TheWell BIOSCIENCE

3D Cell Culture and Beyond

RocketCell™ 3D NSC Xeno-Free Complete Growth Kit

An all-in-one, ready-to-use kit for 3D neural stem cell culture.

Protocol
Catalog #: RC01-CGK

Revision No: v1,0
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RocketCell™ 3D NSC Xeno-Free Complete Growth Kit - RC02-CGK				
Components	**Catalog No.**	**Size**	**Storage Temp**	**Shipping Temp**
VitroGel® NEURON	VHM07	10 mL	4°C	Ambient
RocketCell™ 3D NSC Xeno-Free Suspension Medium	RC01-SM	10 mL	-20°C	Dry Ice
RocketCell™ Cell Viability Enhancer (1000X)	RC02-CV	2 x 50 µL	-20°C	Dry Ice
RocketCell™ NSC Xeno-Free Basal Medium	RC01-BM	500 mL	4°C	Ice Pack
RocketCell™ NSC Xeno-Free Supplement (50X)	RC01-S50	10 mL	-20°C	Dry Ice
CytoGrow™ Recombinant EGF (Human), CHO, Tag Free	CG014-A	10 µg	-20°C	Dry Ice
CytoGrow™ Recombinant bFGF/FGF-2 (Human), E. coli, Tag Free	CG003-A	10 µg	-20°C	Dry Ice

Storage/Stability:

Product	Temperature	Shelf Life
VitroGel® NEURON	2°C - 8°C	24 months
RocketCell™ NSC Xeno-Free Basal Medium	2°C - 8°C, in the Dark	12 months
RocketCell™ 3D NSC Xeno-Free Suspension Medium	-20°C to -80°C	12 months
RocketCell™ NSC Xeno-Free Supplement	-20°C to -80°C	12 months
RocketCell™ Cell Viability Enhancer (1000X)	-20°C	12 months
CytoGrow™ Recombinant EGF (Human), CHO, Tag Free	-20°C	12 months
CytoGrow™ Recombinant bFGF/FGF-2 (Human), E. coli, Tag Free	-20°C	12 months

Growth and Maintenance of Human Induced Pluripotent Stem Cells Derived Neural Stem Cells (NSCs) Using RocketCell™ 3D NSC Xeno-Free Complete Growth Kit

A. Definitions

1. Complete Growth Medium: NSC Basal Medium + 50X Supplement + EGF/FGF-2.
2. Suspension Medium: Specialized medium for resuspension of NSCs when combining in 1:1 ratio with VitroGel® NEURON.
3. Overlay Medium: Complete Growth Medium with 1X RocketCell™ Cell Viability Enhancer.

B. Introduction

RocketCell™ 3D NSC Xeno-Free Complete Growth Kit (TheWell Bioscience, Catalog #: RC01-CGK) is an all-in-one, fully defined, animal-component-free system optimized to support the 3D expansion of iPSC-derived neural stem cells in VitroGel® NEURON hydrogel. The kit includes a phenol-red free basal medium and a 50X supplement and growth factors(EGF/FGF-2) that together forms a Complete Growth Medium for expansion of NSCs in 2D and 3D. In addition, the kit includes VitroGel® NEURON, a synthetic hydrogel tuned for the growth of NSC in 3D; RocketCell™ 3D NSC Xeno-Free Suspension Medium optimized for the resuspension of passaged cell pellets and mixing with VitroGel® NEURON in a 1:1 hydrogel to cell ratio; and RocketCell™ Cell Viability Enhancer (1000X) (Catalog #: RC02-CV), required for the most robust outcomes when passaging cells in 3D.

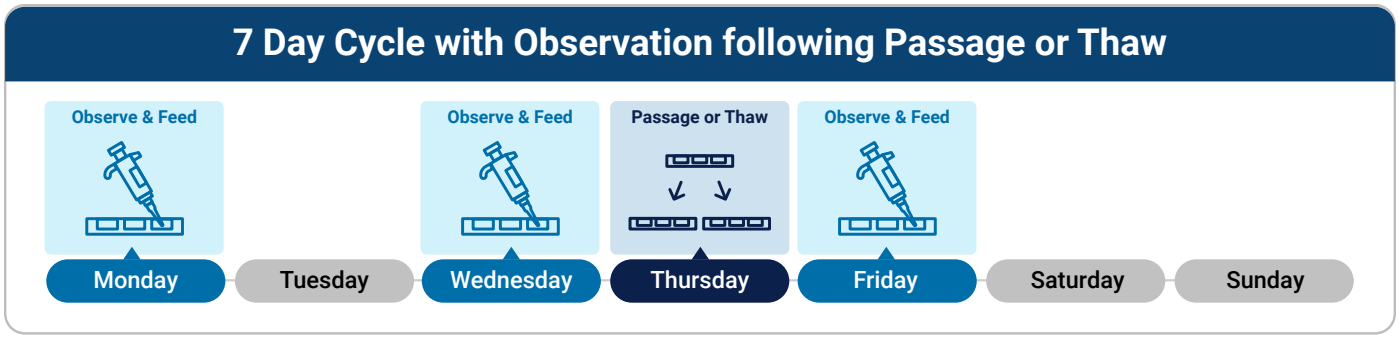
This kit supports the growth in the NSCs as without the need of additional components, as demonstrated by positive immunofluorescence in 2D or 3D with a combination of the following markers: Sox2+/Pax6+/Nestin+. If possible, we recommended that NSCs be expanded in a low-oxygen environment of 5-6% O_2 to optimize stemness, however, this formulation will support growth in NORM-OXY conditions.

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 NSCs 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 NSCs directly into 3D paradigms using vials with validated and high percentage of viable cells.

Routine 7 Day Growth/Passaging cycle

a. Passage (or Thaw) 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

*Cells can also be passaged from existing 3D cultures on Friday depending on empirical observations in the continued presence of the RocketCell™ Cell Viability Enhancer (1000X) and its effect on stemness. We recommend changing out or adding to the cover media on the Friday before weekend-free feeding schedule. Only change 75-80% of the media as per routine with 2D NSC cultures.



*Cells can also be passaged from existing 3D cultures on Friday depending on empirical observations in the continued presence of the RocketCell™ Cell Viability Enhancer and its effect on stemness. We recommend changing out or adding to the cover media on the Friday before weekend-free feeding schedule. Only change 75-80% of the media as per routine with 2D NSC cultures.

C. Components

The 3D kit includes:

- 1 x 10 mL, VitroGel® NEURON
- 10 mL, RocketCell™ 3D NSC Xeno-Free Suspension Medium
- 2 x 50 µL, RocketCell™ Cell Viability Enhancer (1000X)
- 1 x 500 mL, RocketCell™ NSC Xeno-Free Basal Medium
- 1 x 10 mL, RocketCell™ NSC Xeno-Free Supplement (50X)
- 1 x 10 µg, CytoGrow™ Recombinant EGF (Human), CHO, Tag Free
- 1 x 10 µg, CytoGrow™ Recombinant bFGF/FGF-2 (Human), E. coli, Tag Free

D. Recommended Use

Thaw the 50X Supplement at 4°C, and completely mix into the Basal Medium. This is stable for up to 2 weeks. Alternatively, the thawed 50x Supplement may be aliquoted into sterile o-ring screw top tubes in smaller volumes and refrozen. Do not thaw and refreeze more than once. Similarly, the EGF and FGF-2 growth factor vials should be resuspended in 500 uL (20 ug/mL) of sterile cell culture grade water, and used at 1:1000 to supplement the media at a final concentration of 20 ng/mL. As above, the medium may be supplemented entirely, or the growth factors may be aliquoted into sterile o-ring screw top microfuge tubes in smaller volumes to be used as needed.

Once the Complete Growth Medium is made, 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.

Similarly, once thawed, RocketCell™ 3D NSC Xeno-Free Suspension Media should be used within two weeks, otherwise, the user may refreeze smaller aliquots as above. RocketCell™ Cell Viability Enhancer (1000x) should be stored at -20°C, however this solution may not appear to be frozen. Prior to use, bring to RT or warm briefly, and centrifuge the tube to ensure the reagent is at the bottom of the tube.

The media is phenol-red free, however if desired the user may add this pH indicator to a final concentration of 8.1 mg/L to visually monitor the growth and acidification (growth) of the cultures which will help determine optimal growth and feeding regimen. Please note that phenol-red has described estrogenic effects and will also interfere with fluorescent imaging.

E. Materials and Reagents

1. RocketCell™ 3D NSC Complete Growth Kit (RC01-CGK)
   - VitroGel® NEURON, 10 mL (Catalog #: VHM07)
   - RocketCell™ NSC Xeno-Free Basal Medium, 500 mL (Catalog #: RC01-BM)
   - RocketCell™ NSC Xeno-Free Supplement (50X) , 10 mL (Catalog #: RC01-S50)
   - CytoGrow™ Recombinant EGF (Human), CHO, Tag Free, 10 µg (Catalog #: CG014-A)
   - CytoGrow™ Recombinant bFGF/FGF-2 (Human), E. coli, Tag Free, 10 µg (Catalog #: CG003-A)
   - RocketCell™ 3D NSC Xeno-Free Suspension Medium, 10 mL (Catalog #: RC01-SM)
   - RocketCell™ Cell Viability Enhancer (1000X), 2 x 50 µL (Catalog #: RC02-CV)
2. VitroGel® Cell Recovery Solution, 100 mL (Catalog #: MS03-100)
3. VitroGel® Organoid Recovery Solution, 100 mL or 500 mL (Catalog #: MS04)
4. VitroPrime™ 3D Culture and Imaging Plates
5. VitroPrime™ Spread-Attach Plates
6. PBS/EDTA (0.5 mM EDTA)
7. Accutase (for 2D dissociation)
8. Accumax (for 3D dissociation)
9. 40 and 70 micron 50 mL tube top filters
10. P1000 pipette and tips
11. 5 mL glass serological pipettes
12. Refer to Appendix A for additional Materials and Reagents from TheWell Bioscience

F. Preparation of RocketCell™ NSC Complete Growth Medium

1. Defrost the 10 mL frozen RocketCell™ NSC Xeno-Free Supplement (50X) at 4°C or on ice.
2. Aseptically transfer the entire contents of the 50X supplement into the basal medium. Otherwise, divide the 50X supplement 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.
4. Resuspend the vials of EGF (10 µg) and FGF-2 (10 µg) in 500 µL of sterile water to a concentration of 20 µg/mL.
5. Once fully dissolved, transfer the entire contents to sterile o-ring microcentrifuge tubes. If using within two weeks, use 1 tube, otherwise freeze smaller volumes in desired number of tubes and freeze.
6. Add EGF and FGF-2 at a concentration of 20 ng/mL (1:1000) to the Growth Medium to make this Complete Growth Medium. For example, for 50 mL of medium, use 50 µL of each growth factor.
7. Mark bottle or tube to indicate the Complete Growth Medium is growth factor supplemented.

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

1. Thaw the frozen bottle of RocketCell™ 3D NSC Xeno-Free Suspension Medium at 4°C and use within 2 weeks.
2. Users may aliquot the Suspension Medium into sterile o-ring tubes, and refreeze once, as described in *Section D "Recommended Use"*.
3. Prior to using in all protocols detailed below, the RocketCell™ 3D NSC Xeno-Free Suspension Medium must be supplemented with a final of 2X of the RocketCell™ Cell Viability Enhancer.
    a. For example, when resuspending a cell pellet in 1000 µL of Suspension Medium, add 2 µL of RocketCell™ Cell Viability Enhancer. Scale as needed.
    b. As this medium is lower salt than DMEM, use at a ratio of gel to suspension medium 1:1 when combining with VitroGel® NEURON. See *Section M, "Additional tips for Hydrogel Formation"* for optimizing VitroGel® NEURON polymerization.

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

1. Prior to feeding, determine the volume of Complete Growth 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

VitroPrime™ 3D Culture and Imaging Plates			
Plate Size	**Cat#**	**Surface Area**	**Volume of Complete Growth Medium**
96-well	**VP-3D96W5**	0.32 cm^2	100-200 mL
24-well	**VP-3D24W5**	1.9 cm^2	500-1000 mL
6-well	**VP-3D6W5**	9.5 cm^2	2000-6000 mL

VitroPrime™ Spread Attach Plates			
Plate Size	**Cat#**	**Surface Area**	**Volume of Complete Growth Medium**
96-well	**VP-SA96W5**	0.32 cm^2	100-200 mL
48-well	**VP-SA48W5**	0.95 cm^2	200-400 mL
24-well	**VP-SA24W5**	1.9 cm^2	500-1000 mL
12-well	**VP-SA12W5**	3.8 cm^2	1000-2000 mL
6-well	**VP-SA6W5**	9.5 cm^2	2000-6000 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 min for more rapid warming.

I. Initiating Primary 3D Culture using 2D Source

Notes: 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 neuronal stem cells, may alter cellular phenotypes. It is recommended that Primary Initiating 3D Culture passaging be accomplished using Accutase (or similar). It has been demonstrated that these agents allow for gentler passaging, greater viability, and less disruption of cellular phenotypes.

A table of volumes (Table 2) of RocketCell™ 3D NSC Xeno-Free Suspension Medium with RocketCell™ 2X Cell Viability Enhancer, VitroGel® NEURON, and Overlay Media recommended for use with VitroPrime™ Spread Attach and VitroPrime™ 3D Culture and Imaging Plates is seen below.

PLEASE NOTE: That the example below (and see Figure 1) will show exact volumes, however we recommend the user calculate extra amounts for each component according to known level of precision of their pipetting apparatus.

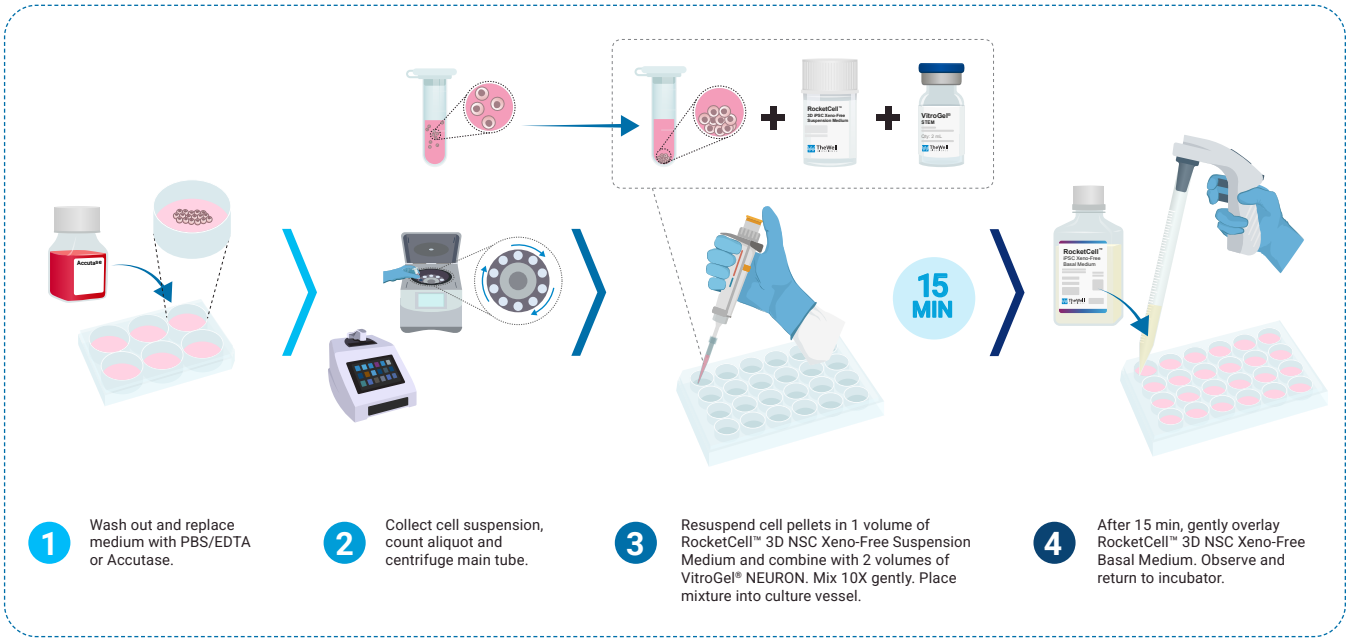


Figure 1: Transitioning from 2D to 3D Cell Culture Workflow

A typical near confluent NSC culture from one well of a 6-well plate may contain between 4-6 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 cells. The density of plating can range from 50,000 to 250,000 cells per well.

Table 2: Suggested Volumes for Cell Suspension, Hydrogel and Overlay Media per Well of **VitroPrime™ Plates**

VitroPrime™ 3D Culture and Imaging Plates				
Plate Size	**Surface Area**	**RocketCell™ 3D NSC Xeno-Free Suspension Medium + 2X RocketCell™ Cell Viability Enhancer**	**VitroGel® NEURON (1:1 ratio)**	**Overlay Media**
96-well	0.32 cm^2	10 µL	10 µL	100 µL
24-well	1.9 cm^2	75 µL	75 µL	300 µL
6-well	9.5 cm^2	600 µL	600 µL	1500 µL

VitroPrime™ Spread Attach Plates				
Plate Size	**Surface Area**	**RocketCell™ 3D NSC Xeno-Free Suspension Medium + 2X RocketCell™ Cell Viability Enhancer**	**VitroGel® NEURON (1:1 ratio)**	**Overlay Media**
96-well	0.32 cm^2	25 µL	25 µL	100 µL
48-well	0.95 cm^2	75 µL	75 µL	150 µL
24-well	1.9 cm^2	150 µL	150 µL	300 µL
12-well	3.8 cm^2	300 µL	300 µL	600 µL
6-well	9.5 cm^2	600 µL	600 µL	1500 µL

Example - Passaging 1 Well of a 6-Well Plate of 2D NSC Cultures to 3D Using a 24-Well Plate:

(A) VitroPrime™ 3D Culture and Imaging Plate or
(B) VitroPrime™ Spread Attach Plate

1. Remove the thawed bottle of **RocketCell™ 3D NSC Xeno-Free Suspension Media** from 4°C storage, spray with disinfectant, wipe, and place in biosafety cabinet. For this example, volumes given are for entire 24-well plate.

   (A) 1.8 mL

   (B) 3.6 mL

2. Remove the tube of **RocketCell™ Cell Viability Enhancer (1000X)** from -20°C, let come to RT for 2-3 min, vortex to mix, centrifuge, and disinfect, and transfer to a Biosafety cabinet.

   For (A) Remove **3.6 µL of RocketCell™ Cell Viability Enhancer** and add to the **1.8 mL** of RocketCell™ 3D NSC Xeno-Free Suspension Medium prepared in Step 1A.

   For (B) Remove **7.2 µL of RocketCell™ Cell Viability Enhancer** and add to the **3.6 mL** of RocketCell™ 3D NSC Xeno-Free Suspension Medium prepared in Step 1B.

3. Place the tube of the **RocketCell™ Cell Viability Enhancer** into -20°C storage.
4. Remove the bottle of VitroGel® NEURON from 4°C storage, spray with disinfectant, wipe, and place in a biosafety cabinet. If new, remove the top blue cover, and peel off the metal retaining ring. For this example:

   (A) 1.8 mL of VitroGel® NEURON will be used.

   (B) 3.6 mL of VitroGel® NEURON will be used.

5. For **each** well of a 6-well plate of 2D cultures to be passaged, **prepare** a tube with 1 mL of RocketCell™ 3D NSC Complete Growth Media containing 1X RocketCell™ Viability Enhancer (1 µL/mL). Alternatively, for 6-wells, combine into a 15 mL tube with 6 mL of the same media. This is used to neutralize the passaging reagent. In this example we assume a yield more than 2.4 million cells/well, and will passage 1 well (1 mL). This total amount of media with 1x RocketCell™ Cell Viability Enahncer can be prepared and pooled in the same tube with the Overlay Media used in the steps below.
6. For each well to be plated in either type of plate, i.e. 24 wells, prepare 300 µL of Overlay Media per well. In this example we will use 24-wells x 300 µL = **7,200 µL Media + 7.2 µL of RocketCell™ Cell Viability Enhancer**. This will be used in Step 22 below.
7. Warm PBS-EDTA to 37°C (or RT), if using an enzyme warm to recommended temperature as per manufacturer.
8. Aspirate medium from one well of the 6-well plate with near confluent NSC cultures.
9. 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.
10. Add 1 mL of Accutase and return to cell culture incubator (37°C) for 5 min. Cells can be passaged with other enzymes of interest, or even non-enzymatic solutions.
11. Remove plate from incubator and observe the passaging well with an inverted microscope. If the cells were confluent, additional time (2-3 min) may be required.
12. Cell layer will begin to retract and dissolve into single cells or small clumps.
13. Once cells have rounded up completely and/or begun to detach, add 1 mL of Complete Growth Medium with 1X RocketCell™ Cell Viability Enhancer and gently triturate cells gently 2-3 times using a 5 mL glass pipette, a fire polished Pasteur pipette, or P1000 pipette.
14. Transfer the dislodged cells into a 15 mL conical tube. The total volume for the 1 well should be approximately 2 mL.

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

15. Remove 10 µL from the cell suspension, combine into new tube with equal amount of Trypan Blue solution (0.4%) and count viable cells.

16. Initiating Primary 3D cultures with 100,000 viable cells per well of both types of the 24-well plate is recommended, but the **initiating** density can range from 50,000 to 250,000 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 new 15 mL conical tube. As above, we recommend accommodation for pipetting error and prepare slightly more than needed. Scale up all solutions.
17. Centrifuge the 15 mL conical tube in at 300 x g for 3-4 min at RT.
18. Carefully aspirate the supernatant, so as not to disturb the pellet.
19. Gently resuspend the pellet with **RocketCell™ 3D NSC Suspension Medium** with **2X Cell Viability Enhancer** using a 5 mL glass pipette or similar.

    (A) 1.8 mL or

    (B) 3.6 mL
20. Add equal **volume** of VitroGel® NEURON, i.e. **(A) 1.8 mL or (B) 3.6 mL** and gently triturate **10** times using a glass 5 mL pipette.
    a. This is a crucial step! NSCs are sensitive to shear force, so use gentle action to homogenize the gel with cells.
21. Once mixed, immediately transfer **(A) 150 µL or (B) 300 µL** of the 1:1 gel:cell mixture per well of a 24-well plate.
    a. If using other plasticware, a quick swirling 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 multichannel reservoir and then dispensed using a multichannel pipette or automated liquid handling device.
22. Set plate aside, cover with the lid and let stand undisturbed for 15 min at RT. It is essential not to move the plate during the polymerization step, as this will lead to loose gel formation, subsequent heterogenous growth pattern of NSCs, and potential cell loss of cells during routine feeding. See *Section M, "Additional tips for Hydrogel Formation"* for additional tips for polymerization.
23. After 15 min (but no longer than 30 min), slowly add 300 µL/well of the **Overlay Medium**. This may be accomplished with a serological pipette on slow speed or a P1000 pipette and running the media down the side of well slowly for (A), or for (B) place medium into the side channel.
24. Observe, and place it in the incubator overnight.
25. Following day use a P1000 pipette to remove and replace 75-80% of the medium with RocketCell™ Complete Growth Medium.
    a. **Optional:** In place of changing cover medium, user may add 1 mL of Complete Growth medium without 1X RocketCell™ Cell Viability Enhancer per well of a 24-well plate for weekend-free culture technique.

J. Maintenance of NSCs in RocketCell™ 3D NSC Xeno-Free Complete Growth Medium

1. Feed cells every day or every other day as with 2D cµLturing techniques by carefully removing 75-80% the media using P1000 or similar pipette and replacing it with fresh room temperature RocketCell™ Complete Growth Media. **See Table 1 for recommended volumes**.
2. Observe daily and proceed with downstream protocols (like passaging, freezing, or immunostaining) as described in sections below after desired amount of time.

K. 3D Subculture from Existing 3D Cultures

Note: As with Initiating **Primary 3D Cultures**, the end user should be mindfµL that overexposure to passaging reagents or digestive enzymes has the potential to damage cells and in the case of pluripotent stem cells, alter cellµLar 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) to break up the neurospheres if needed. We recommend observing the state of the cells following gel recovery to determine if further enzymatic digestion is needed. Refer to Table 2 in *Section I "Initiating Primary 3D Culture using 2D Source"* for volumes of RocketCell™ Suspension Media with 2X RocketCell™ Cell Viability Enhancer, VitroGel® NEURON, and Overlay Medium needed for desired mµLtiwell plate configuration. Growth of NSCs may appear as cells spread throughout the hydrogel, in addition to the appearance of neurospheres. If the user observes heterogenous neurosphere sizes 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 popµLation may lead to heterogenous sizing of NSC colonies, and possible polarization and differentiation.

Loss of some cells may occur, so we recommend counting as best possible to account for both single cells and small spheroids. Alternatively, the user may first 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 initiates cultures as described above, it is expected to obtain roughly 1 million cells/well of 24-well plate after 7 day expansion when starting from 100,000 viable cells. 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 calcµLate in desired amount of extra volume based on precision of pipetting.

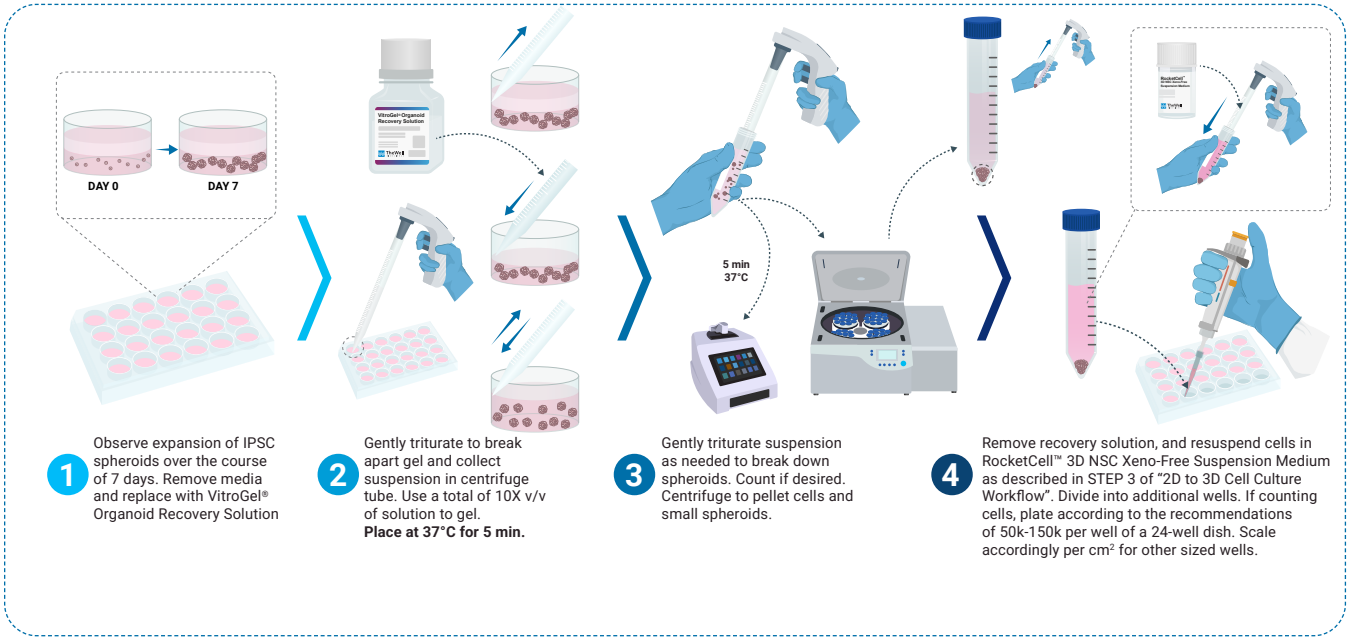


Figure 2: 3D Subculture Workflow

Example; Passaging 4 wells from an Existing 3D Culture from a 24-Well Plate to a New 24-Well Plate;

(A) VitroPrime™ 3D Culture and Imaging Plate or

(B) VitroPrime Spread Attach Plate

1. Prepare needed solutions by following Steps 1-6 in *Section I* "*Initiating Primary 3D Cultures using 2D Source*", but **replacing** volume needed in Step 4 with 4 mL of RocketCell™ Complete Growth Media with 1X RocketCell™ Cell Viability Enhancer. Total volume together with **Overlay Medium 7.2 + 4 = 11.2 mL + 11.2 µL of 1000X RocketCell™ Cell Viability Enhancer**.
2. Warm 10 times the gel volume of 4 wells of the chosen VitroGel® Recovery Solution This is calcµLated for 4 wells of a 24-well plate with (A) 300 µL or (B) 150 µL of hydrogel per well (See Table 2 above).

   For (A) Warm 1.5 mL x 4 wells = 6 mL of VitroGel® Cell Recovery (Catalog #: MS03) or VitroGel® Organoid Recovery (Catalog #: MS04) Solution to 37°C.

   For (B) Warm 3 mL x 4 wells = 12 mL of VitroGel® Cell Recovery Solution (Catalog #: MS03) or VitroGel® Organoid Recovery Solution (Catalog #: MS04) to 37°C.
3. Optional: If user desires to digest neurospheres, warm Accumax solution (0.5 to 1 mL per well used) to recommended temperature as per manufacturer.
4. Optional: If user chooses to filter dissociated neurospheres, label a new 50 mL conical tube for collection of cells and set aside. Set 40 or 70 micron tube top filter aside.
5. Using a P1000 pipette or similar, remove all of the Cover Medium from 4 wells of the 24-well plate after a 7 day growth cycle. With 3D Culture and Imaging plate, vacuum aspiration using the side channel may be used.

   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 o**r** 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. Without triturating the gel/solution, use a 5 mL glass serological pipette, and transfer the hydrogel/solution to the new 15 or 50 mL tube. The entire gel should get sucked into the pipette.
8. For VitroPrime™ Spread Attach Plates, repeat. Add an additional 1.5 mL to the well, and collect into the 15 mL tube.
9. Gently triturate the Gel: Recovery Solution in tube 2-3 times to help breakup the hydrogel. If using a 15 mL tube, 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 tube to a 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 user observes a small gel layer above cell pellet (more likely with VitroGel® Cell Recovery Solution), carefully remove using P1000 or P200 pipette.
14. If cultures contain many neurospheres that require dissociation, add 1-4 mL of Accumax and gently triturate using a glass 5 mL pipette 1-2 times to resuspend spheroids. Otherwise proceed to Step 18.
15. Incubate for 5 min and triturate 3-5 times to help dissociate cells. Observe texture of suspended cells go from larger sandy appearance to a finer silty appearance.
16. Add 1-4 mL of RocketCell™ 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 cells 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:

    For (A) 1.8 mL of RocketCell™ 3D NSC Xeno-Free Suspension Media containing 2X RocketCell™ Cell Viability Enhancer (100,000 cells in 75 µL = density of 1.33 million cells per mL).

    For (B) 3.6 mL of RocketCell™ 3D NSC Xeno-Free Suspension Media with 2X RocketCell™ Cell Viability Enhancer (density of 0.67 million cells per mL).
22. Using a 5 mL glass pipette, add (**A) 1.8 mL or (B) 3.6 mL** of VitroGel® NEURON into the tube, and gently triturate 10 times.
    a. **This is a crucial step!** However, since NSCs are sensitive to shear force, use of gentle action here is required.
23. One properly mixed, transfer **(A) 150 µL or (B) 300 µL** of the 1:1 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 platees, the mixture can be transferred to a standard microwell reservoir and then dispensed using a multichannel pipette or automated liquid handling device.
24. Set plate aside, cover and let stand undisturbed for 15 min at RT. It is **essential** not to move the plate during the polymerization step, as this will lead to loose gel formation, subsequent heterogenous growth pattern of NSCs, and potential cell loss of cells during routine feeding. See *Section M* "A*dditional Tips for Hydrogel Formation*" for additional tips for polymerization.
25. After 15 min (but no longer than 30 min), slowly add growth factor supplemented Overlay Media (300 µL/well of the RocketCell™ Complete Growth Media with 1X RocketCell™ 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 remove 75-80% of the medium using a P1000 pipette and replace with RocketCell™ 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.

L. Initiating 3D Cultures From Cryopreserved NSCs

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 dish (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 2D paradigm using 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™ Suspension Media with 2X 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 I "Initiating Primary 3D Cultures using 2D source"* but replacing Step 4 with 9 mL of growth factor supplemented RocketCell™ Complete Growth Media 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 growth factor supplemented RocketCell™ Complete Growth Media 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 1 mL of growth factor supplemented RocketCell™ Complete Growth Media 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. The user should count cells as above to determine the number of viable cells. 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 K "3D Subculture from Existing 3D Cultures"* for each type of 24-well plate as desired.

M. Additonal Tips for Hydrogel Formation

Many of the VitroGel® Hydrogels indicate mixing at a 2:1 ratio of gel to cells/media. Like RPMI, the NSC base medium has a 40 mM lower salt concentration as compared to DMEM. We have tested the following techniques to increase the efficiency when forming hydrogel;

1. As instructed use VitroGel® NEURON at 1:1 ratio in place of the widely used 2:1 ratio.
2. Using colder temperatures with 1:1 ratio leads to quicker polymerization, therefore the users can keep solutions cool, and/or cool the target plate during 15 min polymerization step.
3. Additional salts may be added to the Suspension Medium to increase final concentration by 40 mM. This will be equivalent to using DMEM medium. After ionic supplementation, users can continue the 1:1 ratio, or try 2:1 ratio This can also be combined with colder temperatures.

N. Immunostaining of NSCs in 3D VitroGel® NEURON Hydrogel in 24-well plate

NOTE: We highly recommend using the VitroPrime™ 3D Culture and Imaging late for routine immunostaining procedures to ensure full integrity of hydrogel during all immunostaining procedure steps. However, with careful technique, other vessels can be used successfully. Users may conduct immunostaining procedures with reagents used with 2D cultures keeping in mind the volume and composition of solution in 3D hydrogel. Therefore, if antibody used is generally diluted 1:1000, we recommend adjusting for final volume of the well. For example, the volume of hydrogel in a well of a VitroPrime™ Spread-Attach 24-well plate is 300 µL, thus adding 300 µL of antibody solution should be diluted now at 1:500. We recommend using equal volume to ensure best incorporation or mixing of solutions into the hydrogel and better outcomes. Further antibody staining can be accomplished with live (1-2 hours at RT) and/or fixed cells (overnight at 4°C). The hydrogel is optically clear and allows for best light penetration. If needed, users can also clarify post-stained cultures as NSC spheroids that are out of focal position can obscure staining of overlapping spheroids. Scale volumes for other sizes of plates.

Optional: To help ensure integrity of hydrogel throughout the procedure, the user may supplement all the solutions mentioned below with additional NaCl. We have tested the addition of 70 mM NaCl using 5M NaCl stock solution. If using antibodies against internal epitopes, include 0.1% Triton X-100 (and/or similar concentrations of NP40, Saponin, or others) with desired blocking and staining solutions. Additionally, if users observe high background fluorescence, they can use 0.1% Tween-20 surfactant in rinse solutions (PBS) to help remove non specific binding to hydrogel.

Fixed Cell Immunostaining

1. Place plate into fridge for 15-30 min to cool.
2. Remove media from well using P1000 or P200 tip.
3. **Optional:** Add cool PBS containing Ca^{2+}/Mg^{2+} or similar buffer to help wash residual proteins that may interfere with fixation procedure. Remove PBS.
4. Add cool 1 mL of 4% neutral buffered formaldehyde.
5. Return plate into 4°C for 1 hour.
6. Remove paraformaldehyde and safely dispose of solution using P1000 tip.
7. Add cool Wash Solution (PBS-Ca^{2+}/Mg^{2+} with 20 mM Tris). The TRIS will help neutralize the paraformaldehyde
8. Wash a total of three times with cool Wash Solution.
9. Add blocking solution (3% BSA in PBS or similar as determined for use with particular antibodies) and return to fridge for 1-2 hours.
10. Remove blocking solution and add diluted primary antibodies. We have tested and validated directly labeled antibodies for detection of pluripotency markers. Alternatively use unlabeled antibodies followed by secondary fluorescently labeled antibodies.

11. If using directly labeled antibodies, the following day remove antibody solution, wash each well three times with PBS-Ca^{2+}/Mg^{2+} (10 min incubation) and proceed with imaging. Otherwise proceed with Step 13.
12. Optional: Prior to imaging remove all wash solutions and use common antifade reagents without or with DAPI or Hoechst 33342 as directed by manufacturer to help preserve the fluorescent signal during imaging techniques, and counterstain the nuclei blue, respectively.
13. If using unlabeled antibodies, remove primary antibody solution, wash three times with PBS-Ca^{2+}/Mg^{2+} (incubate each for 10 min), and proceed with second step antibodies diluted in blocking solution.
14. Incubate at room temperature for 1-2 hours, or at 4°C for 4-6 hours.
15. Proceed as in Step 11 and 12.

Live Cell Immunostaining

1. We have validated several directly antibodies to cell surface epitopes to function in standard live cell immunostaining techniques for NSCs.
2. Remove cell culture media and replace with diluted directly labeled antibodies (use 2X recommended concentration as per manufacturer).
3. Incubate at 37°C for 1 hour, or 4°C for 2hrs.
4. Alternatively, 2X unlabeled antibodies may be used with similar incubation times, followed by three washes with standard base media (like DMEM/F12) as described above, followed by diluted 2X secondary antibodies in Growth Media, and then final three washes.
5. If the user plans to keep cell cultures alive post imaging the final three washes may be in base media, then RocketCell™ Complete Growth Media for imaging.
6. Alternatively, if user wants to preserve staining use PBS-Ca^{2+}/Mg^{2+} for washes, followed by paraformaldehyde fixation, and washes. Further staining of internal epitopes can also be accomplished by further blocking, staining, washes and preservation as detailed above.

Tables

Table 3: Immunophenotyping of NSC populations via immunofluorescence microscopy

Marker	NSC
Nestin	Positive
Pax6	Positive
Sox2	Positive

Appendix A: Other products for culture of NSCs

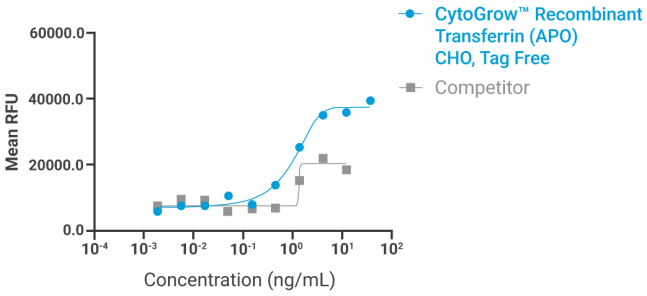
- **VitroGel® NEURON** (VHM07)
- **RocketCell™ NSC Xeno-Free Growth Medium** (RC01-GM)
- **CytoGrow™ Growth Factors**
- **VitroPrime™ 3D Culture and Imaging Plate**
  - » VitroPrime™ 3D Culture and Imaging Plate, 6-Well (VP-3D6W)
  - » VitroPrime™ 3D Culture and Imaging Plate, 24-Well (VP-3D24W)
  - » VitroPrime™ 3D Culture and Imaging Plate, 96-Well (VP-3D96W)
- **VitroPrime™ Spread-Attach Plate**
  - » VitroPrime™ Spread-Attach Plate, 6-Well (VP-SA6W)
  - » VitroPrime™ Spread-Attach Plate, 12-Well (VP-SA12W)
  - » VitroPrime™ Spread-Attach Plate, 96-Well (VP-SA96W)
  - » VitroPrime™ 3D Culture and Imaging Plate, 96-Well (VP-3D96W)
- **Cyto3D® Live-Dead Assay Kit** (BM01)

Appendix B: CytoGrow™ Growth Factors for Neuron Culture

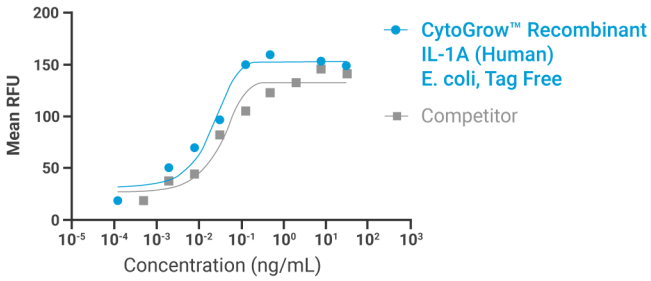
Growth Factor	Cat. No.	Species	Host cell
EGFs (Epidermal Growth Factors)			
EGF	CG014	Human	CHO
NRG1-β1	CG106	Human	E. coli
FGFs (Fibroblast Growth Factors)			
FGF-10/KGF2	CG020	Human	CHO
FGF-8b	CG021	Human	E. coli
FGF-9	CG022	Human	CHO
IGFs (Insulin-like Growth Factors)			
IGF-1	CG120	Human	HEK293
Insulin	CG033	Human	Yeast
LR3 IGF-1	CG054	Human	CHO
NTs (Neurotrophins)			
BDNF	CG002	Human	CHO
NT-3	CG105	Human	HEK293
TGFs (Transforming Growth Factors)			
Activin A	CG001	Human	CHO
Wnt - Related Growth Factors			
Wnt-3a	CG084	Human	CHO
Other Growth Factors			
HGF	CG032	Human	CHO
Noggin	CG059	Human	CHO
OSM	CG061	Human	CHO
Prolactin	CG066	Human	CHO

Growth Factor	Cat. No.	Species	Host cell
Interleukins			
IL-1A	CG036	Human	E. coli
IL-1B	CG037	Human	E. coli
IL-3	CG096	Human	CHO
IL-4	CG040	Human	CHO
IL-6	CG041	Human	CHO
IL-7	CG042	Human	CHO
IL-12	CG043	Human	CHO
IL-13	CG044	Human	CHO
IL-15	CG045	Human	CHO
IL-15(hFc Tag)	CG046	Human	CHO
IL-21	CG048	Human	CHO
IL-21(hFc Tag)	CG049	Human	CHO
IL-23	CG050	Human	CHO
IL-27(His Tag)	CG051	Mouse	HEK293
IL-34(His Tag)	CG052	Human	HEK293
ECMs			
Fibronectin	CG024	Human	CHO
Accessory Proteins (Supplements and Coagulants)			
Insulin-Transferrin-Selenium	CG086		
Transferrin	CG075	Human	CHO
Transferrin-APO	CG076	Human	CHO

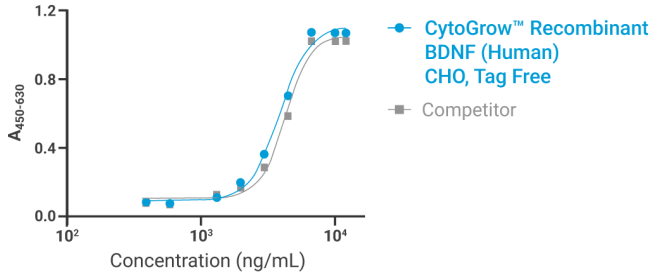
Transferrin (APO) Human, Recombinant Protein, CHO, Tag Free



IL-1A (Human), Recombinant Protein, E. coli, Tag Free



BDNF (Human), Recombinant Protein, CHO, Tag Free



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3D Cell Culture and Beyond

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