Instruction Manual:
Protocol for Growth of Stem Cells on IPS-Spheres in Multi-well Plates

Version 2.0

Overview

This protocol describes the handling and use of IPS-Spheres, a micro-carrier (MC) bead scaffold for the growth, maintenance and passaging of human pluripotent stem cells (hPSC) in culture. Use of IPS-Spheres in cell culture applications will facilitate the growth of high quality and high quantities of naturally-derived or induced pluripotent stem cells (iPSC) in a growth format very similar to embryoid bodies (EB) or spheroids.

Stem cells can be maintained and passaged on IPS-Spheres, and directed into terminal differentiation protocols directly from the cultured (EB) format. This provides consistency and eliminates any intermediate EB formation protocols or stages, thereby saving time, cost and providing greater reproducibility and efficiency.

There are a great many possible combinations of cell culture medium for use with hPSC (natural or induced pluripotent stem cell lines). Furthermore, such cells can exhibit great heterogeneity in growth and differentiation characteristics. Hence, there is no universal protocol that will work each and every time for all cells and applications.

The protocol(s) described here are general guidelines for set-up and use of the IPS-Spheres for hPSC cultivation. Empirical optimization may be required to get the best performance of the system with your chosen cell type and desired medium.

It is strongly recommended that you read and fully understand the entire protocol before beginning your experiments. To maintain sterility, all procedures (except as indicated) should be performed in a sterile biological safety cabinet.

Conditions of Use

This product is for research use only. It should not be used for therapeutic or diagnostic purposes. Sale of this product to a third party, or any other commercial use for this product, is prohibited without prior permission from ReproCELL (Japan) or Brilliant Research (Singapore).

Storage

The IPS-Spheres are suspended in a sterile solution of Phosphate Buffered Saline (PBS) and supplied in a screw-top microfuge vial. The IPS-Spheres should be stored at 4 to 8°C. The product is guaranteed stable for one year after purchase, when handled and stored properly.

Features of the iPS-Spheres

- The IPS-Spheres are cell culture micro-carrier beads optimized for the growth of stem cells or iPS cells under feeder-free culture conditions.
- The IPS-Spheres are made of polystyrene plastic and each approximately 100 µm in diameter.
- The surface of IPS-Spheres are coated with Laminin 521 (extracellular matrix protein) to aid in cell attachment.
- The total surface area of 20 mg of IPS-Spheres is approximately equal to the surface area of one well of a 6-well plate.

General Notes

- Non-adherent / ultra-low-attachment (ULA) plates are required to culture cells on the iPS-Spheres.
- When attached, 3 to 10 iPS cells can probably fully encompass a single MC bead; however, multiple beads are usually aggregated with many cells, forming a 3D structure similar to an embryoid body.
- The initial seeding of dissociated stem cells on IPS-Spheres should be done in the presence of 10 µM ROCK inhibitor to ensure cell viability. Subsequent medium changes do not require ROCK inhibitor.
- Culture plates of iPS cells on IPS-Spheres can be maintained under static conditions, or with gentle circular agitation
- Passaging iPS cells on IPS-Spheres does not require trypsinization or other enzyme treatments; the protocol employs physical disruption by pipetting only.

Brilliant Research IPS-Spheres
(from ReproCELL)

Product Description	Cat. No.	Unit	Storage
IPS-Spheres (*small*)	BR-01S-050	50mg	4°C
IPS-Spheres (*large*)	BR-01S-150	150mg	4°C

Other Reagents and Equipment

Product	Cat. No.	Supplier	Storage
TrypLE™ Select	12563011	Thermo Fisher	-20°C
Y27632 ROCK Inhibitor	04-0012	ReproCELL / Stemgent	-20°C
6- well plate (non-adherent)	3471	Corning	RT
PBS (-). Ca^{2+}- and Mg^{2+}-Free		Cell Culture Reagent Suppliers	4°C
Standard cell culture equipment		Cell Culture Reagent Suppliers	RT

Contents

A) Seeding the iPS cells on the IPS-Spheres

NOTE: Human pluripotent stem cells need only to be dissociated to single cells and seeded on the IPS-Spheres one time. Once a culture on IPS-Spheres is established, the cells can be transferred directly (passaged) between micro-carrier beads by physical means.

1. It is recommended that you aseptically pipette the entire amount of IPS-Spheres suspension to a sterile 15-mL tube.
2. Rinse the vial twice with 1 mL of sterile PBS and add rinse solution to the 15-mL tube containing IPS-Spheres suspension.
3. Once the IPS-Spheres have settled (3-5 min), the PBS may be aseptically removed by careful aspiration or pipetting. Care should be taken not to aspirate the settled pellet of IPS-Spheres.

NOTE: If you do not use all the IPS-Spheres, they can be stored in the PBS buffer (in the 15-mL conical tube) at 4°C for later use.

4. Reconstitute the desired amount of IPS-Spheres in media and add to the plate. *Recommendation*: 20 mg IPS-Spheres for 5 mL culture in 6-well ULA (ultra-low-attachment) plate.

NOTE: It is usually necessary to use ROCK inhibitor (10 µM) in the cell culture medium for the first 24 hours during the initial plating of iPS cells.

5. Prepare a single cell suspension of undifferentiated hPSCs according to your own cell dissociation protocol. *Recommendation*: Gently dissociate the hPSCs by incubating with TrypLE™ Select at 0.5x concentration for ~10 minutes followed by physical disruption with ~10-strokes of a P1000 pipetman.
6. Pellet the cells by centrifugation and remove the supernatant. Resuspend the cells in a small volume of sterile PBS solution (e.g. 1 ml).
7. Count the cells and determine the number of viable cells per milliliter.
8. Add the appropriate volume of viable hPSCs (recommend $1x10^6$ cells) to the 6-well ULA plate containing the IPS-Spheres (step 4). The final cell culture should be 5 mL of medium containing $2x10^5$ cells/mL and 4 mg/mL IPS-Spheres.
9. After adding cells, gently pipette 3 times with 10-mL serological pipette to facilitate the even distribution of cells throughout the culture plate well.
10. Agitate the plate at 100-110 rpm for a minimum of 2 hours in a 37°C incubator with 5% CO_2 and 95% humidity.

NOTE: If a circular agitation device is not available, it is recommended that the plate be swirled (by hand) every 20-30 minutes for a minimum of 2 hours to promote cell attachment and bead clustering.

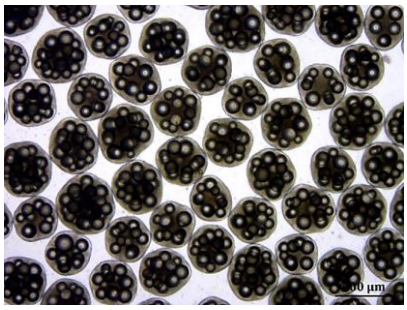
11. After the two hours, the cells can continue to be grown with constant agitation or placed in static culture.
12. Medium changes should be done every day or as needed for your particular cell & medium.

NOTE: The amount of cells (single cells) used to seed, the passage density, and the passage timing (day 5 or day 6 or day 7, etc.) are all variables that need to be

empirically determined with IPS-Spheres. Cell lines and growth medium will impact overall growth rates and final achievable cell densities.

NOTE: Stem cells grown on IPS-Spheres at high density growth under agitation conditions will form clusters of 10-20 micro-carrier beads fully enveloped in cell growth (see Figure 1). These clusters resemble embryoid bodies (EB) in both appearance and function. Since cells are maintained in an EB-like state, they are immediately available for many differentiation procedures that require 3D cultures for optimal performance.

Figure 1 – Human pluripotent stem cell clusters on IPS-Spheres after 4-5 days growth using constant agitation.



B) Changing Culture Medium on IPS-Spheres Grown in Multi-well Cell Culture Plates

The process of changing (refreshing) cell culture medium is simple to perform since cells grown on IPS-Spheres aggregate into clusters that are easily visible by eye, and rapidly settle when stationary.

1. Remove the culture plate from the incubator and place in a biological safety hood.
2. Rest on side of the cell culture plate against an object that is about 20 mm high (e.g. a microfuge tube rack or another 6-well plate), so that the culture plate is tilted, but media does not overflow.
3. Let the plate sit undisturbed for several minutes. Gentle rocking (left to right) or tapping can encourage the IPS-Spheres to settle to the bottom (one-side) of the well.
4. Using a 5 mL pipette and without disrupting the settled beads, remove and discard at least 4 mL of the spent cell culture medium. Withdrawing medium from the "surface" of the liquid will not disrupt the settled aggregates.
5. Add back an equal amount (about 4 mL) of pre-warmed, fresh cell culture medium.

NOTE: For culture medium change on IPS-Spheres, ROCK inhibitor is not required.

C) Passaging iPS cells grown on IPS-Spheres

There are three stages of the passaging process; harvesting, counting, and seeding. The process is conceptually similar to the standard plate-grown method protocol except that with IPS-Spheres, only a small portion of the cells need to be enzymatically dissociated from the micro-carrier beads (for counting purposes only).

1.) Harvesting the IPS-Spheres

1. Remove plate from incubator and place in a Biological Safety (tissue culture) laminar flow hood.
2. With a 5 mL pipet, remove the full contents (culture medium and IPS-Spheres) and place in a 15-mL sterile conical tube.
3. Rinse the well with 5 mL of fresh pre-warmed medium and add it to the same conical tube.
4. Let the aggregated IPS-Spheres settle to the bottom (usually 2-5 min is sufficient).
5. With a 10-mL pipet, remove most of the medium from above the IPS-Spheres settled pellet.
6. With a P1000 or P200 pipetman, remove as much medium as possible without disturbing the pellet. The pellet will occupy about 50 µL volume.
7. Add 950 µL of fresh medium to the pellet, gently mix and set aside in the biological safety hood.

Preparing the fresh IPS-Spheres

It is best to first prepare fresh IPS-Spheres for the seeding stage in advance and as follows:

1. Determine the amount of IPS-Spheres to be used for the experimental set-up. It is recommended to use 20 mg in a single well of a 6-well plate.
2. Remove the total amount of IPS-Spheres to be used and transfer to a sterile microfuge tube. Add about 1 mL of fresh sterile PBS to rinse the beads.
3. Let settle for 5 minutes or centrifuge at low speed to pellet the micro-carrier beads.
4. With a P1000 pipetman, remove as much of the buffer as possible without disturbing the pellet.
5. Add fresh cell culture growth medium to the beads at a concentration of 20 mg (beads) per 100 µL of medium. Set aside at room temperature for later use.

2.) Counting the iPS cells

In order to get an accurate count and prepare the cells for passaging, the harvested cell suspension (from Step 7, page 3) must be physically disrupted to break apart the aggregated IPS-Spheres.

1. Using a P1000 pipetman and with gently pipetting force, physically break the IPS-Sphere cell aggregates into small sizes. Be careful to avoid making bubbles. Usually 35-40 repeated pipetting strokes are required and ideal.

NOTE: Aggregate disruption should be visible to the eye, but can be checked by collecting a small amount (20 µL) and observing under the inverted microscope. Disruption down to single spheres should be evident, with large clumps of cells freely floating in the medium. Pipet only enough to reach the state described and no more. Continued aggressive pipetting (50x or more) will result in high rates of cell mortality.

NOTE: Keep the tube at room temperature in the biological safety hood. Proceed to the next step of cell counting and work swiftly to minimize time.

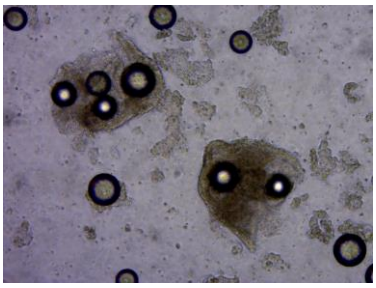
2. With a P200 pipetman set at 100 µL, pipet a few times to get a uniform suspension of cells and IPS-Spheres. When the suspension appears homogeneously suspended, quickly withdraw 100 µL of sample and place in a fresh 15 ml sterile conical tube.
3. To that tube, add 900 µL of prewarmed 0.5x TripLE™ Select cell dissociation solution. Mix by swirling or gentle pipetting. Place the tube in a 37°C incubator for 10 minutes.

NOTE: Usually 5-10 minutes is sufficient to dissociate cells from the micro-carrier beads.

4. After incubation, with a P1000 pipetman, gently disperse the cells by gently pipetting about 10 times. Fully dissociated from the IPS-Spheres and each other, the appearance of the suspension may be slightly milky white.
5. Remove a portion for counting. If using Trypan Blue, remove 10 µL of cells and mix with an equal volume of Trypan Blue solution.
6. Count the cells to derive a concentration of cells per milliliter, and a cell viability assessment.

NOTE: A live cell ratio of around 60-70% is expected for good handling and a healthy culture. For 20 mg of IPS-Spheres, it is common for there to be $0.5x10^7$…to… $1x10^7$ cells yielded from a single well's growth.

Figure 2 – Disrupted aggregates of hPSC (after gentle repetitive pipetting) suitable for passage.



3.) Seeding the Cells

1. Prepare a new non-adherent 6-well plate and label as appropriate.
2. Add 5 ml of fresh medium (containing 10 µM of ROCK Inhibitor) to the wells as needed.
3. Also add 20 mg of fresh (new) IPS-Spheres that were rinsed (in sterile PBS) and suspended in cell culture medium (100 µL) as described in step 5 on the prior page.
4. Back calculate the total number of live cells in the original 1 ml of harvested sample (from Step 7, page 3).

NOTE: For cell passaging, the cell-seeding density is dependent on your cell line, culture medium and desired amplification. For rich medium such as StemFit AK02N, as little as $5x10^4$ cells can be used to seed a fresh well in a 6-well plate. Seeding with $1x10^6$ cells is likely the upper limit.

5. Determine the volume of the cell suspension that is needed to achieve your desired seed quantity. With a pipetman set for the required quantity, gently pipet repeatedly to homogenously suspend the cells and micro-carrier beads. Then quickly remove the required amount to seed each well on the new plate.

NOTE: Cells on IPS-Spheres are not enzymatically dissociated from the IPS-Spheres during passage; they are simply mechanically stripped. This is less disruptive to their surface proteins.

6. Place the newly passaged plate on a rotating platform for at a minimum of 2 hours to allow cells and beads to interact and exchange.

NOTE: If observed under the microscope, almost immediately, you can see fresh individual IPS-Spheres beginning to associate with cell clusters and other beads.

7. Incubate and change the medium routinely, such as every day until the cells are ready for passage again. No ROCK inhibitor is required for medium changes.

Frequently Asked Questions

Q. **Can the IPS-Spheres remain at room temperature for prolonged periods?**
A. Although it is recommended to store the IPS-Spheres at 4°C to 8°C, leaving them at room temperature for several hours does not damage the beads.

Q. **Can iPS-Spheres be used in a Bioreactor (spinner-flask) or shake-flask for scale-up?**
A. Yes, it is possible. A concentration of 4 mg/mL of micro-carrier beads is recommended. Medium change in Bioreactors with IPS-Spheres is simple since the cell aggregates will easily settle to the bottom of the vessel and medium can be easily removed from above. Furthermore, compared to spheroid culture, in the micro-carrier format, the agitation speed is not so critical for EB formation on IPS-Spheres. Even a gently shaking Erlenmeyer-format culture flask can be used. Reproducibility, yields and success are frequently better when using micro-carriers such as IPS-Spheres in bioreactors than without.

Q. **Can the IPS-Spheres be used for cultivation of Mesenchymal Stem Cells (MSC) or other cells?**
A. IPS-Spheres are not recommended for MSC culture. The bead size (100 µm) of the IPS-Spheres is optimal for iPS cells, but believed to be too small for effective culturing of MSC. Furthermore, the extracellular matrix protein is also not optimal for MSC. A slightly larger bead size is believed to be better for MSC culture. Brilliant Research is currently developing MSC-Spheres for commercial launch at some future date. The ability to grow other cells on IPS-Spheres is largely unknown.

Q. **Is it possible to remove IPS-Spheres from a cell culture and collect the cells?**
A. Yes, IPS-Spheres can be removed from a cell culture by first enzymatically dissociating cells from the micro-carrier beads and then filtering through a 40 micron mesh cell culture filter. Beads are generally around 100 microns in size, and hence easily retained by the filter. Individual cells or small clumps of cells will easily pass through the mesh filter.

Q. **What kind of cell growth or population expansion can I expect from the IPS-Spheres?**
A. Population growth kinetics vary according to the choice of cells and medium. However, it is typical to achieve ~$1x10^6$ to $2x10^6$ cells/ml when growing in 6-well plates using 20 mg of IPS-Spheres per well. The yield in bioreactors can exceed plate cell densities, but is dependent on many other factors.

References

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