA12W)

VitroPrime™ Spread Attach Plate, 24-Well (VP-SA24W)

VitroPrime™ Spread Attach Plate, 48-Well (VP-SA48W)

VitroPrime™ Spread Attach Plate, 96-Well (VP-SA96W)

VitroPrime™ 3D Culture and Imaging Plate, 6-Well (VP-3D6W)

VitroPrime™ 3D Culture and Imaging Plate, 24-Well (VP-3D24W)

VitroPrime™ 3D Culture and Imaging Plate, 96-Well (VP-3D96W)

TheWell BIOSCIENCE

3D Cell Culture and Beyond

Revision No: v1.0
Revision Date: 12/2025

For research use only. Not intended for use in diagnostics or therapeutic procedures.

TheWell Bioscience Inc.,
1 Deerpark Dr, Ste C Monmouth Junction, NJ 08852

www.thewellbio.com
support@thewellbio.com

US TF: +1 866.3D. CELLS
TEL: +1 973.855.4955