Applied StemCell, Inc.
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Datasheet

MyEZGel™ 3D-iPSC Matrix (hiPSC Using Guide)

Product Information

Catalog Number

ASR-3055

Description

The MyEZGel™ 3D-iPSC Matrix is a powerful tool for *in vitro* 3D human induced pluripotent stem cell (hiPSC) culture, with more accurate *in vivo* predictions for life science research and development. The MyEZGel™ 3D-iPSC Matrix kit consists of a vial of MyEZGel™ Matrix Solution, a patented peptide nanofiber solution; a vial of matrix trigger, MyEZGel™ Kicker Solution; and a vial of MyEZGel™ Gro. The MyEZGel™ nanofibrils are formulated into a basic or a customer desired cell culture medium in neutral pH. A 3D microenvironment can be formed accordingly for hiPSC growth. With MyEZGel™, cells no longer suffer acidic or chill conditions; cultured cells are easily harvested from the matrix; all operating and growth procedures can be completed at room temperature or 37°C in neutral pH.

Advantages of MyEZGel™ 3D-iPSC Matrix:

- Enables xeno-free cell culture
- No acidic or chill pre-gel solution
- Mimics cell microenvironment
- Cells are easily encapsulated
- Fast hydrogel formation (~30 min)
- Purified Synthetic Peptide in neutral pH
- Works with standard methodologies
- Cell suspension at room temperature
- Easy for cell harvest after 3D culture

Quantity

ASR-3053: 2 mL
ASR-3054: 6 mL
ASR-3055: 10 mL
ASR-3056: 20 mL

Components

	ASR-3053	ASR-3054	ASR-3055	ASR-3056
MyEZGel™ Matrix Solution	2 mL (ASR-3053-1)	6 mL (ASR-3054-1)	10 mL (ASR-3055-1)	20 mL (ASR-3056-1)
MyEZGel™ Kicker Solution	0.3 mL (ASR-3053-2)	0.3 mL (ASR-3054-2)	0.5 mL (ASR-3055-2)	1.0 mL (ASR-3056-2)
MyEZGel™ Gro Solution	50 µL (ASR-3053-3)	150 µL (ASR-3054-3)	250 µL (ASR-3055-3)	500 µL (ASR-3056-3)

Source

Synthetic peptides from amino acids

Shipping

Dry Ice

Storage and Stability

MyEZGel™ Matrix Solution and Kicker Solution should be stored at 4°C; MyEZGel™ Gro solution should be stored at -20°C.

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Quality Control	Each lot of MyEZGel™ 3D-iPSC Matrix is subject to QA/QC procedures to ensure consistency and quality. Certificate of Analysis is available upon request.
Safety Precaution	Please wear the appropriate Personal Protection Equipment (lab coat, gloves, safety goggles) when handling the cells and MyEZGel™ 3D-iPSC Matrix.
Restricted Use	Products described here are for research use only and not intended for human or animal diagnostic or therapeutic uses.

Additional Reagents Required but not Provided

- Control 2D hiPSC, Cat# ASE-9211 (or) Control 3D-iPSC Line, Cat# ASE-9050; Applied StemCell
- mTeSR™1 Feeder Free Media, STEMCELL Technologies, Cat# 05850
- Dulbecco's Phosphate-Buffered Saline (DPBS), Sigma-Aldrich, Cat# D8537
- 1X PBS, ThermoFisher, Cat# 15575020
- EDTA, MidSci, Cat# IB70185
- DMSO, Sigma-Aldrich, Cat# D8418
- 10% Neutral Buffered Formalin, Fisher Scientific, Cat# 23-305510
- Triton X-100, Sigma-Aldrich, Cat# T8787
- Cold Water Fish Gelatin (Teleostean Gelatin), Sigma-Aldrich, G7765
- Normal Rabbit Serum, ThermoFisher Scientific, Cat# 31883
- Goat Oct3/4 Antibody (N-19) 100 µg/mL, Santa Cruz Biotechnology, Cat# sc-8628
- Rabbit Anti-Goat IgG(H+L) 2° Antibody Alexa Fluor 488, ThermoFisher Scientific, Cat# A-11078

Protocol

Before Using the MyEZGel™ 3D-iPSC Matrix, Please Read the Following Two Messages Carefully:

Message# 1: Mixing Ratio Notice

The hiPSC-qualified 3D hydrogel matrix solution (MyEZGel™ Matrix Solution) contains 1% W/V stock solution of standard peptide. If you are a first-time user, we recommend using different mixing ratios in the range of 0.3-0.6% W/V final peptide concentration for hiPSC encapsulation (hydrogel concentration), in order to identify the best mixing ratio for your cells. The Table 2 presents examples of two mixing ratios at 0.3% and 0.5% concentration. Please use the following table as a reference to mix the MyEZGel™ Matrix Solution and cell suspension.

Note:

- *MyEZGel™ Kicker solution is always 2% of the final plating volume of MyEZGel™ 3D-iPSC Matrix + cell suspension together. Example: Final plating volume for one well of a 12-well plate is 1000 µL.*
- *For hiPSC culture, 0.5% is suitable for all sizes listed, while 0.3% can be used for 48-well and 96-well plates.*
- *Add the MyEZGel™ Kicker Solution to your cell suspension FIRST, before you mix the MyEZGel™ Matrix Solution with your cell suspension. If you still have questions, please contact technical support at info@appliedstemcell.com.*
- *The 3D-iPSC matrix should be prepared fresh for use each time. Do not pre-mix the Matrix Solution and Kicker Solution and store.*

Message# 2: Add Medium on the Top of Hydrogel to Prevent Drying and for Long Term Culture

After hydrogel formation (30 minutes but not longer than 1 hour, at 37°C after mixing), cell medium needs to be added to the top of the gel to provide fresh nutrition and prevent drying for long term culture. Media should be changed every day from day 2 of culture. Refer to Table 3 for the recommended volume of medium to be added to each well based on different well sizes.

1. Cell Encapsulation and Culture

Note:

- *The recommended seeding cell density for hiPSC from subculture is (1.8 – 2) x 10^5 cells/ mL in a 0.5% W/V concentration of hydrogel matrix, MyEZGel™ 3D-iPSC Matrix.*
- *Seeding cell density for directly thawing cryopreserved hiPSC in a 0.5% MyEZGel™ 3D-iPSC Matrix is (3.6 – 4) x 10^5 cells/ mL. It is recommended to have a 1-2 times higher seeding density for frozen cells, in order to maintain good cell viability and proliferation.*

1.1 Warm the MyEZGel™ Matrix Solution and MyEZGel™ Kicker Solution to room temperature (15 - 25°C) or 37 °C (37°C water bath). Pre-warm mTeSR1 complete medium to room temperature.

1.2 To prepare cell medium stock solution, thaw MyEZGel™ Gro**,** and add it into mTeSR1 complete medium at ratio 1:1000 v/v (MyEZGel™ Gro: mTeSR1 complete medium).
Note: MyEZGel™ Gro is used as a cell culture supplement and should be diluted into mTeSR1 medium immediately before use; use within two weeks after dilution. Medium used for cell culture in this protocol are all supplemented with MyEZGel™ Gro.

1.3 To make a cell suspension from subculture:
1.3.1 Harvest cells from your 2D or 3D subculture and count the cells.
1.3.2 Centrifuge the cells at 200g for 5 minutes.
1.3.3 Proceed to step #1.5.

1.4 To make a cell suspension from cryopreserved cells:
1.4.1 Thaw the vial containing frozen hiPSC by gently agitating in a 37 °C water bath. To reduce the possibility of contamination, keep the O-ring and cap out of the water.
1.4.2 Remove the vial from the water bath as soon as the contents start to thaw and place on ice.
1.4.3 Add 1 mL of pre-warmed mTeSR1 complete medium to the hiPSC vial and pipette until the cell suspension is completely thawed.
1.4.4 Transfer the cell suspension to a 15 mL conical tube. Use another 1 mL of mTeSR1 complete medium to rinse the vial for the remaining cells and add to the cell suspension in the conical tube.
1.4.5 Dilute the mixture to 10 mL using mTeSR1 complete medium. Count the cells.
1.4.6 Centrifuge at 200 g for 5 minutes.
1.4.7 Proceed to step #1.5

1.5 Aspirate the supernatant and resuspend cells in mTeSR1 complete media supplemented with MyEZGel™ Gro such that the final seeding cell density in MyEZGel™ 3D-iPSC Matrix+ mTeSR™ complete media for plating is (1.8 - 2) x 10^5 cell/ mL for cells from subculture or (3.6 – 4) x 10^5 cells/ mL for cryopreserved cells.
Note:

- *Make sure your starting cell suspension (= stock cell suspension) has the adequate amount of cells to prepare your desired final seeding density.*
- *The stock cell suspension density will vary depending on the percentage of hydrogel matrix to be used.*
- *Calculate stock cell suspension density using the below formula:*

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$$\text{Stock cell suspension} = \frac{\text{Final seeding cell density (cell/mL)}}{\text{Volume of cells/ well (mL)}^{**}}$$

***Refer to Table 2 for examples of volume of cells/ well for 0.3% and 0.5% matrix. Calculate volume of cells/ well for other hydrogel % based on this table.*

- *Table 1 provides examples of stock cell suspension density for 0.3% - 0.6% MyEZGel™ 3D-iPSC Matrix (hydrogel) calculated for a final seeding density of 2 x 10^5 cells/ mL (for hiPSC from subculture) and 4 x 10^5 cells/mL (for cryopreserved hiPSC).*

Table 1. Examples of stock cell suspension density for 0.3% - 0.6% MyEZGel™ Matrix*

Final Seeding Density (cells/mL)	Hydrogel Concentration (%)			
	0.3%	0.4%	0.5%	0.6%
2 x 10^5 cells/mL (Subculture)	3 x 10^5	3.5 x 10^5	4.2 x 10^5	5.3 x 10^5
4 x 10^5 cells/mL (Frozen cells)	6 x 10^5	7 x 10^5	8.4 x 10^5	10.6 x 10^5

** Stock cell suspension is calculated based on a final seeding density of 2 x 10^5 cells/ mL. For a different final density, please calculate the stock cell suspension density using the given formula.*

1.6 Then, add MyEZGel™ Kicker Solution to the cell suspension, according to the Mixing Ratio in Table 2 (pipet well without introducing air bubbles by always immersing pipet tip into cell solution to avoid introducing air bubbles).

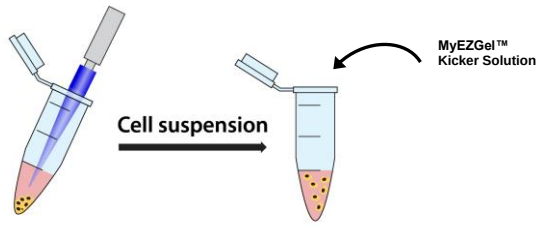


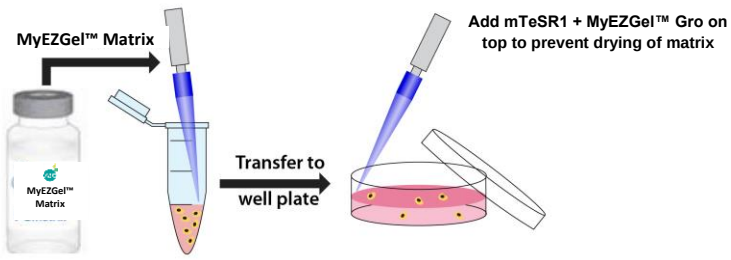
Table 2. Examples of mixing ratios of MyEZGel™ Matrix Solution, cell suspension and MyEZGel™ Kicker Solution*, and maximum plating volume for different well sizes

Well Plate	0.3% W/V			0.5% W/V			Maximum Plating Volume for 1 Well (µL)
	Cell Suspension (µL)	MyEZGel™ Kicker Solution (µL)	MyEZGel™ Matrix 1% Solution (µL)	Cell Suspension (µL)	MyEZGel™ Kicker Solution (µL)	MyEZGel™ Matrix Solution (µL)	
12	680	20	300	480	20	500	1000
24	340	10	150	240	10	250	500
48	170	5	75	120	5	125	250
96	68	2	30	48	2	50	100

**MyEZGel™ Kicker solution is always 2% of the final plating volume of MyEZGel™ 3D-iPSC Matrix + cell suspension together. Example: Final plating volume for one well of a 12-well plate is 1000 µL.*

1.7 Mix the MyEZGel™ Matrix Solution carefully into the cell suspension of step #1.6 at the mixing ratio indicated in Table 2 (pipet well without introducing air bubbles).

1.8 Transfer the mixture into the center of each well, then swirl the plate to cover the entire well bottom surface.



1.9 Incubate the plate at 37°C (5% CO_2) for 30 minutes or longer (but not more than 1 hour) to complete the gelation.

1.10 After gelation, add cell medium (mTeSR1 complete medium supplemented with MyEZGel™ Gro) on top of the gel in each well to prevent the matrix from drying (recommended volume of each well is listed in Table 2).
Note: Add the cell culture medium gently along the wall of each well on the top surface without disturbing the hydrogel).

Table 3. The recommended volume of medium to add to the hydrogel to prevent drying

	12-well	24-well	48-well	96-well
Volume to add (µL)	2000	1000	500	200

1.11 Change the medium very gently by adding or replacing part or all of the medium above the gel with fresh medium every day, starting from day 2 of each passage.
Note:
- *Usually, 60% of the medium is replaced at each time to avoid disruption of the gel surface.*
- *Cells will not perform well without appropriate growth factors, it is users' preference what growth factors are needed for their cells, or contact Applied StemCell, Inc. for suggestions.*

2. Cell Recovery for Passaging, Counting and Downstream Applications

2.1 **Gel disruption:** Remove the upper layer of cell medium and add Dulbecco's Phosphate-Buffered Saline (DPBS, without Mg2+ / Ca2+) at a volume equal to that of the plated MyEZGel™ Matrix gel.

2.2 Mechanically disrupt the gel THOROUGHLY by repeatedly pipetting the gel and DPBS mixture about 20 times to dissolve the gel completely.
Note: Maintaining DPBS:MyEZGel™ 3D Matrix gel ratio and thorough gel disruption through pipetting are critical steps for cell isolation from the gel. If not performed properly, gel may have issues dissolving and cell harvesting will be difficult.

2.3 Transfer the mixture to a conical centrifuge tube (i.e., 15 mL tube for one well of 24-well plate, or a 50 mL tube for handling up to 3 wells of 3D hiPSC of 24-well plate after full dilution). The recommended conical centrifuge tube size vs. well-plate are listed in Table 4.

Table 4*. Recommended conical centrifuge size vs. well-plate size for gel dilution

	12-well	24-well	48-well	96-well
Gel plating volume	1000 µL	500 µL	250 µL	100 µL
Minimum final volume of diluted gel and cell mixture from one well (20 folds)	20 mL	10 mL	5 mL	2 mL
Suggested conical tube size	50 mL	15 mL	15 mL	2 mL or 15 mL

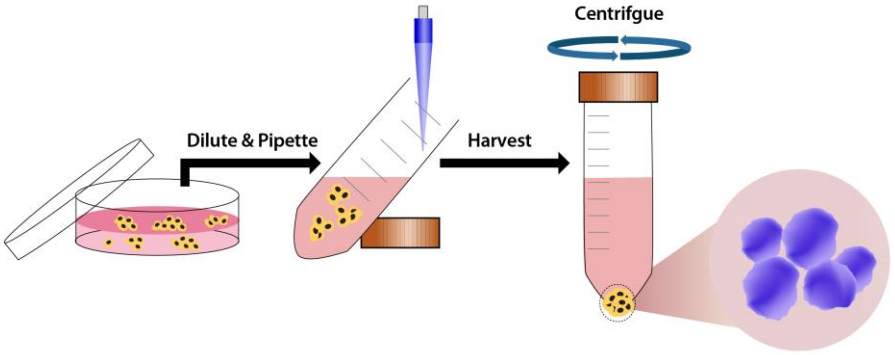
** The conical tube size suggested here is for harvesting cultured cells from one well of a culture dish. If harvesting more than one well of cultured cells at the same time, the tube size needs to be appropriately larger. For example, a 50 mL conical tube if recommended for harvesting cells from 3 wells of a 24-well plate (3x10 mL).*

2.4 **Rinse:** Use DPBS to rinse the well and combine the solution to the centrifuge tube. Recommended volume is double the maximum plating volume for each well listed in Table 1 (i.e., 200 µL for each well of a 96-well plate or 1000 µL for a 24-well plate).

2.5 **Gel dilution:** Pipette the mixture THOROUGHLY and add additional DPBS to further dilute the mixture by at least 20 folds (see Table 4.) of the original plating volume (i.e., 20 x 500 µL for one well of a 24-well plate), and mix well. (For example, dilute 3 wells of cells and gel mixture from a 24-well plateto a total volume of 30 mL or higher).

2.6 **Centrifuge:** Centrifuge at 200g for 5-6 minutes, depending on the amount of diluted gel solution in the conical tubes (Recommended: 6 minutes for more than 30 mL diluted gel solution).
2.7 Discard supernatant and collect the cell pellet.
2.8 **Colonies breakup:** To break up the colonies, add 0.5 mM EDTA solution to the cell pellet. The volume of EDTA depends on the number of cells (For example, hiPSC pellet harvested from 2-3 wells of a 24-well plate on Day 5 and containing 2-3 x10^6 cells, needs 1 mL 0.5mM EDTA solution to break up colonies).

2.8.1 Mix gently to disrupt the pellet, then incubate the tubes at 37°C (5% CO_2) for 9 minutes.
2.8.2 After incubation, add mTeSR1 complete medium equal to half the volume of 0.5 mM EDTA solution used in each tube (i.e., 2-3 x10^6 cells need 0.5 mL mTeSR1).
2.8.3 Centrifuge at 200g for 5 minutes.
2.8.4 Discard supernatant and resuspend the pellet in mTeSR1 complete medium supplemented with MyEZGel™ Gro for cell counting, passage, cryopreservation or downstream applications.



3. Cryopreserving 3D Cultured iPSCs

3.1 Resuspend hiPSC pellet in freezing medium or complete growth medium with 5-10% DMSO to a concentration of 1 x 10^6 to 5 x 10^6 cells/ mL.
3.2 Allow the cells to sit at room temperature for 15 minutes, so the cryoprotectant can diffuse into the cells.
3.3 Cells should be frozen slowly at 1°C/ min.
Note: This can be achieved by using a programmable cooler or by using cold isopropanol in a Mr. Frosty freezing container. Isopropanol should be chilled in a -80°C freezer for 24 hours.

3.4 Quickly transfer the vial to liquid nitrogen or -130°C freezer for long term storage.

4. Immuno-Staining

4.1 Gently remove the “free” medium on top of the gel in the culture well.
4.2 Gently rinse the gel surface once with DPBS (without Ca2+ and Mg2+ ions).
4.3 Fix cells in gels with 10% neutral buffered formalin for 30-40 minutes.
4.4 Wash the formalin out twice with DPBS (without Ca2+ and Mg2+ ions), 15 minutes for each rinsing.
4.5 Prepare washing buffer and blocking solution.
4.1.1 Washing buffer: DPBS (with Ca2+ and Mg2+ ions) + 0.2% triton X-100 + 0.1% cold water fish gelatin.
4.1.2 Blocking solution: washing buffer + 10% serum from the same source animal as the second antibody.
4.1.3 Pipette and mix blocking solution thoroughly.
4.1.4 Filter through a syringe filter (Millex GP .22 micrometer PES membrane). Since some fluid will be lost due to filter clogging, mix at least one-more-mL more than you actually need.
Note: Blocking solution should be prepared just before applying it to the sample.

4.6 Remove the DPBS on top of the gel and add washing buffer.
4.7 Incubate at room temperature for 10 minutes. Repeat once.

Note: If the sample is not blocked and stained immediately, it can be stored after adding washing buffer. To store the sample, the plate should be sealed with parafilm and stored at 4°C.

4.8 Remove the DPBS on top of the gel. Add washing buffer and incubate at room temperature for 10 minutes. Wash twice.
Note: If the sample is not blocked and stained immediately, it can be stored after adding washing buffer. To store the sample, the plate should be sealed with parafilm and stored at 4°C.

4.9 **Day 1: Blocking:** Remove the washing buffer, add blocking solution to the fixed samples (i.e., 500-800 µL per well of blocking solution for a 24-well plate). Let set overnight (or > 12 hours).

4.10 **Day 2: Primary Antibody (1° Ab)**

4.10.1 Prepare 1° Ab solution by adding the primary antibody solution to washing buffer. The final 1°Ab concentration for 3D staining should be similar or higher than that used for 2D culture staining [9].
Note: Typically, dilute 1°Ab with washing buffer at a ratio of 1:100 (1.e., Goat Oct3/4 antibody N-19 with final concentration of 3 µg/mL was used for hiPSC staining directly in MyEZGel™ 3D Matrix (Figure 1).

4.10.2 Add 1° Ab solution to the gel, incubate at room temperature overnight.
Note: Use sufficient volume of antibody solution to completely soak the gel (i.e., 1 mL for gel in 24-well plate or 500 µL for gel in a 48-well plate).

4.10.3 Process one sample without 1° Ab as a negative control to assess background staining.

4.11 **Day 3: Secondary antibody (2° Ab)**

4.11.1 Rinse with washing buffer 4 times (2 hours per wash).

4.11.2 Prepare 2° Ab solution by adding the secondary antibody solution to washing buffer. The final concentration of 2°Ab should be similar or higher than that used for 2D culture staining.
Note: Typically, using the concentration recommended by the vendor or determined empirically [9]. That is, Rabbit anti-goat IgG (H+L) 2°Ab Alexa flour 488 with final concentration of 5 µg/mL was used for staining hiPSC directly in MyEZGel™ 3D Matrix.

4.11.3 Wrap the plate with aluminum foil, incubate at room temperature, overnight (or >12 hours).

4.12 **Day 4: Rinse and Imaging**

4.12.1 Rinse at least 6 times (1 hour per wash) with washing buffer.

4.12.2 Replace washing buffer with glycerin for imaging.

4.12.3 Procced with imaging.

Note:

- *To achieve a strong and specific signal, it is necessary to thoroughly block non-specific binding of antibodies to the MyEZGel™ matrix, as well as, allowing diffusion of antibodies through the gel matrix. Therefore, extended the blocking and incubation times, higher antibody concentrations, and multiple washes are required.*
- *Since it is a 3D system, the background is influenced by other cells at different planate positions (Figure 1.). Therefore, post-processing of images may be needed to reduce background brightness.*

Supporting Data

Characterization of iPSCs Cultured in MyEZGel™ 3D Matrix

Pluripotency Marker Analysis

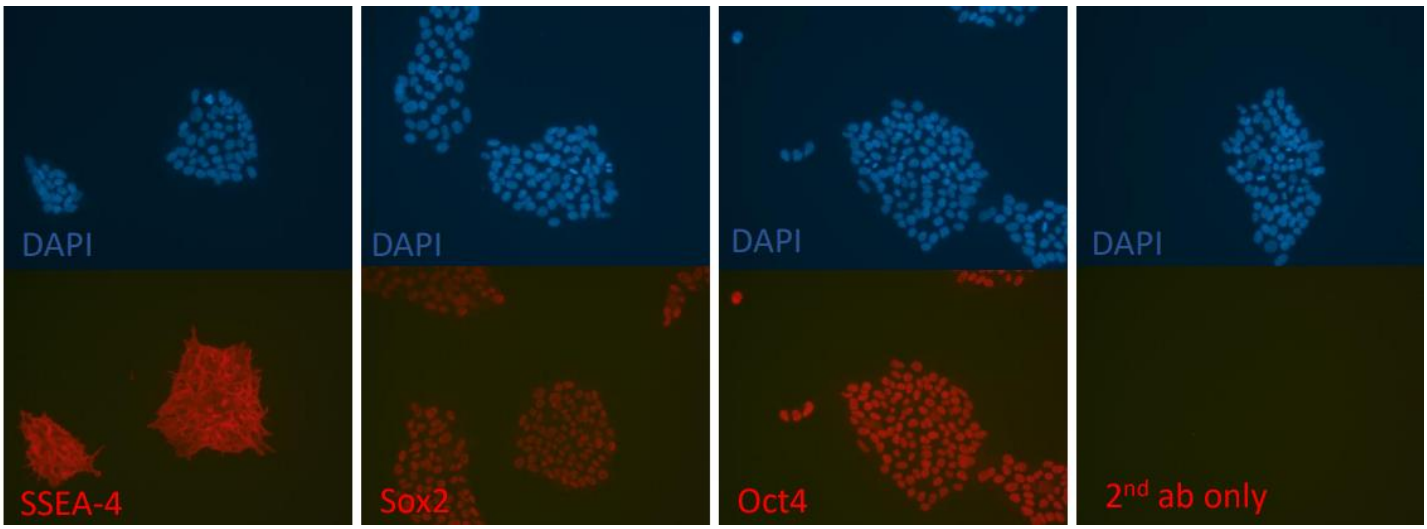
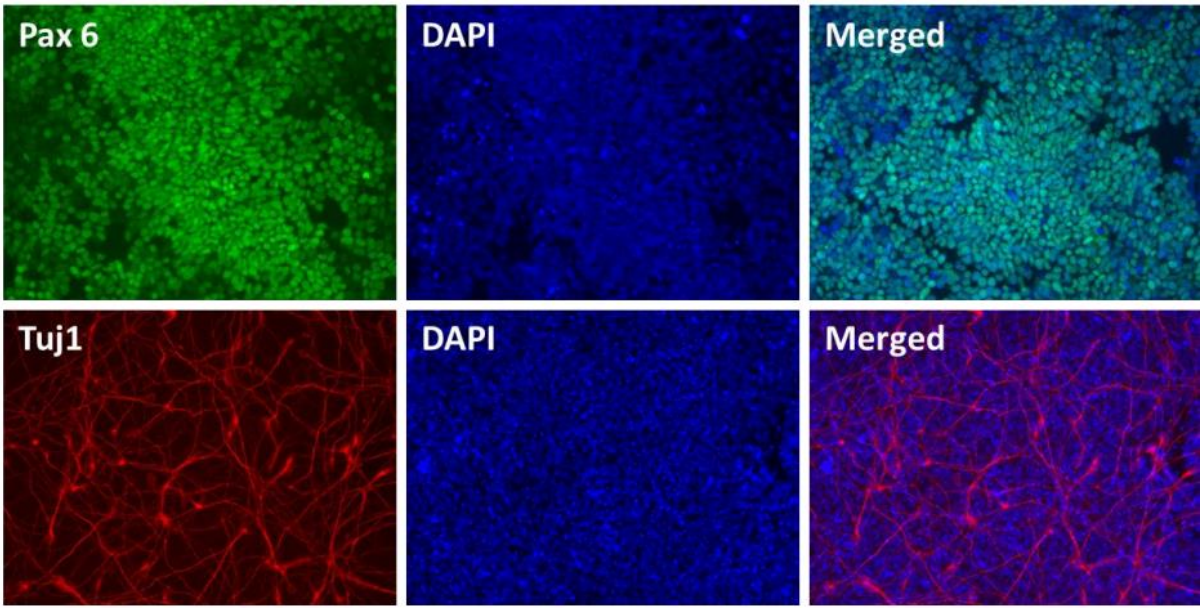


Figure 1. Immunostaining of iPSCs cultured in MyEzGel™ 3D Matrix for pluripotency markers. The 3D-iPSCs expressed common iPSC biomarkers. Image: Nuclear marker: DAPI, Pluripotency markers: SSEA-4, Sox2, Oct4, and 2nd ab only.

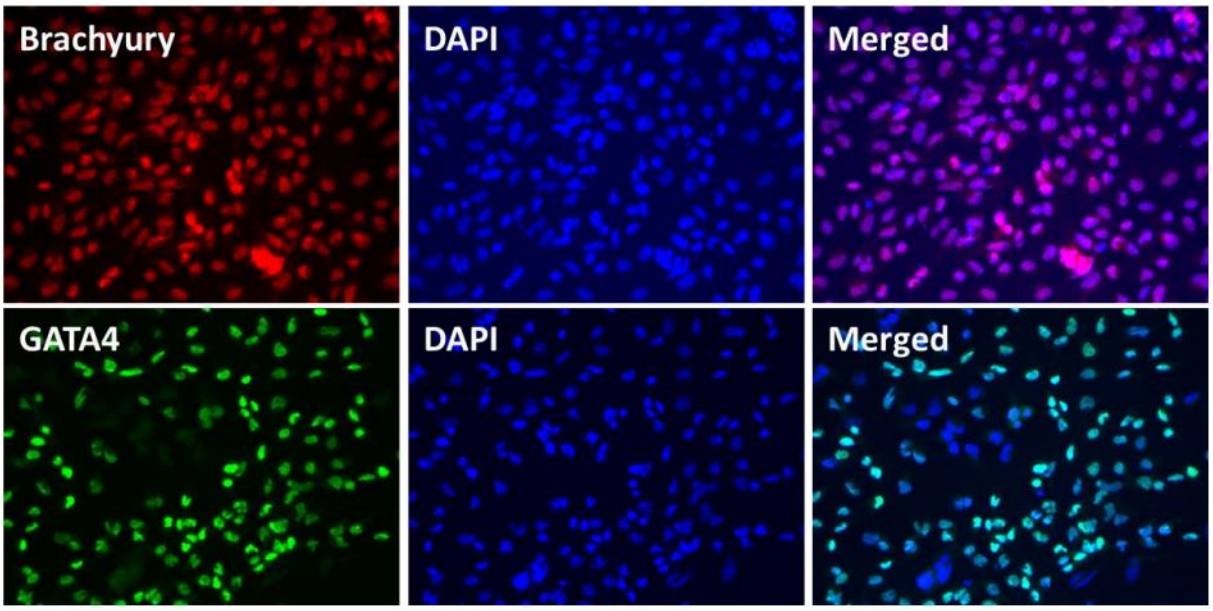
In Vitro Directed Differentiation to the Three Germ Layers

The iPSCs were recovered and expanded using mTeSR on a Matrigel-coated plate. The cells were disassociated and seeded into a 6-well plate. For the differentiation process, the iPSCs were first treated with Knockout DMEM supplemented with Activin and Wnt3 for Endoderm induction, with BMP4 and Activin for Mesoderm induction, or with Dorsomorphin, SB431542, and Noggin for Ectoderm induction. The staining confirmed the presence of all three germ layers (ectoderm, mesoderm, and endoderm) and the differentiation potential of the 3D cultured iPSCs to all three germ layers.

Ectoderm



Mesoderm



Endoderm

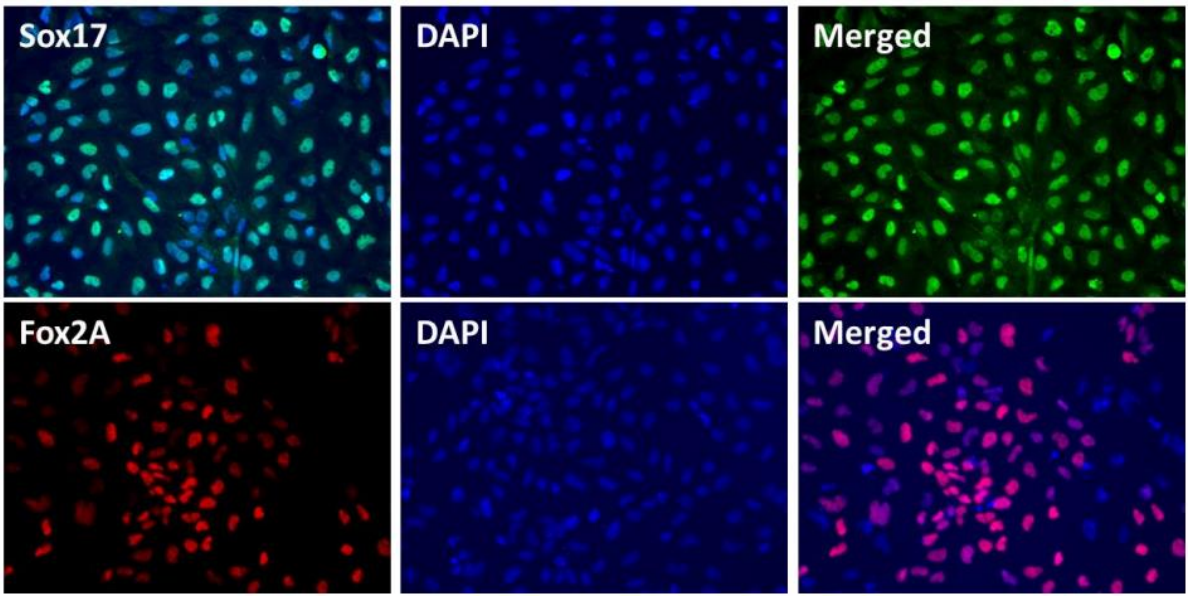


Figure 2. *In vitro* direct differentiation of MyEZGel™ 3D cultured iPSCs to the three germ layers. Immunofluorescent staining for lineage-specific biomarkers of the 3 germ layers following direct differentiation of the 3D cultured iPSCs. Immunostaining confirmed the differentiation potential of the iPSCs to all 3 germ layers. Images: Nuclear marker: DAPI (blue); Ectoderm markers: Pax6 (green) and Tuji 1 (red); Mesoderm markers: Brachyury (red) and GATA4 (green); Endoderm Sox17 (green-blue) and Fox2A (red).

Reference

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