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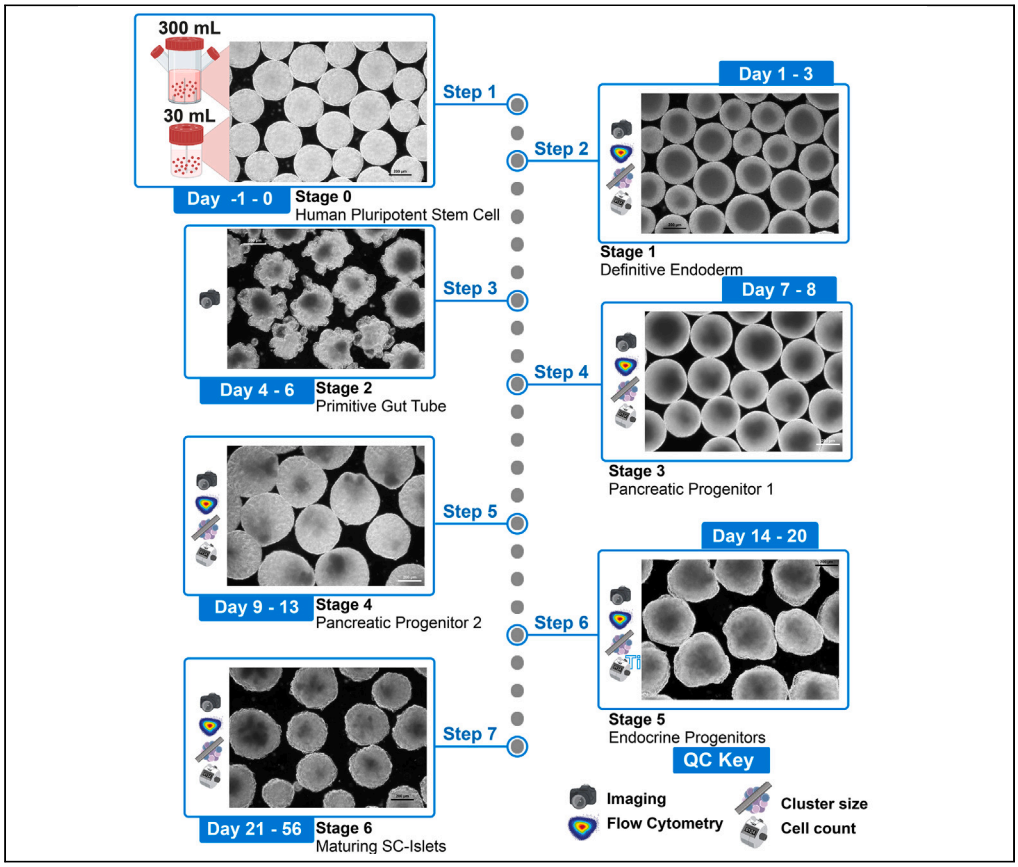
Scalable generation of 3D pancreatic islet organoids from human pluripotent stem cells in suspension bioreactors



Here, we present a protocol for producing 3D pancreatic-like organoids from human pluripotent stem cells in suspension bioreactors. We describe scalable techniques for generating 10,000–100,000 organoids that further mature in 4–5 weeks into α- and β-like cells with glucose-responsive insulin and glucagon release. We detail procedures for culturing, passaging, and cryopreserving stem cells as suspended clusters and specify growth media and differentiation factors for differentiation. Finally, we discuss functional assays for research applications.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Scalable production of 10^4–10^5 hPSC-islet organoids entirely in suspension bioreactors

Steps and quality control for culture and cryopreservation of hPSCs as 3D clusters

6-stage, 20-day directed differentiation protocol generates 3D islet-like organoids

4–5 week maturation phase yields cells with improved glucose-responsive insulin/glucagon release

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Scalable generation of 3D pancreatic islet organoids from human pluripotent stem cells in suspension bioreactors

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SUMMARY

Here, we present a protocol for producing 3D pancreatic-like organoids from human pluripotent stem cells in suspension bioreactors. We describe scalable techniques for generating 10,000–100,000 organoids that further mature in 4–5 weeks into α- and β-like cells with glucose-responsive insulin and glucagon release. We detail procedures for culturing, passaging, and cryopreserving stem cells as suspended clusters and specify growth media and differentiation factors for differentiation. Finally, we discuss functional assays for research applications.

For complete details on the use and execution of this protocol, please refer to Alvarez-Dominguez et al.[1]

BEFORE YOU BEGIN

The diverse applications for human pluripotent stem cell-derived islets (SC-islets) in research, pharmacology, and transplantation necessitate their production at both small and large scales.[2–6] Given the widespread potential to treat millions of diabetics, a scalable culture method becomes necessary. Traditional culture formats cannot meet this need. Standard two-dimensional culture formats, such as polystyrene tissue culture plates, limit the three-dimensional morphogenesis of peninsular structures that form islets *in vivo*.[7–9] Here, we utilize rotary suspension bioreactor systems to offer a realistic, feasible, and consistent route to scalable three-dimensional SC-islet formation. Our protocol involves bioreactors of 30 mL and 300 mL culture volumes, which yield tens and hundreds of millions of SC-islet cells per bioreactor, respectively.

The methods below were optimized for the human embryonic stem cell line Hues 8, and comparable outcomes can be obtained for gene-modified subclones.[1] Although variability between differentiations is expected, we outline steps that minimize variability, and discuss quality checks and potential solutions to issues encountered during the differentiation process. Three-dimensional suspension culture is a robust method for generating functional SC-islets; however, further studies are needed to fully understand the influence of mechanical aspects in bioreactor systems, such as effects of fluid shear.[10,11]

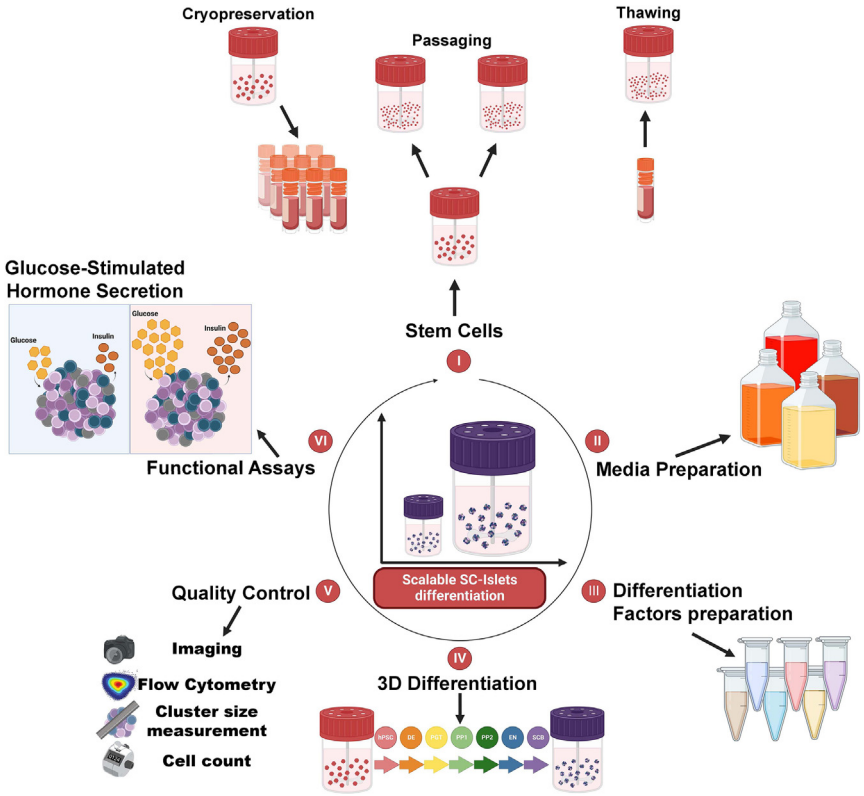

Figure 1. Overview of the necessary phases to complete and verify differentiation

Guide for the necessary phases involved in the differentiation of stem cells into stem-cell derived islets. The process includes the culturing and maintenance of stem cells, media preparation, differentiation factor preparation, 3D differentiation protocol, step by step quality controls, and functional assays to confirm differentiation has been achieved.

This protocol was developed from earlier SC-islet differentiation methods,[1,12–14] by refining and standardizing cell culture, differentiation, and phenotyping protocols to suspension bioreactors of both 30 mL and 300 mL volumes.

For convenience, the protocol is divided into independent sections covering all necessary steps for deriving pancreatic islet-like organoids from human embryonic stem cells (Figure 1). We first address the 3D culturing of human pluripotent stem cells (hPSCs), including adaptation, passaging, and cryopreservation as clusters in 3D suspension culture. We then cover the preparation of 4 types of media used for the stepwise differentiation protocol, and describe reconstitution, aliquoting, and storage conditions for the small molecules and growth factors used for direct differentiation. The section "step-by-step method details" provides the stepwise protocol by which hPSCs are directed to differentiate toward the pancreatic islet fate, including media changes and quality controls performed at each stage. We also provide a guide for assessing cell function by way of glucose-stimulated insulin and glucagon secretion.

Institutional permissions

Use of the human PSC line Hues 8 (NIH registry #0021) in this protocol was approved by the University of Pennsylvania Institutional Review Board and Human Stem Cell Research Advisory Committee. All research involving hPSCs should be conducted with permission of the appropriate institutional regulatory body and in accordance with its relevant guidelines.

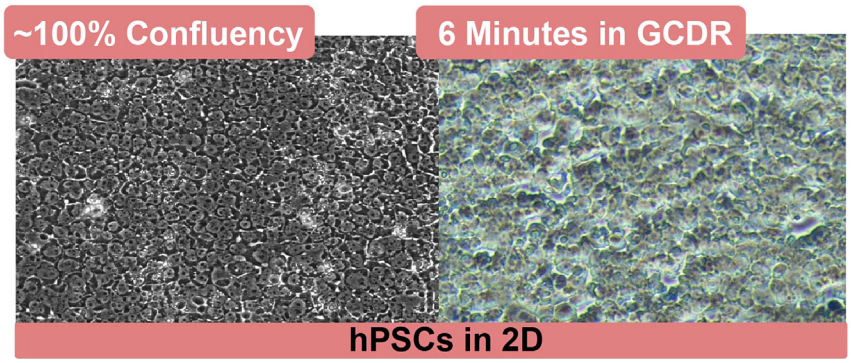

Figure 2. 2D hPSC proper morphology at 100% confluency, and after 6 min in GCDR

At 100% confluency, 2D hPSCs should be tightly packed, with rounded cell borders. Variability or irregularities between cells should be minimal. Rapid division of cells means that many should have multiple nucleoli. After incubating in GCDR, gaps should appear at the edges of the cells. Under the microscope, these will appear as white areas between cells. Cells may not completely dissociate into single cells, but instead into small aggregates. Cells should become more oval shaped as they dissociate, exhibiting a smooth appearance and uniform distribution. Minimal cell death or membrane damage should occur.

Pluripotent stem cell culture

Timing: 1–3 weeks

Timing: ~20 min (for step 1)

Timing: ~20 min (for step 2)

Timing: 3–14 days (for step 3)

Timing: ~30 min (for step 4)

These steps are prerequisite for 3D differentiation and describe hPSC culture and maintenance of pluripotent stem cells, including adaptation, culture, and passage as clusters in 3D suspension culture.

Note: Human pluripotent stem cell (hPSC) clusters should be cultured in complete mTeSR1 medium (see materials and equipment for Growth media preparation) prior to differentiation.

Note: hPSC clusters must remain highly undifferentiated (see expected outcomes for flow cytometry results for pluripotency markers, and for expected pluripotent morphology).

Note: All reagents and vessels must remain sterile. Perform all work inside a Biological Safety Cabinet (BSC). Disinfect all surfaces with 70% ethanol prior to beginning work. Spray and wipe down with 70% ethanol all objects placed inside BSC, except for cell culture dishes.

Note: For pluripotent stem cell culture, ROCK inhibitor is always used from a stock concentration of 10 μM and is always used in a dilution of 1:1000.

1. 2D to 2D single cell passaging with TrypLE.

Note: hPSCs should be passaged once they reach 90%–100% confluency in 2D planar culture (Figure 2) (see expected outcomes for confluency expectations).

Note: To seed a 30 mL bioreactor at 0.6 × 10^6 cells/mL, plate hPSCs in 100 mm plates. For larger bioreactors, use a larger plate or use multiple 100 mm plates.

a. Prepare Matrigel-coated 100 mm plate(s) for hPSC culture (see materials and equipment for Matrigel stock preparation).
   i. Dilute Matrigel to 0.02 mg/mL in cold DMEM/F12.

⚠ CRITICAL: Matrigel must remain cold to avoid premature polymerization

   ii. Coat the plate with 1 mL per 10 cm^2 of plate growth area.
b. Aspirate culture media from plate with cells, wash once with 1× PBS and aspirate.

Note: for a wash, use approximately 0.5 mL/cm^2

c. Add TrypLE to dissociate cells.

Note: approximately 1 mL TrypLE/50 cm^2 growth area

   i. Incubate for 3.5–4 min at 37°C.

⚠ CRITICAL: Stop dissociation as soon as cells appear round when looked at under a microscope, as this is the point when gaps will begin to form between cells(Figure 2).

   ii. Pre-mix 50 mL of complete mTeSR1 medium and 50 μL of 10 μM ROCK Inhibitor in a 50 mL conical tube during the incubation time.
d. Quench dissociation reaction by adding complete mTeSR1 medium + ROCK Inhibitor (Y) directly onto the cells to begin dislodging them.

Note: Coat the plate with approximately 1 mL/10 cm^2 growth area.

e. Dislodge any remaining adherent cells using a cell scraper.
f. Using a serological pipette, collect the cells in a new 50 mL conical tube.
   i. Add 10 mL mTeSR1+ ROCK Inhibitor (Y) to the dish to collect remaining detached cells, wash and transfer to the conical tube.
g. Centrifuge for 5 min at 350 g to pellet single cells.
h. Aspirate the supernatant and resuspend the pellet in 1 mL of complete mTeSR 1 medium with ROCK Inhibitor (Y).

⚠ CRITICAL: To obtain a homogenous single-cell suspension, use a P1000 tip and pipette up and down ~15 times.

⚠ CRITICAL: avoid bubbles, which can contribute to loss of cell viability.

i. Count cells and resuspend to desired cell seeding concentration.
   i. Calculate how many cells will need to be plated (the seeding density is 2.00 × 10^5 viable cells per cm^2 for Hues 8).
   ii. Resuspend at the desired 2D seeding density with mTeSR1+ ROCK Inhibitor (Y).
j. Aspirate Matrigel media from new pre-coated plates prepared in step 1a (there will be a thin film of Matrigel coating the plate, avoid touching it).
k. Distribute the cell suspension evenly across the plate, place in the incubator, and swirl the plate in a Figure 8 motion to distribute the cells evenly.

2. 2D to 3D Clump Passaging with Gentle Cell Dissociation Reagent (GCDR)

⚠ CRITICAL: once cells are confluent in the plate, you may move to 3D to begin adapting to 3D and preparing for differentiation.

a. Recover plate from incubator,
   i. Aspirate old culture media.
   ii. Add enough GCDR to cover the plate.

Note: Approximately 1 mL/10 cm^2 growth area.

   iii. Carefully return lid to plate.

Note: Take care to avoid any contact that may cause contamination.

b. Incubate cells in GCDR at room temperature (20°C–25°C) until they begin to dissociate and loosen (approximately 6 min for the Hues 8 cell line).

Note: Cells are sufficiently dissociated when small gaps begin to appear between cells at the edges of confluent areas, appearing as fuzzy white boundaries (Figure 2). At this time, proceed to next step immediately.

⚠ CRITICAL: incubation time must be optimized for each cell line to achieve sufficient dissociation.

c. Aspirate GCDR and quench by pouring fresh mTeSR1 + 1:1000 ROCK Inhibitor (Y) directly onto cells.

⚠ CRITICAL: Add enough volume to cover the plate (approximately 1 mL/10 cm^2 growth area).

⚠ CRITICAL: Cells should remain attached and should not detach when aspirating GCDR.

d. Hold scraper in one hand, and with the other gently elevate the far edge of the plate so that the media pools in the rim closest to you.
   i. Gently scrape across the entire bottom of the plate in fluid motions.
   ii. Turn the plate 90 degrees and repeat the process until all cells are lifted. At this point, cells should be clustered in relatively large clumps.
   iii. Continue tilting the plate toward you so that the remains pool in the rim closest to you.
   iv. Disperse cells into smaller clumps by using a 5 mL serological pipette to gently pipette up and down 3–5 times in the pooled media.

e. Transfer cell solution to a 50 mL conical tube,
   i. dilute with 5 mL quench (mTeSR1 + ROCK Inhibitor (Y))
   ii. gently resuspend cell mixture.
   iii. Once homogenous, count cells, and seed at desired density by placing in bioreactor (0.6 million cells/mL for Hues 8 cells adapted to growth as 3D clusters).

⚠ CRITICAL: if you have fewer than the 18 million cells required for the full 30 mL, you may seed at a lower volume (generally as few as 7 million cells.)

Note: upon seeding, the media will appear cloudy, and no clusters will be visible. Clusters begin to form in the hours after clump passaging.

⚠ CRITICAL: 3D seeding density must be optimized for each cell line (if adapting for the first time, see next section).

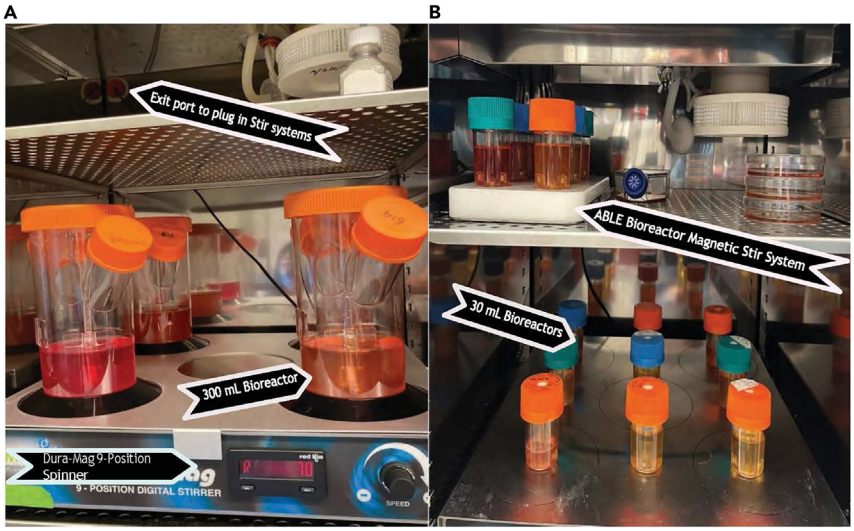

Figure 3. Incubator setup for 30 mL and 300 mL stirred suspension bioreactors

(A) 300 mL bioreactors are placed on a Dura-Mag 9-Position Digital Stirrer and must be properly centered. Cables should be threaded through the indicated exit port in the back of the incubator.

(B) 30 mL bioreactors can be placed on both the Dura-Mag 9-Position Digital Stirrer and the ABLE bioreactor Magnetic Stir System, with its digital controller placed outside of the incubator.

3. Adapting hPSCs to 3D Suspension Culture.

   These steps should allow for the adaptation of hPSC from 2D culture to 3D culture, ensuring the desired morphology and size by the end of the adaptation process.

   a. Day 0.

      i. Using the 2D to 3D clump passaging method described above, harvest cells and resuspend in mTeSR1+ 1:1000 ROCK Inhibitor (Y).

      ii. Add the appropriate amount of mTeSR1+Y to the bioreactor to bring cells to a density of 1 million cells/mL.

      iii. Add cells to media in the bioreactor, and transfer to incubator for culture in Dura-Mag magnetic stir system (Figure 3) set at optimal rpm (e.g., 70 rpm for Hues 8 cells in 300 mL bioreactor).

   ⚠ CRITICAL: the rpm must be optimized for each cell line, bioreactor volume, and magnetic stir system to achieve the desired growth rate. Optimal rpm ensures that clusters display round, uniform shape, distinct borders, and reach the desired size after 72 h.

   b. Day 1.

      i. After 24 h, transfer the bioreactor to the BSC, and wait 5–10 min for the newly formed cell clusters to settle by gravity.

      ii. Without disturbing cell clusters, gently aspirate half the volume of spent media, and replace with fresh complete mTeSR1 media without Y. Return bioreactor to incubator.

   c. Day 2.

      i. Transfer bioreactor to the BSC and wait 5–10 min for the cell clusters to settle.

      ii. Tilt the bioreactor and slowly aspirate media getting as close as possible to the clusters without disturbing them (Figure 4).

      iii. Replace media with fresh mTeSR1 without Y. Return bioreactor to incubator.

   d. Day 3–7.

      Feed every other day as described on Day 2.

      i. Monitor the size of cell clusters each day by taking an aliquot and measuring under a microscope (see quality controls).

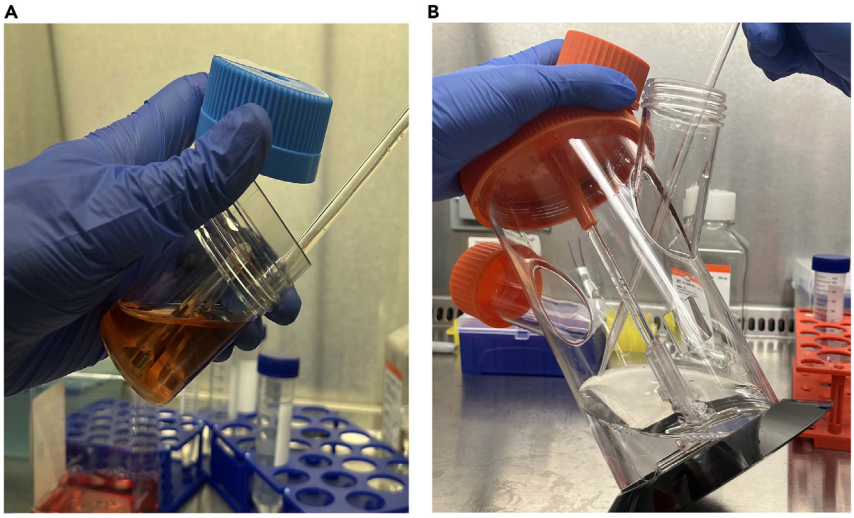

Figure 4. Change of media for 30 mL and 300 mL stirred suspension bioreactors

Bioreactors are tipped at approximately a 45° angle, with the impeller perpendicular to the tipping axis, to ensure that the clusters are not disturbed when tipping.

(A) For 30 mL bioreactors, open lid and keep open with one hand as shown. Keep aspirator tip on the meniscus of the media, and slowly follow it down as media is aspirated.

(B) For 300 mL bioreactors, open one of the side arms, tip, and keep aspirator tip on the meniscus of the media, slowly following it down as media is aspirated. The tipping angle may be increased as the level lowers.

ii. Wait until they reach a diameter between 200–300 μm to passage them as described in step 4, seeding cells at 0.8–1 × 10^6/mL. If cluster growth stops after day 5, do not wait further and proceed to passage.

⚠ CRITICAL: the 3D seeding density after passaging must be optimized during 3D adaptation of each new cell line to achieve the target cell cluster diameter.

e. Repeat steps a-d for subsequent passages,
   i. aiming to progressively reduce the passage cycle, until achieving passage every 3 days.
   ii. Reduce the 3D cell seeding density to 0.6–0.8×10^6 cells/mL once passaging every 3 days.

⚠ CRITICAL: Fully 3D adapted cells should, after 72 h, increase at least 2–3-fold in number and form clusters whose diameter average minus one standard deviation is greater than 200 μm within that period.

4. Clump Passaging hPSCs in 3D Culture.
   These steps describe the method by which hPSCs in 3D culture are passaged.

   Note: Bioreactors may be re-used for around 5 passages of the same undifferentiated hPSC line.

   Note: To prevent cross-contamination via aerosolized cells, only passage one bioreactor at a time, and never work with bioreactors from different cell lines in the BSC at the same time. Do not re-use pipette tips between cell lines or bioreactors.

   Note: Work quickly and deliberately. Avoid leaving cells in cell strainers or in any media other than mTeSR1 for prolonged times. Only leave cells in strainers for the amount of time it takes to complete the step.

Note: hPSC clusters should be acclimated to incubator conditions (5% CO_2, 37°C), and reach an average diameter >200 μm after 72 h of culture for at least 3 passages prior to differentiation.

a. Set up.
   i. Warm complete mTeSR1 medium and GCDR to room temperature (20°C–25°C).
   ii. Label each 50 mL conical tube with one of the following: #1; #2; #3; Quench (Q) + ROCK inhibitor (Y); Waste (W).
   iii. Open a 300 μm cell strainer and place in tube #1.
   iv. Open a 37 μm cell strainer and place in waste tube, with the narrower mouth (top) of the strainer facing upwards (arrow pointing up) and the wider mouth (bottom) facing downwards (inside conical tube). This will be used to collect the clusters.
   v. To make Quench media, add 30 μL ROCK inhibitor (Y) to 30 mL complete mTeSR1 (1:1000) to the Q + Y tube.
b. Remove bioreactor from incubator.
   i. Spray entire outer surface with 70% ethanol.
   ii. Place bioreactor inside BSC and wipe dry with tissue paper.
c. Partially unscrew bioreactor lid (which should keep covering the bioreactor, but loose enough so that media is not disturbed when proceeding to aspirate in the next step). Wait 2–4 min until cell clusters have completely settled.

⚠ CRITICAL: If cell clusters take >4 min to settle, this may indicate that cells are differentiating and/or dying, in which case the 3D culture should be discarded, and a new stock of cells be thawed or re-adapted from 2D culture.

d. For 300 mL bioreactors, tilt bioreactor and slowly aspirate media, getting as close as possible to the cell clusters without disturbing them (Figure 4). Leave enough media (~30 mL) to transfer. For 30 mL bioreactors, do not aspirate, and instead continue to the next step.
e. Using a 25 mL serological pipette,
   i. resuspend cell clusters inside bioreactor, draw up entire culture volume and pass through the 300 μm cell strainer into tube #1.
   ii. Wash bioreactor with ~10–20 mL of fresh mTeSR1 to collect any remaining cell clusters and pass through strainer.

⚠ CRITICAL: The tip of the serological pipette should not touch the cell strainer. Place the tip of the pipette gently against the side wall of the strainer, not directly on the bottom filter.

f. Discard cell strainer, close 50 mL conical tube and wait for the cell clusters to completely settle (2–4 min). During this time,
   i. rinse the bioreactor with the full bioreactor volume of PBS: swirl to wash, and aspirate to clean any leftover debris from bioreactor walls and impeller.
   ii. Refill with original volume of PBS, close lid, and set aside.
g. Once cell clusters settle in tube #1, aspirate old media without disturbing the clusters.

⚠ CRITICAL: Aspirate along meniscus, slowly approaching the clusters with the tip. Once the clusters start to move slightly, stop aspirating to ensure no clusters are removed. 1–2 mL of media can remain.

h. Using a 50 mL serological pipette, draw up 30 mL PBS.
   i. Use 2 mL to wet the 37 μm cell strainer placed in the waste tube (arrow pointing up) and add the remaining 28 mL PBS to tube #1.

ii. With the same pipette, draw up PBS + clusters and pass through 37 μm cell strainer into the waste tube. PBS, along with any single cells and debris, will pass through the strainer, while clusters are retained.

⚠ CRITICAL: The cell strainer may become clogged due to a dense layer of cell clusters. Use the 50 mL pipette to gently draw clusters up and down to expel remaining PBS.

⚠ CRITICAL: The tip of the serological pipette should not touch the cell strainer. Place the tip of the pipette gently against the side wall of the strainer, not directly on the bottom filter.

i. Remove 37 μm cell strainer from waste tube, and invert into tube #2 so that the narrow side faces down, and the clusters fall into tube #2. Leave the wider mouth of the strainer facing upwards and discard the waste tube.

j. Using a 50 mL serological pipette, pass 30 mL of GCDR through the wide mouth of the upside-down 37 μm cell strainer to dislodge cells clusters into tube #2. For smaller volumes of cell clusters, 15–20 mL of GCDR will suffice.

⚠ CRITICAL: gently press the tip of the pipette against the filter to dislodge clusters. Every ~5 mL, check strainer to locate remaining clusters.

k. Invert the 37 μm filter once more so that the narrow mouth faces upwards again and place in tube #3. Cover with tube lid to be used later.

l. Screw the cap on tube #2 (containing clusters + GCDR), and
   i. place in a 37°C water bath for 6 min.
   ii. After 2 min, swirl the tube gently to resuspend clusters without letting them stick to the tube walls, and place back in water bath.

⚠ CRITICAL: Work briskly to ensure that cells do not remain in GCDR for too long.

m. During the 6-min incubation, continue to wash the bioreactor.
   i. For 300 mL bioreactors from step 4f, swirl PBS, carefully remove the lid with impeller and turn it upside down.
   ii. Aspirate debris from external blade and/or the inner chamber. Ensure *nothing* touches the impeller except for a clean aspiration tip. Aspirate PBS in bioreactor, clean any remaining drops, replace lid, and set aside.
   iii. For 30 mL bioreactors, swirl, aspirate PBS, clean any remaining drops, close, and set aside.

n. After 6 min of GCDR incubation at 37°C
   i. gently remove tube #2 from the 37°C water bath
   ii. sanitize with 70% ethanol, and place in BSC.

⚠ CRITICAL: Cluster density decreases following GCDR incubation. Clusters will displace more easily and take longer to settle.

Note: Take care to leave the pellet as undisturbed as possible while transferring back to BSC, to avoid losing clusters in the next step.

o. Once most clusters settle, aspirate GCDR.

⚠ CRITICAL: A handful of clusters will typically not settle and are lost during aspiration. Once again, aspirate until clusters start to move, and then immediately stop. Do not aspirate any clusters. ~1–2 mL of GCDR can remain.

p. Using a 50 mL serological pipette add 30 mL of Quench media to tube #2 and gently resuspend clusters 2× to break up the pellet and mix evenly.
q. Draw up 10 mL of the Quench + cluster suspension into the 50 mL pipette.
    i. place the pipette at a 90° angle with the tip directly against the 37 μm cell strainer in tube #3.
    ii. press down firmly.
    iii. Break up clusters by passing the cluster suspension through the 37 μm strainer into tube #3 by *slowly* applying pressure to the trigger of the serological pipette controller. The ideal speed is ~0.5 mL per second.
    iv. Repeat with remaining suspension volume.

⚠ CRITICAL: Dissociating clusters in smaller volumes (~10 mL), rather than the full volume at once, makes it easier to pass through the filter, as clusters have less time to sink to the bottom of the pipette tip and create a jam at the opening. If passage through the 37 μm cell strainer slows significantly, maintain pressure with the pipette, and slide to a new area of the filter that has not yet been used to break up clusters.

r. Discard 37 μm cell strainer. Using a new serological pipette,
    i. slowly draw the cell clumps up to measure the total volume,
    ii. resuspend them 2 times slowly and gently.
    iii. Obtain a cell count now.
s. While counting, calculate the volume of cell solution required to seed a new bioreactor at the target density. For Hues 8 cells, this 3D seeding density is 0.6 million cells per mL. Expected cell viability is ~95%.
t. Using a 50 mL serological pipette.
    i. add the necessary complete mTeSR1 medium + ROCK inhibitor at 1:1000 dilution.
    ii. Gently mix cell solution 2 times to get an even suspension, then seed the appropriate volume into bioreactor.
    iii. Pipette up and down once to remove any cells that may stick to the inside walls of the serological pipette.
u. Return bioreactor to incubator and continue to incubate at optimal rpm (e.g., 70 rpm for Hues 8 cells in 300 mL bioreactor).

Media change in bioreactors

Timing: 10 min (for step 5)

5. Step-by-Step Media Change in Bioreactors.
This section explains how to change media in bioreactors.

Note: Thaw the factors you will be using at 20°C–25°C (keep in the dark if they are light sensitive).

Note: Transfer necessary volume (enough to replace old media) of basal differentiation media (media without factors) to a sterile, autoclaved glass bottle or a conical tube and pre-warm in 37°C water bath for 30 min to 1 h

Note: Once media is warmed and factors are thawed, transfer to BSC and, using sterile technique, add factors to basal media to prepare complete differentiation media. Swirl to ensure media is mixed thoroughly.

Note: It is not necessary to remove all media when exchanging media. For 300 mL bioreactors, expect to leave behind ~15 mL. For 30 mL bioreactors, expect to leave behind ~2–3 mL.

a. Remove bioreactor from incubator. Close cap, spray with 70% ethanol, dry thoroughly with Kimwipe, and place in BSC. Leave bioreactor undisturbed while cell clusters settle.

⚠ CRITICAL: Settling time depends on cell line and cluster size. New lines should be assessed individually, and settling time should be noted for each stage. The following is a reference for cluster settling time by stage for Hues 8 cell clusters:

24 h after passage: 5–6 min.
48 h after passage: 3–4 min.
72 h after passage (Stage 1 day 1): 2–3 min.
Stage 2 day 1: 6–7 min.
Stage 3 day 1: 7–9 min.
Stage 4 day 1: 3–4 min.
Stage 5 day 1: 3–4 min.
Stage 6 day 1: 2–3 min.
Stage 6 middle days: 1–2 min

⚠ CRITICAL: do not leave cell clusters undisturbed for longer than needed to settle, as they will begin to clump together or stick to the bottom of the bioreactor.

b. Once cell clusters settle,
   i. unscrew the bioreactor cap, tilt away from you, and,
   ii. insert a fresh aspirator as shown in Figure 4.
   iii. Begin to aspirate media. As the level of the liquid lowers, tilt further to accommodate. Remove as much media as possible without disturbing the cell clusters.
c. Return bioreactor to flat, resting position.
   i. Thoroughly mix fresh differentiation media again.
   ii. Pour new media *gently* so that media runs down the side of the bioreactor and not directly onto clusters.
d. Replace bioreactor cap and tighten. If using a larger bioreactor with side-caps, unscrew/loosen both side caps roughly 3–4 times to let air in.
e. Return bioreactor to incubator. Position properly on stirring plate so that the impeller spins smoothly (Figure 3).

Cryopreserving and thawing 3D hPSC clumps

Timing: 1 h

These steps detail cryopreservation of hPSCs as 3D clumps, and subsequent thawing.

6. Cryopreserving 3D hPSC clumps.

Note: Print cryo-safe labels including cell line name, passage number, freezing media, cell count, and whether cells are 2D or 3D adapted. Pre-label cryovials.

Note: Acclimate freezing container to 4°C by placing in refrigerator 5–16 h.

a. 2–3 bioreactors can be handled at once to minimize the amount of total time cells are under stress. Perform clump passaging as described in step 4: Clump Passaging hPSCs in 3D Culture.
b. After passage (see Step 4), pool clumps together, mix well, and count cells.
c. Split mixed clumps into multiple 50 mL conical tubes, spin down at 200 g for 2 min at 20°C.

d. Aspirate media and resuspend in mFreSR + 1:1000 ROCK Inhibitor (Y) on ice at an appropriate concentration (e.g., 20 M cells/mL in a single cryovial for seeding 30 mL bioreactors), transfer to cryotubes.

⚠ CRITICAL: Keep mFreSR cold. Work quickly as cryopreservation media is toxic to cells at room temperature. Freeze 5–25 million cells/mL.

e. Place cryotubes into Mr. Frosty freezing container. Transfer the freezing container to −80°C freezer (where it will freeze slowly at −1°C/min). After 24 h, transfer cryovials on dry ice to liquid nitrogen for long-term storage.

7. Thawing cryopreserved 3D hPSC clumps.

Note: 3D culture and propagation of hPSCs begins by thawing cryopreserved cells, and seeding at an appropriate density (e.g., 0.6 million cells per mL for 3D adapted Hues 8 cells).

Note: Prepare bioreactors by placing them in BSC and labeling appropriately.

Note: **Complete** mTeSR1 medium and ROCK inhibitor should be warmed to room temperature (20°C–25°C) prior to use.

a. Remove frozen cryovials from liquid nitrogen and place on dry ice to transport. Immerse cryovial in 37°C water bath for approximately 1.5 min, until there is only a small ice cluster remaining.

⚠ CRITICAL: do not allow the cryovial to thaw completely in 37°C water bath (cells should spend as little time in thawed mFreSR medium as possible).

b. Spray cryovial with ethanol and dry. Do not allow ethanol to enter the vial when opening.
c. Using a P1000 pipette, transfer 1 mL of mTeSR1 + 1:1000 ROCK inhibitor medium dropwise into cryovial.
d. Using the same P1000 pipette, transfer 1 mL of cell suspension dropwise from cryovial into a 15 mL conical tube with 9 mL mTeSR1 + 1:1000 ROCK inhibitor.
e. Centrifuge clumps at 200 g for 2 min. Aspirate media and resuspend in 1–5 mL of mTeSR1 + 1:1000 ROCK inhibitor.
f. Perform a cell count and resuspend clumps in the target volume of mTeSR1 + 1:1000 ROCK inhibitor (e.g., 0.6 million cells per mL). Transfer to bioreactor and incubate in magnetic stir system at optimal rpm (e.g., 70 rpm for Hues 8 cells in 300 mL bioreactor).

Quality controls

Timing: 1 h

Taking quality controls ensures that proper differentiation is taking place, in order to monitor the progress of each passage, and to allow the user to discard the passage if it is not looking good.

Note: Quality control samples are collected upon each stage's completion (on the first day of the following stage).

8. Quality control (QC) sample collection,

Note: Cell concentration fluctuates from stage to stage. These numbers can be changed if not enough cells are collected from these volumes.

Note: Calculate the total volume of cells you will require for QC as follows:

Morphology	0.35 mL.
Cluster size	0.35 mL.
Flow cytometry	0.5 mL.
Cell count	0.35 mL.

a. Remove bioreactor from the incubator. Ensure all caps are closed. Spray with ethanol, dry, and place in BSC.
b. Evenly resuspend clusters. This can be done by gently spinning the impeller with a wide bore P1000 pipette tip. Once an even distribution of clusters is achieved throughout the bioreactor (after several spins of the impeller) draw up the desired volume of media (see Note in step 8) from the center of the bioreactor, where the mixture will be most accurately representative.
c. Transfer representative sample to either a 24-well plate or a 1.5 mL centrifuge tube for cell cluster size and count or flow cytometry, respectively.
d. Return bioreactor to incubator.

9. Bright-field imaging (morphology, cell cluster size QC).
   a. After sample collection, take 24-well plate to microscope.
   b. Set up the microscope as follows: 4× magnification, Phase 2
      i. Using the focus knob, ensure that cell cluster edges are in focus for an accurate diameter measurement.
   c. Take pictures and measure the diameter of clusters.

⚠ CRITICAL: Take enough pictures to capture approximately 100 cell clusters, to ensure an accurate calculation of their average diameter.

10. Flow cytometry sample collection.
    a. Spin the 0.5 mL sample of cell clusters in a 1.5 mL microcentrifuge tube at 200 g for 3 min for clusters to settle.
    b. Transfer to BSC and remove supernatant with pipette.
    c. Add 0.5 mL room temp (20°C–25°C) TrypLE to tube.

⚠ CRITICAL: if diluting, take note of the dilution factor. This is important for cell count measurements.

    d. Incubate 7–15 min in 37°C water bath, gently swirling tube once after 5 min to resuspend clusters.

⚠ CRITICAL: depending on cell cluster size, dissociation may happen sooner, or may take the full 15 min. If the sample becomes cloudy after swirling, it is most likely ready.

    e. Manually break up clusters by gently pipetting up and down (approximately 20 times) with a P1000. Clusters should disperse easily.

⚠ CRITICAL: If collecting a cell count, transfer an appropriate volume of single cell suspension to another centrifuge tube, stain with trypan blue, and perform cell count.

    f. Fill up with 1 mL 1× PBS + 2% FBS wash and spin down to pellet cells (400 g for 3 min).
    g. Aspirate wash and add 1 mL of 4% PFA, resuspending pellet gently with the P1000 pipette.
    h. Fix cells at 4°C for 30–60 min. After fixation, fill the remainder of tube with 1× PBS + 2% FBS wash and spin down (400 g for 3 min).
    i. Aspirate and add 500 µL 1× PBS + 2% FBS. Use P1000 to gently resuspend pellet.
    j. Store at 4°C until staining (see expected outcomes and expected results for QC by stage for staining antibodies). Aim to stain and run within 1–2 weeks post-collection.

11. Cell count sample collection.

Note: if also collecting flow cytometry sample, use single cell suspension from flow cytometry sample and skip to step e If not, see below.

a. Transfer 0.5 mL of clusters to 1.5 mL centrifuge tube.
b. Spin cell clusters at 200 g for 3 min, aspirate supernatant and replace with 0.5 mL pre-warmed TrypLE (always 1:1).
c. Incubate 7–15 min in 37°C water bath, swirling tube after 5 min to resuspend clusters.

⚠ CRITICAL: Clusters of different sizes may dissociate sooner than 15 min if small, or may take the full 15 min if large. If not sure how long, check to see if the solution becomes cloudy after swirling.

d. Manually break up clusters by gently pipetting up and down (approximately 20 times) with a P1000 pipette. Clusters should disperse easily.
e. Mix x μL cell suspension with an equal volume of trypan blue by pipetting up and down gently multiple times, and count cells. Cells may be counted manually with a hemocytometer or with an automated cell counter.

Media and factor preparation

Timing: 1 h (for step 12)

Timing: ~20 min per factor (for step 13)

⚠ CRITICAL: Differentiation media is created in two separate phases: mixing and filtering.

⚠ CRITICAL: This protocol describes how to make 10 L of the desired media. If less media is necessary, use the table above to modify the amounts to produce desired volume of media. Each medium should be prepared separately.

12. Differentiation Media Preparation.
a. Spray down 500 mL MCDB131 bottles with 70% ethanol.
b. Add 10 out of the 20 MCBD131 bottles to be prepared to carboy with stir bar and begin stirring. Keep empty bottles to transfer media into vacuum filters.
c. Add all reagents needed for the specific Media being made, except FAF-BSA

⚠ CRITICAL: FAF-BSA foams up when added to media. Do not add until later to ensure even mixing of other reagents.

d. Mix for about 10 min, or until all reagents are thoroughly dissolved. Stop stirring.
e. Remove Carboy from stirrer, add FAF-BSA, and manually give the Carboy a swish, to help dissolve FAF-BSA. Return Carboy back to stir plate and stir for about 10 min.

⚠ CRITICAL: if FAF-BSA is not dissolving in a timely manner, the medium may be gently heated to make dissolving easier.

f. Add the remaining 10 bottles of MCDB131 to Carboy and allow mixing for a final 10 min. Keep empty bottles to transfer media into vacuum filters.
g. After fully mixed, add media from Carboy back to the saved 500 mL MCDB131 bottles, to begin the filtering process.
h. Filtering.

i. Spray MCDB131 bottles with newly made media and 1 L TC bottles with 70% ethanol and place inside BSC.
ii. Attach vacuum filters to each sterile TC bottle.
iii. Pour media from MCDB131 bottles through vacuum filter and into TC bottles (one filter per liter of media) until all media has been filtered.
iv. Label and store media at 4°C for up to six months.

13. Preparation of differentiation factors.

Note: For each differentiation factor, make aliquots with approximately 10% more volume than needed for a 30 mL or 300 mL bioreactor. For aliquots smaller than 10 μL use an additional microliter. This is to ensure that there is enough of each differentiation factor.

Note: Perform aliquoting in BSC using sterile technique, with lights off for differentiation factors that are light sensitive

Note: While aliquoting, constantly pipette the mixture up and down to maintain homogeneity. Solubility of some factors is low. Pipette to maintain constant amount of dissolved factor.

Note: Avoid repeated freezing/thawing of factors

a. **Activin A (10 μg/mL Single-Use Aliquot Concentration)** *R&D Systems 338-AC-500.* Dilute Activin A to 10 μg/mL using 0.1% BSA in sterile 1× PBS solution up to 50 mL. For 300 mL differentiations, aliquot 3.2 mL into sterile 15 mL conical tubes. For differentiations in 30 mL differentiations, aliquot 320 μL into 1 mL microcentrifuge tubes.

⚠ CRITICAL: store original vial at −20 to −70°C for up to 1 year. After reconstitution store at −20 to −70°C under sterile conditions for up to 3 months.

b. **Alk5i II (100 mM Single-Use Aliquot Concentration)** *DNSK International DNSK-ALK5-02.* Dissolve 500 mg of Alk5i II (1 vial) in DMSO up to 17.4 mL. For 300 mL differentiations, transfer 40 μL aliquots to sterile, autoclaved microcentrifuge tubes. For 30 mL differentiations, aliquot to 4 μL. To avoid having to create hundreds at a time, aliquot 3 mL at a time, and keep the rest at to −70°C.

⚠ CRITICAL: store original vial at −20 to −70°C for up to 2 years. After reconstitution store at −20 to −70°C for up to 3 months under sterile conditions.

c. **Betacellulin (100 μg/mL Single-Use Aliquot Concentration)** *R&D Systems 261-CE-250.* Thaw one 250 μg vial of Betacellulin and add up to 2.5 mL of 0.1% BSA in PBS. For 300 mL differentiations, transfer 70 μL aliquots to sterile, autoclaved microcentrifuge tubes. For 30 mL differentiations, aliquot to 7 μL.

⚠ CRITICAL: store original vial at −20 to −70°C for up to 1 year. After reconstitution store at −20 to −70°C for up to 3 months under sterile conditions.

d. **CHIR99021 (10 mM Single-Use Aliquot Concentration)** *Tocris 4423.* Dissolve 10 mg of CHIR99021 in 2.15 mL of DMSO. For 300 mL differentiations, transfer 100 μL aliquots to sterile, autoclaved microcentrifuge tubes. For 30 mL differentiations, aliquot to 10 μL.

⚠ CRITICAL: store powder at −20 to −70°C, protected from light. After reconstitution, store at −20 to −70°C for up to 6 months. It is important to protect the product from light during aliquot preparation, storage, and use.

e. KGF (50 μg/mL Single-Use Aliquot Concentration) R&D (#251-KG/CF). Dissolve 1 mg of KGF in 1 mL of 0.1% BSA in PBS Solution. Transfer to a sterile 50 mL conical tube and add 19 mL of 0.1% BSA in PBS Solution. For 300 mL differentiations, transfer 350 μL and 650 μL aliquots to sterile, autoclaved microcentrifuge tubes. For 30 mL differentiations, aliquot to 35 and 65 μL.

⚠ CRITICAL: store original vial at −20 to −70°C, tightly closed, for up to 2 years. Store aliquots at −20 to −70°C for up to 6 months under sterile conditions.

f. LDN (1 mM Single-Use Aliquot Concentration) *Sigma*-Aldrich *SML0559-5MG*. Dissolve 5 mg of LDN in 1.23 mL of DMSO (10 mM). Aliquot into 12 tubes of 100 μL and add 900 μL DMSO to each tube (1 mM). For 300 mL differentiations, thaw and transfer 70 μL aliquots to sterile, autoclaved microcentrifuge tubes. For 30 mL differentiations, aliquot to 7 μL.

⚠ CRITICAL: store powder at −20 to −70°C for up to 1 year. After reconstitution, store aliquots at −20 to −70°C for up to 6 months, protected from light.

g. PDBU (1 mM Single-Use Aliquot Concentration) *EMD Millipore 524390-5MG*. Dissolve 5 mg of PdbU in 9.9 mL of DMSO. For 300 mL differentiations, transfer 160 μL aliquots to sterile, autoclaved microcentrifuge tubes. For 30 mL differentiations, aliquot to 17 μL.

⚠ CRITICAL: store original vial at −20 to −70°C desiccated for up to 2 years. After reconstitution store at −20 to −70°C for up to 3 months.

h. Retinoic Acid (10 mM Single-Use Aliquot Concentration) *Sigma-Aldrich R2625-50 mg*. Dissolve 50 mg of RA in 16.64 mL of DMSO. For 300 mL differentiations, transfer 75 μL aliquots to sterile, autoclaved black opaque microcentrifuge tubes. For 30 mL differentiations, aliquot into tubes of 7 μL and 1 μL.

⚠ CRITICAL: store aliquots at −80°C for up to six months. Protect from light during aliquot preparation, storage, and use.

i. ROCK inhibitor (Y-27632) (10 mM Single-Use Aliquot Concentration) *DNSK International DNSK-KI-15-02*. Dissolve 250 mg Y-27632 in 78 mL of molecular grade H2O. Filter sterilize, then make equal numbers of 350, 650, and 950 μL aliquots in sterile, autoclaved microcentrifuge tubes.

⚠ CRITICAL: Store the original vial tightly sealed below −20°C for up to 1 year. After reconstitution store the aliquots at −20°C tightly sealed for up to 6 months. Protect from light during aliquot preparation, storage, and use.

⚠ CRITICAL: Due to the frequency of use of ROCK inhibitor, aliquots can be kept at 4°C for up to a week.

j. SANT1 (1 mM Single-Use Aliquot Concentration) *Sigma-Aldrich S4572-5MG*. Dissolve 5 mg of SANT1 in 13.4 mL of DMSO. For 300 mL differentiations, transfer 85 μL (single aliquots) or 170 μL (double aliquots) to sterile, autoclaved microcentrifuge tubes. For 30 mL differentiations, aliquot to 8.5 μL.

⚠ CRITICAL: Store the original item at −80°C for up to 1 year, and store aliquots at −20°C for up to 3 months.

k. T3 (10 mM Single-Use Aliquot Concentration) *EMD Millipore 64245-250 mg*. Dissolve 250 mg of T3 in 37.1 mL of DMSO. For 300 mL differentiations, transfer 40 μL aliquots to sterile microcentrifuge tubes. For 300 mL differentiations, aliquot to 4 μL.

⚠ CRITICAL: Store the aliquots at −20°C / −80°C for up to 6 months.

l. **XXI (10 mM Single-Use Aliquot Concentration).** *EMD Millipore 565790-1MG*. Dissolve 1 mg of XXI in 204 µL of DMSO. For 300 mL differentiations, transfer 40 µL aliquots to sterile, autoclaved microcentrifuge tubes. For 30 mL differentiations, aliquot to 4 µL.

⚠ CRITICAL: Store the aliquots at −20°C for up to 6 months.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Oct4 (mouse) (1:100)	BD Biosciences	561589
Sox17 (goat) (1:500)	R&D Systems	AF1924
Pdx1 (goat) (1:500)	R&D Systems	AF2419
Nkx6.1 (mouse) (1:500)	Iowa DSHB	F55A12
C-peptide (rat) (1:500)	Iowa DSHB	GN-ID4
Glucagon (mouse) (1:500)	Santa Cruz	sc-514592
Chemicals, peptides, and recombinant proteins		
mTeSR1 Basal Medium	STEMCELL Technologies	85850
Activin A	R&D Systems	338-AC-500
Alk5i II	DNSK International	DNSK-ALK5-02
Betacellulin	R&D Systems	261-CE-250
Bovine serum albumin solution	Millipore Sigma	A1595-50ML
Calcium chloride dihydrate	Sigma-Aldrich	C3306
CHIR99021	Tocris	4423
Corning Basal Cell Culture Liquid Media - DMEM and Ham's F-12, 50/50 Mix	Corning	10092CV
D-(+)-glucose	Sigma-Aldrich	G7528
Dimethyl sulfoxide (DMSO)	Millipore Sigma	D2650
Fatty acid-free bovine serum albumin (FAF-BSA)	Proliant Biologicals	68700
Gentle cell dissociation reagent (GCDR)	STEMCELL Technologies	100-0485
Gibco TrypLE Select Enzyme (1×), no phenol red	Fisher Scientific	50-591-419
glutaGRO supplement	Corning	25-015-CI
Heparin sodium salt	Sigma-Aldrich	H3149
HEPES buffer solution (1 M)	Gibco	15630-056
Insulin-transferrin-selenium-ethanolamine (ITS-X) (100×)	Fisher Scientific	51500056
LDN193189 hydrochloride	Sigma-Aldrich	SML0559-5MG
Magnesium sulfate	Sigma-Aldrich	M2643
Matrigel hESC-qualified matrix	Corning	354277
MCDB131 medium	Corning	15-100-CV
mFreSR cryopreservation medium	STEMCELL Technologies	5854
M-PER Mammalian Protein Extraction Reagent	Thermo Fisher Scientific	78501
Paraformaldehyde 16% aqueous solution	Electron Microscopy Sciences	15710
PBS 1×	Corning	21-040
Penicillin-Streptomycin	Corning	30-001-CI
Phorbol 12,13-dibutyrate (PDBU)	EMD Millipore	524390-5MG
Potassium chloride	Sigma-Aldrich	P9541
Potassium phosphate monobasic	Sigma-Aldrich	P5655
Recombinant human KGF (FGF-7)	PeproTech	100-19-1MG
Retinoic acid	Sigma-Aldrich	R2625-50MG
ROCK inhibitor (Y-27632)	DNSK International	DNSK-KI-15-02
SANT1	Sigma-Aldrich	S4572-5MG
Sodium bicarbonate	Sigma-Aldrich	S5761
Sodium bicarbonate ($NaHCO_3$)	Sigma-Aldrich	S5761

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Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Sodium chloride	Sigma-Aldrich	S3014
Sodium phosphate dibasic	Sigma-Aldrich	S0876
T3	EMD Millipore	64245-250MG
Vitamin C	Sigma-Aldrich	A4544
XXI	EMD Millipore	565790-1MG
Experimental models: Cell lines		
Human embryonic stem cell line 8 (XY)	NIH	Registry #0021
Other		
37 micron reversible strainers	STEMCELL Technologies	27250
50 mL conical centrifuge tubes	MidSci	C50B
ABLE 30 mL bioreactor	ReproCELL	ABBWVS03A-6
ABLE bioreactor magnetic stir system base 30 mL and 100 mL	ReproCELL	ABBWBP03N0S-6
ABLE bioreactor system controller and motor	ReproCELL	ABBWDW-1013
ART Wide Bore Filtered Pipette Tips	Fisher Scientific	2079GPK
Bottle-top vacuum filters	Corning	431097
Corning Falcon Cell Scrapers	Corning	353085
Disposable spinner bioreactor 300 mL	Corning	3578
Freezing Container, Nalgene Mr. Frosty	Millipore Sigma	C1562
Human glucagon ELISA	Mercodia	10-1271-01
Millicell cell culture insert, 12 mm, polycarbonate, 12 µm	Sigma-Aldrich	PIXP01250
Nunc cryotube 1.8 mL	Fisher Scientific	377267
pluriStrainer 300 µm (cell strainer)	pluriSelect	3-50300-03
Stirrer, magnetic, nine position, Dura-Mag, 120 V, 500 mL	Chemglass	CLS-4100-A9
Thermo Scientific Forma Series II Water-Jacketed CO_2 Incubator	Fisher Scientific	13-998-076

MATERIALS AND EQUIPMENT

KREBs buffer

	Stock concentration	Final concentration	Volume (mL)
Reagent	mM	mM	500
MilliQ H2O			467.9
NaCl [5 M]	5000	128	12.8
KCl [2 M]	2000	5	1.25
$CaCl_2 \cdot 2H_2O$ [1 M]	1000	2.7	1.35
$MgSO_4$ [1 M]	1000	1.2	0.6
Na_2HPO_4 [0.1 M]	100	1	5
KH_2PO_4 [1 M]	1000	1.2	0.6
$NaHCO_3$ [1 M]	1000	5	2.5
HEPES [1 M]	1000	10	5
BSA	10%	0.10%	5

Note: KREBs may be stored at 4°C for up to a month

Note: For 500 mL of KREBS buffer, put 467.9 mL of deionized water into a sterile 500 mL bottle, and combine the above reagents in order, mix and filter using a vacuum filter

Complete mTeSR 1 Medium

Reagent	Final concentration	Amount
mTeSR1 Basal Medium	N/A	400 mL
mTeSR1 5× supplement	2.442 mM	0.22 g

Note: Growth medium may be stored at 4°C for up to two weeks

S1 Medium		
Reagent	Final concentration	Amount
MCDB131	N/A	500 mL
Glucose	2.442 mM	0.22 g
NaHCO3	29.28 mM	1.23 g
FAF-BSA stock concentration	2%	10 g
ITS-X stock concentration	0.002×	10 μL
Glutamax stock concentration	1×	5 mL
Vitamin C	0.25 mM	0.022 g
P/S stock concentration	1×	5 mL

Note: Store differentiation media at 4°C for up to six months

Note: Differentiation media must be vacuum filtered and remain sterile

S2 Medium		
Reagent	Final concentration	Amount
MCDB131	N/A	500 mL
Glucose	2.442 mM	0.22 g
NaHCO3	14.64 mM	0.615 g
FAF-BSA stock concentration	2%	10 g
ITS-X stock concentration	0.002×	10 μL
Glutamax stock concentration	1×	5 mL
Vitamin C	0.25 mM	0.022 g
P/S stock concentration	1×	5 mL

Note: Store differentiation media at 4°C for up to six months

Note: Differentiation media must be vacuum filtered and remain sterile

S3 Medium		
Reagent	Final concentration	Amount
MCDB131	N/A	500 mL
Glucose	2.442 mM	0.22 g
NaHCO3	14.64 mM	0.615 g
FAF-BSA	2%	10 g
ITS-X	0.5×	2.5 mL
Glutamax	1×	5 mL
Vitamin C	0.25 mM	.022 g
P/S	1×	5 mL

Note: Store differentiation media at 4°C for up to six months

Note: Differentiation media must be vacuum filtered and remain sterile

BE5 Medium		
Reagent	Final concentration	Amount
MCDB131	N/A	500 mL
Glucose	19.99 mM	1.8 g

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Continued		
Reagent	Final concentration	Amount
NaHCO3	20.89 mM	0.877 g
FAF-BSA stock concentration	2%	10 g
ITS-X stock concentration	0.5×	2.5 mL
Glutamax stock concentration	1×	5 mL
Vitamin C	0.25 mM	.022 g
Heparin	0.667 μM	5 mg
P/S stock concentration	1×	5 mL

Note: Store differentiation media at 4°C for up to six months.

Note: Differentiation media must be vacuum filtered and remain sterile

STEP-BY-STEP METHOD DETAILS

The differentiation protocol takes the hPSCs through the 6-stage media protocol. See Figure 5 for the overall directed differentiation path, as well as quality controls, media, and factors used at each differentiation step.

SC-islet differentiation in 3D bioreactors

Timing: 2 days of incubation, 20 days of differentiation, 5 weeks of maturation

Day-by-Day Differentiation Guide							
Day	Stage	Media	Factors	Stock Conc.	Final Conc.	Vol/30 mL	Vol/300 mL
−1	S0d1 (24 h)	mTeSR1 Half media change	–		–	–	–
0	S0d2 (48 h)	mTeSR11	–		–	–	–
1	S1d1	S1	Activin A	10 μg/mL	100 ng/mL	0.3 mL	3.0 mL
			CHIR99021	10 mM	3 μM	9 μL	90 μL
2	S1d2	S1	Activin A	10 μg/mL	100 ng/mL	0.3 mL	3.0 mL
3	S1d3	No media change	–		–	–	–
4	S2d1	S2	KGF	50 μg/mL	50 ng/mL	30 μL	300 μL
5	S2d2	No media change	–		–	–	–
6	S2d3	S2	KGF	50 μg/mL	50 ng/mL	30 μL	300 μL
7	S3d1	S3	KGF	50 μg/mL	50 ng/mL	30 μL	300 μL
			LDN193189	1 mM	200 nM	6 μL	60 μL
			Sant-1	1 mM	0.25 μM	7.5 μL	75 μL
			RA	10 mM	2 μM	6 μL	60 μL
			PDBU	1 mM	500 nM	15 μL	150 μL
			ROCK Ing	10 mM	10 μM	30 μL	300 μL
8	S3d2	S3	KGF	50 μg/mL	50 ng/mL	30 μL	300 μL
			Sant-1	1 mM	0.25 μM	7.5 μL	75 μL
			RA	10 mM	2 μM	6 μL	60 μL
			PDBU	1 mM	500 nM	15 μL	150 μL
			ROCK Inh	10 mM	10 μM	30 μL	300 μL
9	S4d1	S3	KGF	50 μg/mL	50 ng/mL	30 μL	300 μL
			Sant-1	1 mM	0.25 μM	7.5 μL	75 μL
			RA	10 mM	0.1 μM	0.3 μL	3.0 μL
			ROCK Inh	10 mM	10 μM	30 μL	300 μL
			Activin A	10 μg/mL	5 ng/mL	15 μL	150 μL
10	S4d2	No media change	–		–	–	–

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Day	Stage	Media	Factors	Stock Conc.	Final Conc.	Vol/30 mL	Vol/300 mL
11	S4d3	S3	KGF	50 µg/mL	50 ng/mL	30 µL	300 µL
			Sant-1	1 mM	0.25 µM	7.5 µL	75 µL
			RA	10 mM	0.1 µM	0.3 µL	3.0 µL
			ROCK Inh	10 mM	10 µM	30 µL	300 µL
			Activin A	10 µg/mL	5 ng/mL	15 µL	150 µL
12	S4d4	No media change	–		–	–	–
13	S4d5	S3	KGF	50 µg/mL	50 ng/mL	30 µL	300 µL
			Sant-1	1 mM	0.25 µM	7.5 µL	75 µL
			RA	10 mM	0.1 µM	0.3 µL	3.0 µL
			ROCK Inh	10 mM	10 µM	30 µL	300 µL
			Activin A	10 µg/mL	5 ng/mL	15 µL	150 µL
14	S5d1	BE5	Sant-1	1 mM	0.25 µM	7.5 µL	75 µL
			Betacellulin	**100 µg/mL**	20 ng/mL	6 µL	60 µL
			XXI	10 mM	1 µM	3 µL	30 µL
			Alk5i	100 mM	10 µM	3 µL	30 µL
			T3	10 mM	1 µM	3 µL	30 µL
			RA	10 mM	0.1 µM	0.3 µL	3.0 µL
14	S5d2	No media change	–		–	–	–
16	S5d3	BE5	Sant-1	1 mM	0.25 µM	7.5 µL	75 µL
			Betacellulin	100 µg/mL	20 ng/mL	6 µL	60 µL
			XXI	10 mM	1 µM	3 µL	30 µL
			Alk5i	100 mM	10 µM	3 µL	30 µL
			T3	10 mM	1 µM	3 µL	30 µL
			RA	10 mM	0.1 µM	0.3 µL	3.0 µL
17	S5d4	No media change	–		–	–	–
18	S5d5	BE5	Betacellulin	100 µg/mL	20 ng/mL	6 µL	60 µL
			XXI	10 mM	1 µM	3 µL	30 µL
			Alk5i	100 mM	10 µM	3 µL	30 µL
			T3	10 mM	1 µM	3 µL	30 µL
			RA	10 mM	0.025 µM	0.075 µL	0.75 µL
19	S5d6	No media change	–		–	–	–
20	S5d7	BE5	Betacellulin	100 µg/mL	20 ng/mL	6 µL	60 µL
			XXI	10 mM	1 µM	3 µL	30 µL
			Alk5i	100 mM	10 µM	3 µL	30 µL
			T3	10 mM	1 µM	3 µL	30 µL
			RA	10 mM	0.025 µM	0.075 µL	0.75 µL
21+	S6 Odd days	S3	None		–	–	–
22+	S6 Even days	No media change	–		–	–	–

Stage 0- pluripotent stem cells

Note: Stage 0 begins on the first day after clump passaging in 3D (stage 0 day 0) differentiation preparation.

Note: Stage 0 is the only stage when no Penicillin-Streptomycin is added to the media, to prevent loss of pluripotency.[15] Follow strict sterile protocol to avoid contamination during this time.

Note: To prepare complete mTeSR1 medium (basal medium + 5× supplement), thaw 5× mTeSR1 supplement at 4°C (do not place in water bath), then add to mTeSR1 basal medium.

Note: The day after splitting (24 h half-feed), complete mTeSR1 should contain ROCK inhibitor. The next day (48 h full feed) it should not contain any ROCK inhibitor.

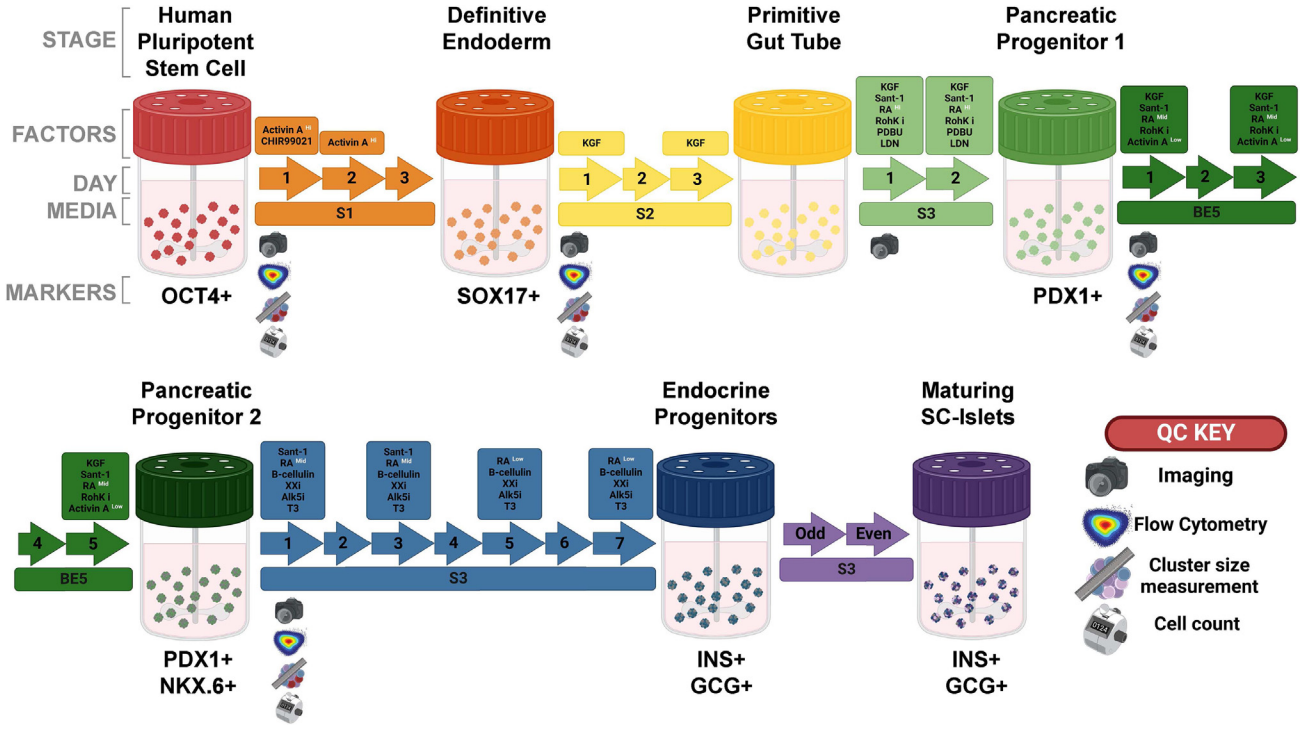

Figure 5. Overview of the six-step 3D differentiation protocol

The schematic follows the cells through their consecutive fates, induced by the steps of the protocol, along the journey from stem cell to maturing islet. Shown are the number of days at each step, the necessary media and factors added each day, the quality checks performed at the beginning of each step, and the protein markers to be assessed for completion of each differentiation stage.

1. *Day 0*: Passage maintenance line as described in step 4: Clump Passaging hPSCs in 3D Culture". Reseed the maintenance line, start a parallel line for later differentiation. Seed at optimal density (e.g., 0.6 × 10^6 cells/mL in mTeSR1 + 1:1000 ROCK inhibitor for Hues8). For 30 mL bioreactors, 18 M cells in 30 mL is typical. Take a sample for analysis of pluripotency by flow cytometry (see before you begin step 8: QC sample collection).
2. 24 h (Day 1): After 24 h, perform a half-media change as described below:
   a. Remove bioreactor from incubator, clean the outer surface with 70% ethanol and place in BSC. Wipe dry with a Kim wipe.
   b. Wait 3–5 min until cell clusters have mostly settled.

   ⚠ CRITICAL: Clusters should be small at this point but will settle to the bottom third of the bioreactor if given enough time. Visually confirm the absence of particles in the top half of the bioreactor.

   c. Open the bioreactor cap and aspirate half the volume (15 mL for the 30 mL bioreactor or 150 mL for the 300 mL bioreactor). Be careful not to aspirate any clusters.

   ⚠ CRITICAL: Keep the aspiration tip as close to the surface of the media as possible to minimize cell loss.

   d. Return culture to full volume by replacing with either 15 mL or 150 mL fresh complete mTeSR1 medium (no ROCK inhibitor). Replace and tighten cap. Return bioreactor to the incubator.
3. 48 h (Day 2): After all cell clusters have settled, perform a full-media change as described in step 11: Step-by-Step Media Change in Bioreactors. Replace spent media with fresh complete mTeSR1.

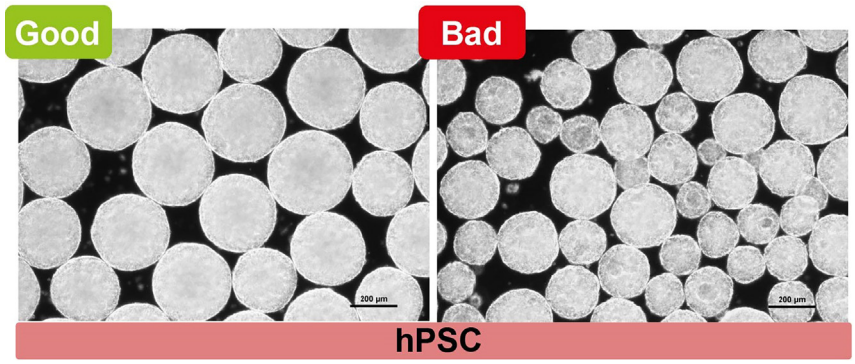

Figure 6. Good and bad morphology for 3D human pluripotent stem cell clusters
3D hPSC clusters entering stage 1 should be round and uniform in morphology, with clear, sharp edges. An average cluster diameter in the 200–270 µm range is expected. Poor clusters are not uniform in size or morphology, lack sharp edges, and can present a hollow core and cell shedding (resulting in cloudier media).

Stage 1- definitive endoderm

Timing: Days 1–3

In stage 1 of this protocol, stem cells are differentiated into definitive endoderm using the transforming growth factor Activin A (AA) and the GSK-3 inhibitor/Wnt activator CHIR9902.

Note: Stage 1 media is exchanged over two consecutive days: stage 1 day 1 uses Activin A and CHIR99021, and stage 1 day 2 uses only Activin A (see day-by-day differentiation guide for concentrations).

4. S1d1 (Day 1)
   a. Collect QC for the completion of stage 0:
      i. Use a P1000 pipette to spin the impeller and draw up a representative sample.
      ii. Transfer 0.5 mL of cell clusters to a 24-well plate to take bright field images.
      iii. Measure cluster size, then use for flow cytometry QC.

   Note: see before you begin step 8: QC sample collection and expected outcomes for collection, flow cytometry markers, and morphology.

   Note: see Figures 6 and 7 for expected hPSC flow cytometry marker results and cluster morphology.

   b. Perform a full-media change to s1d1 media (S1 Medium + Activin A and CHIR99021) as described in step 11: Step-by-step media change in bioreactors. Try to remove all spent media without aspirating clusters.
5. S1d2 (Day 2): Wait a full 24 h after s1d1 media change to perform a full-media change to s1d2 media (S1 Medium + Activin A) as described in step 11: Step-by-step media change in bioreactors.
6. S1d3 (Day 3): No media change. Bioreactor remains in culture undisturbed.

Stage 2- primitive gut tube

Timing: Days 4–6

Primitive gut tube is induced in stage 2 using keratinocyte growth factor (KGF).

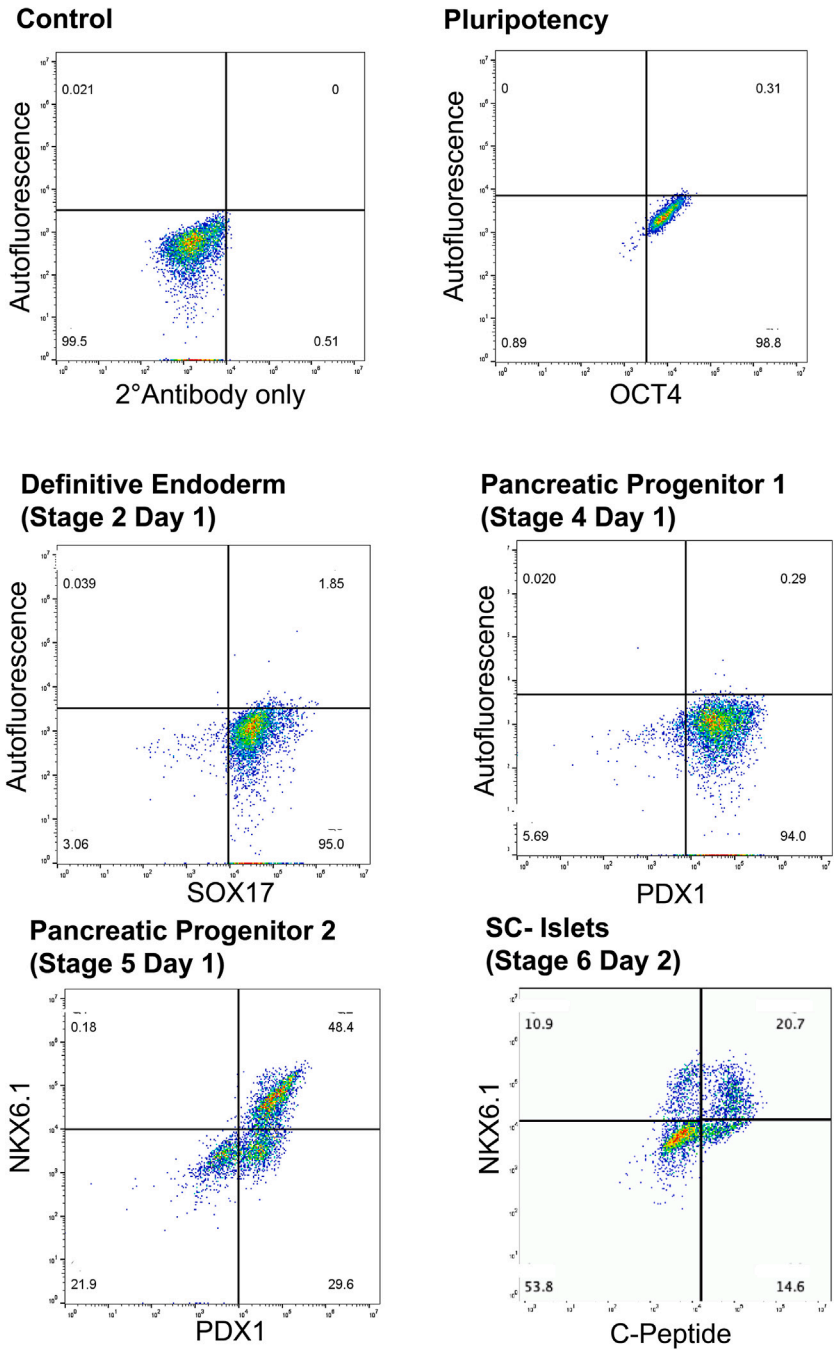

Figure 7. Example flow cytometry quantification of stage-specific markers

Upon quantification by flow cytometry, hPSC clusters should be >85% OCT4+; DE clusters >85% SOX17+; PP1 >85% PDX1+; PP2 35–60% NKX6.1+/PDX1+; SC-islets 15%–30% NKX6.1+/C-peptide+ and 15%–30% NKX6.1+/C-peptide+. An unstained sample (secondary antibody only) is shown as control for flow cytometry gating. Antibodies used are shown in the key resources table.

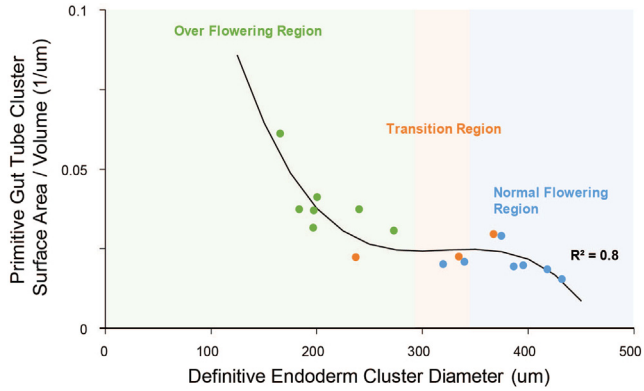

Figure 8. Success of primitive gut tube morphogenesis depends on initial definitive endoderm cluster diameter

A cubic model predicts morphogenesis during differentiation from DE to PGT. The surface area to volume ratio of PGT clusters as a function of their diameter at the DE stage is shown. Smaller DE clusters, which have excess surface area relative to volume, are easier to fold and thus more susceptible to excessive folding into a "flower" circular morphology with fluffy edges (over-flowering, green). Larger clusters, with lower surface area to volume ratios, undergo normal flowering (blue). A transition region (orange) exists where normal flowering morphology and over-flowering morphology co-exist in the same differentiation.

Note: Stage 2 uses KGF on its first and third day.

Note: There is no flow cytometry QC taken upon stage 2 completion.

Note: See Figure 8 for the expected relationship between definitive endoderm cluster diameter and primitive gut tube surface area/volume, for differentiations conducted in 30 mL bioreactors in a magnetic stir culture system set to 60 RPM. Larger clusters are necessary for proper flowering in lower bioreactors volumes, likely due to increased shear forces.

7. S2d1 (Day 4):
   a. Collect QC for the completion of stage 1 (stage 1c):
      i. Use a P1000 pipette to spin the impeller and draw up a representative sample.
      ii. Transfer 0.5 mL clusters into a 24-well to take bright field images.
      iii. Measure cluster size, then use for flow cytometry QC.

Note: (see before you begin step 8: QC sample collection). See Figures 7 and 9 for expected Definitive Endoderm flow cytometry marker results and cluster morphology.

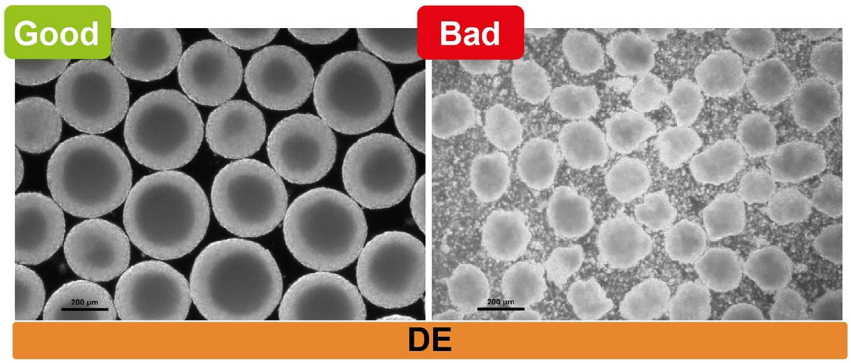

Figure 9. Good and bad morphology for 3D definitive endoderm clusters

3D DE clusters entering stage 2 should be round and uniform in size and can present rough edges or a hollow core. Poor clusters are smaller and less uniform in morphology due to excessive cell shedding (resulting in cloudier media).

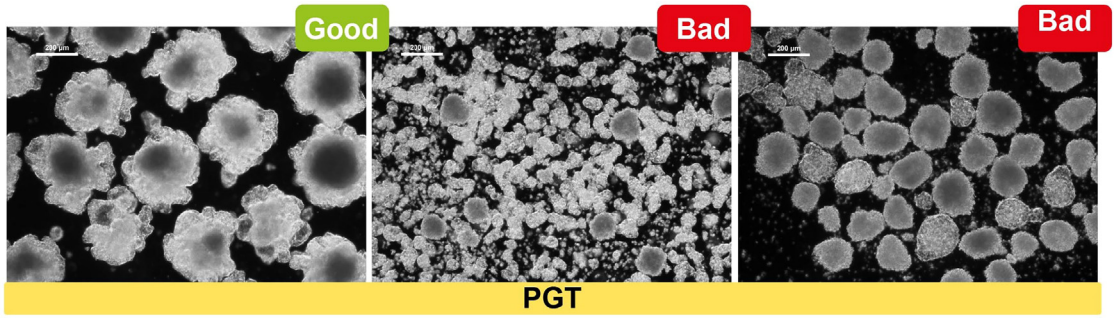

Figure 10. Good and bad morphology for primitive gut tube clusters

3D PGT clusters entering stage 3 should show a uniform "flower" circular morphology with fluffy edges. Poor clusters show over-flowering, leading to excessive cell shedding, or underflowering, indicating failed differentiation.

b. Perform a full-media change to s2d1 media (S2 Medium + KGF) as described in step 11: step-by-step media change in bioreactors. It is difficult to tell when clusters have settled at this point due to cloudiness in the media. However, slowly lifting the bioreactor should allow you to see settled clusters. Typically, this takes 4–5 min.

8. S2d2 (Day 5):
   a. No media change. Bioreactor remains in culture undisturbed.
9. S2d3 (Day 6):
   a. Perform a full-media change to s2d3 media (S2 Medium + KGF) as described in step 11: step-by-step media change in bioreactors.

Note: The clusters take longer to settle at this stage due to flowering (up to ~7–9 min). If there are a few clusters suspended in media after this time, aspirate them to keep the sunken cells from sticking to the bioreactor.

Stage 3- pancreatic progenitors 1

Timing: Days 7 and 8

In Stage 3 early PDX1+ pancreatic endoderm progenitors (PP1) are induced using a combination of KGF and hedgehog inhibitor (SANT1), along with a BMP inhibitor (LDN193189), the PKC activator Phorbol 12,13-dibutyrate (PDBU), and retinoic acid (RA)

Note: Stage 3 involves feeding every 24 h. The first day requires KGF, Sant1, LDN, RA, ROCK inhibitor, and PDBU. The second day requires all the same factors except LDN (see day-by-day differentiation guide).

10. S3d1 (Day 7):
    a. Collect QC for the completion of stage 2 (stage 2c):
       i. use a P1000 pipette to spin the impeller and draw up a representative sample.
       ii. Collect 0.35 mL into a 24-well to take bright field images and measure cluster size (no flow cytometry sample).

Note: (see before you begin step 8: QC sample collection).

Note: See Figure 10 for expected Primitive Gut Tube cluster morphology.

b. Perform a full-media change to s3d1 media (S3 Medium + KGF, LDN193189, Sant-1 RA, PDBU, ROCK inhibitor) as described in step 11: step-by-step media change in bioreactors.

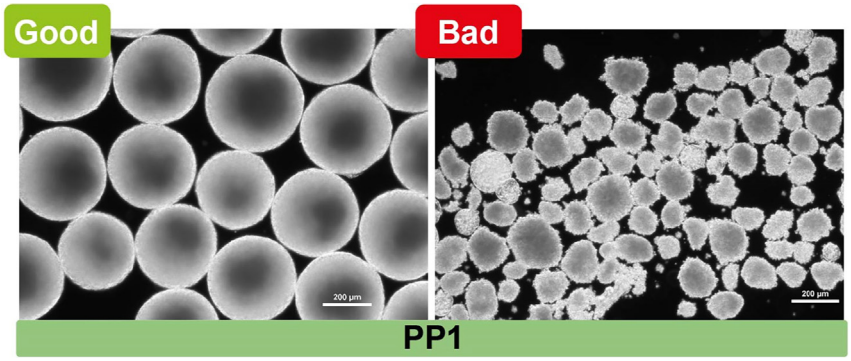

Figure 11. Good and bad morphology for early pancreatic progenitors

3D PP1 clusters entering stage 4 should show a uniform round morphology, a solid core, and be relatively even in size. Poor clusters are smaller and less uniform in morphology.

Note: The clusters take longer to settle at this stage due to flowering, up to ~7–9 min. If there are a few clusters suspended in media after this time, aspirate them to keep the sunken cells from sticking to the bioreactor.

11. S3d2 (Day 8):
    a. Perform a full-media change to s3d2 media (S3 Medium + KGF, Sant-1 RA, PDBU, ROCK inhibitor) as described in step 11: step-by-step media change in bioreactors. The clusters should settle much faster than on s3d1, and the medium is clear after clusters settle.

Stage 4- pancreatic progenitors 2

Timing: Days 9–13

In Stage 4 late PDX1+ NkX6.1+ pancreatic endoderm progenitors (PP2) are induced using a combination of KGF and hedgehog inhibitor (SANT1), along with a BMP inhibitor (LDN193189), the PKC activator Phorbol 12,13-dibutyrate (PDBU), and retinoic acid (RA), and AA.

Note: Stage 4 requires a wash on the first day, due to a decrease in the retinoic acid concentration.

Note: Stage 4 feedings differ from stage 3 in that they require Activin A, but not LDN191189 or PDBU, and the RA concentration drops (see day-by-day differentiation guide).

12. S4d1 (Day 9):
    a. Collect QC for the completion of stage 3(stage 3c):
        i. use a P1000 pipette to spin the impeller and draw up a representative sample.
        ii. Transfer 0.5 mL clusters into a 24-well to take bright field images.
        iii. Measure cluster size, then use for flow cytometry QC.

    Note: (see QC sample collection).

    Note: See Figures 7 and 11 for expected Early Pancreatic Progenitors flow cytometry marker results and cluster morphology.

    b. In addition to the volume required for media change, prepare an extra half volume of basal S3 media (no factors) on this day for wash.
    c. Mandatory Wash:

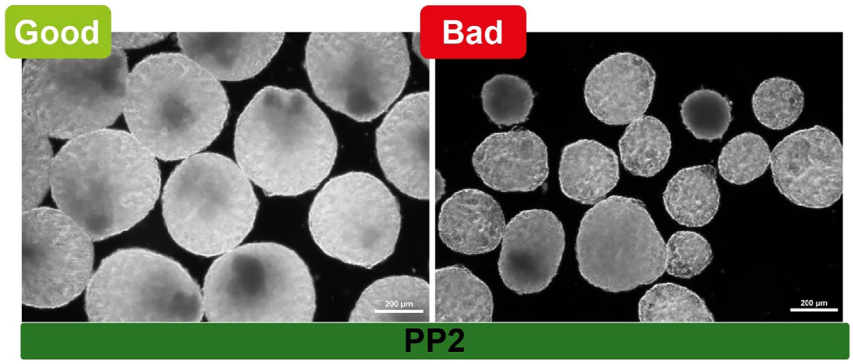

Figure 12. Good and bad morphology for late pancreatic progenitors
3D PP2 clusters entering stage 5 should show a uniform "crater"-like appearance and may present small lumps. Poor clusters are smaller and less uniform in morphology and may present over-lumping.

i. Perform a full-media change as described in step 11: step-by-step media change in bioreactors, through step 2.
ii. Instead of adding fresh differentiation media, add half culture volume of fresh basal S3 media (no factors) and gently swirl bioreactor to mix.
iii. Wait 2–4 min for clusters to settle, then aspirate away basal S3 media.

⚠ CRITICAL: this is required to remove residual retinoic acid from stage 3.

d. Proceed to step 3 of "step-by-step media change in bioreactors and feed with s4d1 media (S3 Medium + KGF, Sant-1 RA, ROCK inhibitor, Activin A).

13. S4d2 (Day 10):
    a. No media change. Bioreactor remains in culture undisturbed.
14. S4d3 (Day 11):
    a. Perform a full-media change to s4d3 media (S3 Medium + KGF, Sant-1 RA, ROCK inhibitor, Activin A) as described in step 11: step-by-step media change in bioreactors.
15. S4d4 (Day 12):
    a. No media change. Bioreactor remains in culture undisturbed.
16. S4d5 (Day 13):
    a. Perform a full-media change to s4d5 media (S3 Medium + KGF, Sant-1 RA, ROCK inhibitor, Activin A) as described in step 11: step-by-step media change in bioreactors.

Stage 5- endocrine progenitors

Timing: Days 14–20

Stage 5 achieves endocrine differentiation using the epidermal growth factor betacellulin, the γ-secretase inhibitor XXI, thyroid hormone (T3), the ALK5 transforming growth factor inhibitor II, SANT1, and RA.

17. S5d1 (Day 14):
    a. Collect QC:
        i. use a P1000 pipette to spin the impeller and draw up a representative sample.
        ii. Transfer 0.5 mL clusters into a 24-well to take bright field images.
        iii. Measure cluster size, then use for flow cytometry QC.

Note: See before you begin step 8: QC sample collection.

Note: See Figures 7 and 12 for expected Late Pancreatic Progenitors flow cytometry marker results and cluster morphology.

b. Perform a full-media change to s5d1 media (BE5 Medium + Sant-1, Betacellulin, XXI, Alk5i, T3, RA) as described in step 11: step-by-step media change in bioreactors.
c. Cloudy media may develop later in this stage, as with stage 1.

18. S5d2 (Day 15):
    a. No media change. Bioreactor remains in culture undisturbed.
19. S5d3 (Day 16):
    a. Perform a full-media change to s5d3 media (BE5 Medium + Sant-1, Betacellulin, XXI, Alk5i, T3, RA) as described in step 11: step-by-step media change in bioreactors.
20. S5d4 (Day 17):
    a. No media change. Bioreactor remains in culture undisturbed.
21. S5d5 (Day 18):
    a. Perform a full-media change to s5d5 media (BE5 Medium + Betacellulin, XXI, Alk5i, T3, RA) as described in step 11: step-by-step media change in bioreactors.

⚠ CRITICAL: Pay attention to the difference in media composition between days 3 and 5. After day 3, Sant1 is no longer added, and the concentration of retinoic acid is reduced.

22. S5d6 (Day 19):
    a. No media change. Bioreactor remains in culture undisturbed.
23. S5d7 (Day 20):
    a. Perform a full-media change to s5d7 media (BE5 Medium + Betacellulin, XXI, Alk5i, T3, RA) as described in step 11: step-by-step media change in bioreactors.

Stage 6- maturing SC-Islets

Timing: Days 21–56

Timing: ~4 h (for step 27)

Stage 6 has no exogenously added factors and yields SC-islets that gain increasing levels of NKX6.1 and C-peptide along with robust glucose-stimulated insulin and glucagon secretion function over 4–5 weeks of extended culture.

24. S6d1 (Day 21):
    a. Collect QC:
        i. use a P1000 pipette to spin the impeller and draw up a representative sample.
        ii. Transfer 0.5 mL clusters into a 24-well to take bright field images.
        iii. Measure cluster size, then use for flow cytometry QC.
        iv. Use clusters for flow cytometry QC.

Note: see before you begin step 8: QC sample collection.

Note: See Figures 7 and 13 for expected Endocrine Progenitors flow cytometry marker results and cluster morphology.

Note: Reference Figures 7 and 15 for expected flow cytometry results by stage and by week.

b. Perform a full-media change with S3 media (no differentiation factors) as described in step 11: step-by-step media change in bioreactors.

25. S6d2 (Day 22):

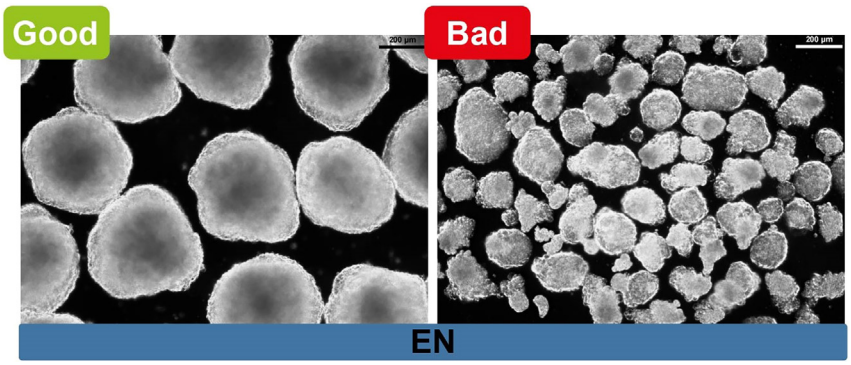

Figure 13. Good and bad morphology for endocrine progenitors

3D EN clusters entering stage 6 should show a less uniform, lumpy, and somewhat uneven appearance, including craters and buds containing endocrine cells. Poor clusters are smaller in size and show greater variability.

a. No media change. Bioreactor remains in culture undisturbed.

26. Rest of Stage 6:
    a. Every other day for the rest of Stage 6 (e.g., Stage 6 day 1, 3, 5), perform a full-media change to fresh S3 media as described in step 11: step-by-step media change in bioreactors.

⚠ CRITICAL: if necessary, the feeding regimen can be shifted to e.g., even instead of odd days by performing a media change two days in a row.

b. Collect QC weekly to track the level of Nkx6.1/c-peptide copositive cells.

Note: See Figures 14 and 15 for expected maturing SC-islet cluster morphology and flow cytometry marker results.

Note: The percentage of Nkx6.1/c-peptide copositive cells typically peaks between s6d7-s6d25. Glucose-stimulated hormone secretion function typically peaks between weeks 3–5 of extended culture (see Figure 16 for expected results).

27. Glucose-stimulated Hormone Secretion.
    Glucose-stimulated Hormone secretion assays are a method of quality checking the function of sc-islets as they move through the maturation phase. Steps to measure SC-islet hormone secretion through serial static incubations are described below. See (Alvarez-Dominguez et al., 2020) for dynamic SC-islet hormone secretion measurements using a perfusion instrument.

Note: Use freshly made 20 mM glucose, 2.8 mM glucose and 30 mM KCl in 2.8 mM glucose buffers.

Note: Store KREBS buffer at 4°C for up to 1 week.

Note: Use wide bore P1000 pipette tips to safely transfer cell clusters without accidental trituration.

Note: 0.5 mL samples should contain about 1,000 cell clusters (about 1 million cells). Typical cluster pellet sizes for 1,000 cell clusters in a 15 mL conical tube is about 5 mm.

Note: This preparation is sufficient for assaying glucose-stimulated hormone secretion in triplicate for each of 4 samples. If you have more samples, prepare more plates accordingly.

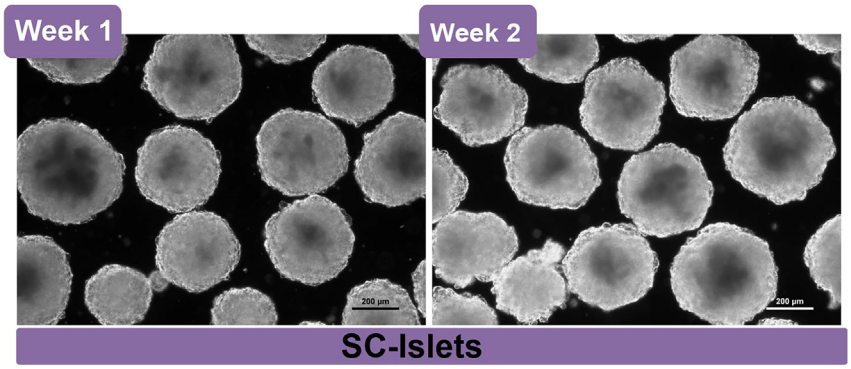

Figure 14. Expected morphology for maturing SC-islets

3D SC-islets cultured for several weeks in stage 6 should maintain an even morphology and may increase size due to larger endocrine buds.

a. Set Up.
   i. Make 2.8 mM glucose (LOW) buffer: dilute 1 M glucose 1:357 into KREBs buffer. 20 mM glucose (HIGH) buffer: dilute 1 M glucose 1:50 into KREBs buffer. 30 mM KCl buffer: dilute 2 M KCl into LOW glucose buffer with 0.75:50 dilution.

$$\text{Total LOW glucose buffer needed} = (5\ \text{mL} * X) + (6\ \text{mL} * 3 * X)$$

$$\text{Total HIGH glucose buffer needed} = (2\ \text{mL} * 3 * X)$$

$$\text{Total KCl in LOW glucose buffer needed} = (1\text{mL} * 3 * X)$$

   X = sample number (samples are run in triplicate).
   ii. Each sample is tested in triplicate (3 inserts/sample). Place 12 inserts into the top two rows of a low-attachment 24-well plate (Figure 17.) The bottom two rows will be used for wicking.
   iii. Pre-fill wells containing inserts with 0.5 mL of LOW glucose Buffer, inside and outside the insert by adding about 250 µL on each. Then pre-fill the top 12 wells of 4 additional 24-well plates without inserts: one with 1 mL/well LOW glucose buffer, another with 1 mL/well HIGH glucose buffer, another with 1 mL/well KCl buffer, and the last one with TrypLE (1 mL/well) (Figure 17).
   iv. Place pre-filled plates in a 37°C incubator to equilibrate for at least 15 min.
   v. Collect 5 mL of each bioreactor.
   vi. Wash each sample with 2 mL of LOW glucose buffer in a 15 mL conical tube, spin down at 200 g and resuspend in 2 mL of LOW glucose buffer for distribution to the 24-well plate.

⚠ CRITICAL: out of 2 mL, 3 × 0.5 mL volume is used for assaying hormone secretion in triplicate, and the remaining 0.5 mL volume can be used for intracellular insulin content estimation.

b. Fasting step.
   i. Distribute 0.5 mL from each sample into each of the 3 replicate inserts/sample (final volume per well is 1.0 mL) using wide bore P1000 pipette tips, then place the plate in the 37°C incubator for 1 h. If you have more than one 24-well plate, process one plate at a time.

c. Insulin Content Preparation.
   i. After placing plates into the incubator for the fasting step, aspirate LOW glucose buffer from the 0.5 mL leftover of each sample in the 15 mL conical tube and add 1 mL of M-Per mammalian protein extraction reagent to lyse cells.

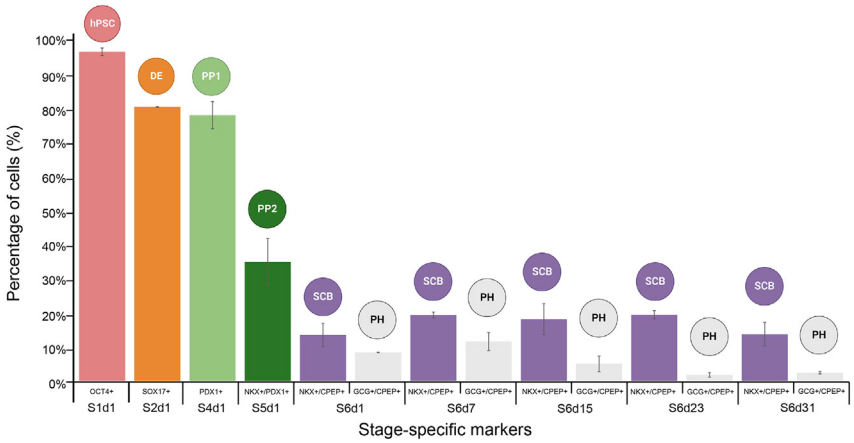

Figure 15. Expected stage-specific marker results

Expected average results and the standard error of the mean for stage-specific protein markers, quantified by flow cytometry, during directed SC-islet differentiation. hPSC, human pluripotent stem cells (OCT4+); DE, definitive endoderm (SOX17+); PP1, early pancreatic progenitors (PDX1+); PP2, late pancreatic progenitors (PDX1+ NKX6.1+); EN, endocrine progenitors (NGN3+); SC-β, stem-cell-derived β cells (NKX6.1+ C-peptide+); PH, polyhormonal cells (NKX6.1+ C-peptide+). Antibodies used are shown in the key resources table.

ii. Incubate samples at room temperature (20°C–25°C) for about 15 min.

iii. Using a P1000 pipette, triturate clusters by pipetting up and down until all the cells are lysed. Transfer 1 mL of insulin content sample into a 1.5 mL microcentrifuge tube and store at −80°C until the time of the ELISA assay.

d. LOW glucose challenge step.

i. After 1 h of LOW glucose buffer incubation, remove plate containing 'glucose starved' cell clusters from the incubator, as well as the other LOW glucose prefilled plate.

ii. Using forceps, gently take out the insert and shake it, then transfer each insert into an empty well in the bottom two rows and let LOW buffer drain from insert into the well by wicking (Figure 17, B).

iii. Promptly pass inserts into the "LOW" plate and place it in the 37°C incubator for 1 h. This is the start of the LOW glucose challenge.

⚠ CRITICAL: when transferring inserts, push each gently into the bottom of the well ensuring that there are no air bubbles blocking buffer flow into the insert.

iv. After 1 h of the last LOW glucose buffer incubation, remove the plate containing the inserts as well as the HIGH glucose buffer prefilled plate.

e. HIGH glucose challenge step.

i. Repeat step 21dii of the LOW glucose challenge and transfer inserts into the HIGH glucose buffer containing plate and place the plate in the incubator for 1 h. This is the start of the HIGH glucose challenge.

⚠ CRITICAL: When transferring inserts, push each gently into the bottom of the well ensuring that there are no air bubbles blocking buffer flow into the insert.

ii. To collect the first LOW glucose incubation supernatant, remove the 'wicked' liquid from the 24-well plate and combine it with the liquid in the original well from which the insert was removed. Mix using a P200 pipette then transfer 0.2 mL into a low-attachment 96-well plate and store at −20 to −70°C. Store for up to 6 months.

iii. After 1 h of HIGH glucose buffer incubation, remove the plate containing the inserts as well as the KCl plate.

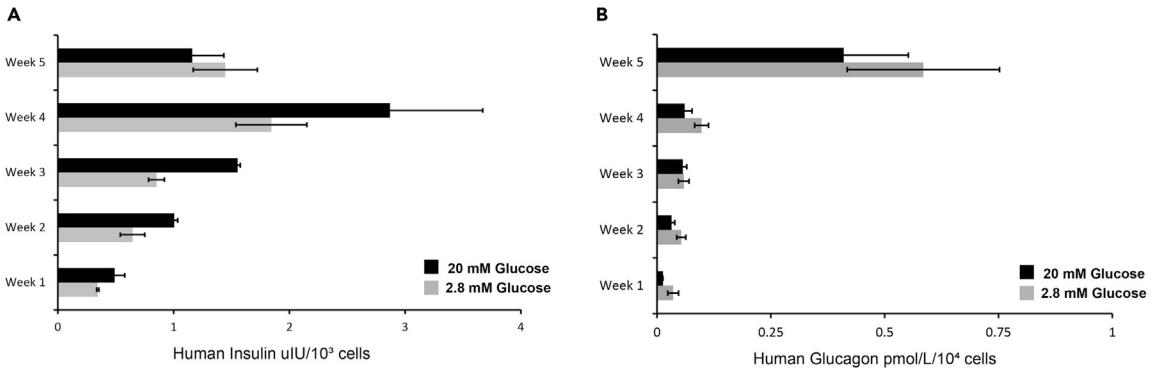

Figure 16. Expected glucose-stimulated hormone secretion function

This figure represents the average and standard error of the mean of weekly glucose-stimulated hormone secretion. 3D SC-islets cultured for several weeks in stage 6 should progressively amplify their insulin (A) and glucagon (B) secretion fold-change in 20 mM glucose relative to 2.8 mM glucose and expand their absolute secretion capacity. Peak glucose-stimulated hormone secretion function is observed after 3–4 weeks of culture.

f. KCl challenge step.
   i. Repeat step 21dii of the LOW glucose challenge and transfer inserts into the KCl buffer containing plate. This is the start of the KCl challenge.

⚠ CRITICAL: When transferring inserts, push each gently into the bottom of the well ensuring that there are no air bubbles blocking buffer flow into the insert.

   ii. To collect the HIGH glucose incubation supernatant, follow step 21eii of the HIGH glucose challenge step.
   iii. After 1 h of KCl buffer incubation, remove the plate containing the inserts as well as the TrypLE prefilled plate.

g. Cell cluster dispersion and cell counting step.
   i. Repeat step 21dii of the LOW glucose challenge, transfer inserts into the TrypLE containing plate and incubate in the 37°C incubator for 30 min. This is the start of the cluster dissociation step.

⚠ CRITICAL: When transferring inserts, push each gently into the bottom of the well ensuring that there are no air bubbles blocking buffer flow into the insert.

   ii. To collect KCl incubation supernatant, follow step 21eii of the HIGH glucose challenge step.
   iii. After 30 min of TrypLE incubation, triturate clusters by pipetting up and down using a P1000 pipette, removing as many cells from the insert mesh as possible without rupturing the mesh. Transfer 0.5 mL of dispersed cells into a microcentrifuge tube (Figure 17, C1).
   iv. Remove the remaining 0.5 mL volume from the mesh by puncturing through it with a P1000 pipette and transferring liquid into the same microcentrifuge tube (Figure 17, C2). Each microcentrifuge tube should now contain 1 mL of cells + TrypLE.
   v. Count the total number of cells. The total cell count will be used to normalize insulin secretion level from each replicate measurement.

h. Human Hormone level measurement by protein ELISA.
   i. Dilute the collected samples using x mL KREBs buffer for x number of cells.

⚠ CRITICAL: Sample dilution must be optimized according to the number of cells assayed and their hormone content / secretion level. For Hues 8 SC-islets, we typically

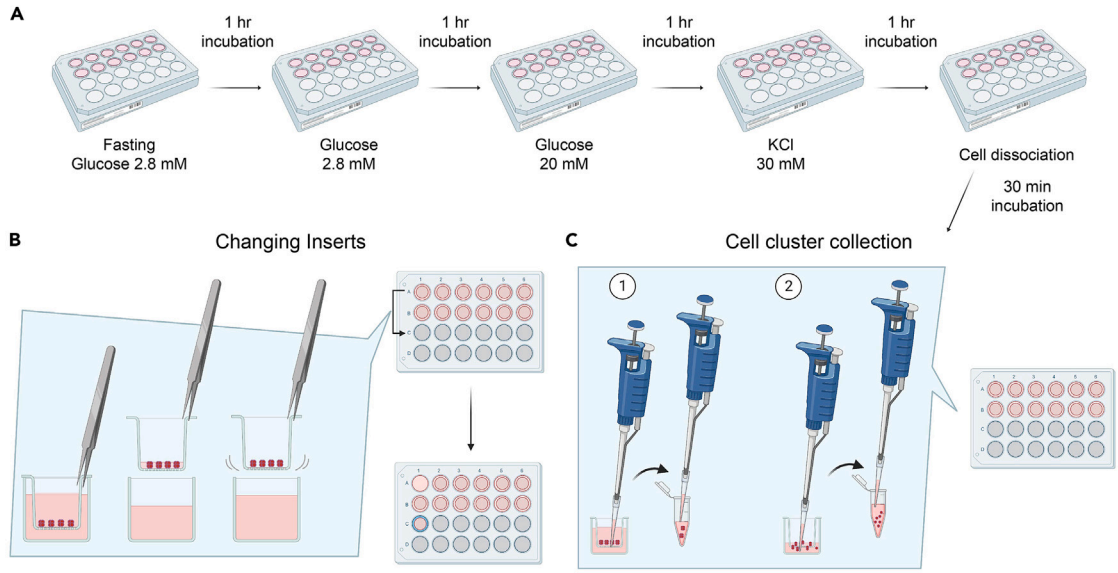

Figure 17. Overview of protocol for measuring glucose-stimulated hormone secretion

(A) 3D SC-islets are subjected to sequential challenges with low and high glucose concentrations, followed by a challenge with KCl and subsequent dissociation to obtain a cell count.

(B) Procedure for changing inserts.

(C) Procedure for SC-islet cluster collection and mechanical dissociation.

use a 1:10 dilution for assaying glucagon secretion, 1:50 dilution for insulin secretion, and 1:5000 dilution for intracellular insulin content.

ii. Measure human insulin and glucagon levels using human insulin ELISA (ALPCO Diagnostics; 80-INSHUU-E01.1) and Glucagon ELISA (Mercodia; 10-1271-01), respectively, according to the manufacturer's instructions. (https://www.alpco.com/insulin-elisa.html , https://www.mercodia.com/products/glucagon-elisa/)

iii. Divide the measured hormone concentration by the total cell number in the sample, calculate the average from the triplicates, and express secretion results per 1000 cells (uIU/mL/1000 cells (insulin) or pmol/L/10000 cells (glucagon).

EXPECTED OUTCOMES

For flow cytometry outcomes at each stage as well as visual outcomes at each stage, see quantification and statistical analysis and Figures 5, 6, 7, 9, 10, 11, 12, 13, 14, and 15.

2D hPSC outcomes: hPSCs should form tightly packed, rounded, and smooth looking cells. They should maintain distinct cell borders, lacking visible signs of irregularities between cells. Due to rapid division of hPSCs, multiple nucleoli should be observable in many of the cells. See Figure 2 for an example of ideal 2D hPSC morphology at 100% confluency.

Stage 0 Expected Outcomes: Upon stage 0 completion, cell clusters should be round and uniform (Figure 6). For 300 mL bioreactors, the expected cluster diameter is 200–300 μm. For 30 mL bioreactors, shear forces are larger, thus a diameter of >350 μm is desirable. hPSCs at this stage should remain almost entirely pluripotent, with >95% cells positive for the pluripotency marker OCT4, as measured by flow cytometry (Figure 7).

Stage 1 Expected Outcomes: Upon stage 1 completion, cell clusters should be round and uniform, with occasionally more fluffy edges (Figure 9). Cluster size should be relatively consistent with the previous stage, though it may become slightly smaller. Cluster cores may become hollow. Clusters

shed significant numbers of cells at the end of stage 1, and as a result the media becomes cloudy. This can be mistaken for contamination but is not an immediate cause for alarm. Check the supernatant for excessive presence of single cells, which marks poor differentiation outcomes. Flow cytometry results should indicate that >85% of cells express SOX17 upon stage 1 completion (Figure 7). Slightly lower results may not indicate differentiation failure.

Stage 2 Expected Outcomes: Clusters "flower" (Figure 10). Each cluster should appear fluffy and amorphous, having lost its uniform appearance.

Stage 3 Expected Outcomes: Clusters completing stage 3 are uniform and round with a solid core (Figure 11), and >50% of cells should express PDX1.

Stage 4 Expected Outcomes: Stage 4 is a point where visual signs of a failed differentiation can appear. Watch for cloudy media, significant cell loss, breaking up of clusters, or poor morphology (Figure 12) as these may indicate a potential problem or poor differentiation outcome. Expect 35%–60% NKX6.1/PDX1 double positive cells.

Stage 5 Expected Outcomes: Expect Lumpy, variable "peanut-like" clusters (Figures 13 and 14). 10%–30% NKX6.1/C-peptide co-positive cells are expected at the end of this stage, and should increase in stage 6, peaking around 3 weeks.

QUANTIFICATION AND STATISTICAL ANALYSIS

Expected results for QC by stage

Stage	QC collected	Vol.	Cluster morphology	Cell count (# viable cells/300 mL)	Expected FACS results
End of Stage 0 (S1d1)	Picture, Cluster Diameter, Flow cytometry	0.5 mL	Round, uniform clusters	Approx. 450–550 million	>90% Oct4+ cells
End of Stage 1 (S2d1)	Picture, Diameter, Flow cytometry, count	0.5 mL			>80% Sox17+ cells
End of Stage 2 (S3d1)	Picture	0.35 mL	Flowering	N/A	N/A
End of Stage 3 (S4d1)	Picture, Diameter, Flow cytometry, count	0.5 mL	Round, uniform clusters.	Approx. 400–500 million	>80% Pdx1+ cells
End of Stage 4 (S5d1)	Picture, Diameter, Flow cytometry, count	0.5 mL	Budding	Approx. 500–700 million	25%–50% Nkx6.1+/Pdx1+ cells
End of Stage 5 (S6d1)	Picture, Diameter, Flow cytometry, count	0.5 mL	Lumpy, often "peanut-like"	Approx. 300–600 million	>10% Nkx6.1+/c-pep+ cells
Stage 6 (week 1–4)	Picture, Diameter, Flow cytometry, count	0.5 mL	Bumpier, fluffier edges		>20% Nkx6.1+/c-pep+ cells

LIMITATIONS

This scalable protocol was optimized to robustly generate 3D SC-islets with the Hues 8 hPSC line and gene-modified subclones,[1,12–14] but further optimization may be required to extend it to different hPSC lines (see troubleshooting). Distinct pancreatic lineage differentiation propensities have been reported for embryonically derived lines,[9,16] and induced patient-derived lines are known to show varying differentiation competence and yields across different SC-islet differentiation protocols.[17–20]

We have outlined steps to minimize variability between differentiations and discussed potential evidence-based solutions to address poor differentiation outcomes (see troubleshooting). Yet, variables such as passage number, karyotype instability and copy number variation may still cause uncontrolled variability and need to be addressed for each maintenance line individually.

SC-islets differ in cell composition from natural counterparts. They contain fewer monohormonal insulin-producing cells, and harbor polyhormonal (including alpha- and delta-like) and ductal-, acinar-, and enteroendocrine-like cells.[14,21] To form SC-islets free of non-native and misdifferentiated lineages, additional steps for cell purification[22–27] and reaggregation[13,14] may become necessary.

Suspension bioreactors are a robust method to yield SC-islets that perform glucose-stimulated insulin and glucagon secretion. However, SC-islets generally lack the precision, kinetics, and magnitude of insulin/glucagon secretion that natural islets show during adult life, even after extended *in vitro* maturation.[1,13,14,28,29] The mechanisms underlying islet functional maturation are an active field of study,[30–33] and harnessing them will be necessary to generate fully mature *in vitro* SC-islet products.

TROUBLESHOOTING

Problem 1

Poor morphology of 3D hPSC clusters (before you begin step 4, Figure 6).

Potential solution

Ensure proper 3D clump passage, cryopreservation, and thawing technique, working quickly, deliberately, and carefully (pluripotent stem cell culture). Do not incubate hPSC clusters in GCDR longer than necessary for subsequent dissociation. Consider thawing a new cell maintenance line if <90% are pluripotent, as measured by flow cytometry.

Problem 2

Cells become stuck in cell strainer during hPSC clump passage (before you begin, step 4q).

Potential solution

Wet filter with 2 mL PBS before passing 3D hPSC clusters through the 37 μm strainer to break them up into clumps. Gently pipette cells up and down inside of the cell strainer. Use a new cell strainer to break up remaining clumps if necessary.

Problem 3

Difficulty adapting hPSCs to 3D suspension culture (before you begin, step 3).

Potential solution

The 3D adaptation steps must be optimized for each new cell line (Adapting hPSCs to 3D suspension culture). Optimize the hPSC seeding density (try different densities between 0.4 million and 1 million cells per mL), the rotation speed of the magnetic stir system, and the passage cycle until achieving the target growth rate and cluster size. Different cell lines may also benefit from different incubation times in GCDR (see step 4: clump passaging hPSCs in 3D culture). If 3D adaptation continues to fail, consider thawing a new cell maintenance line.

Problem 4

FAF-BSA not dissolving during preparation of differentiation media (before you begin, step 12e).

Potential solution

Warm basal media prior to preparation. If stirrers are not working, shake container vigorously and warm in water bath. Leave for 20 min and shake again until all FAF-BSA has dissolved.

Problem 5

Excessive shedding during differentiation of 3D definitive endoderm clusters (Steps 4–6).

Potential solution

Increase the starting size of 3D hPSC clusters entering differentiation. This can be achieved by lowering the RPM of the magnetic stir system, or by waiting longer than 72 h after seeding hPSC clusters before adding differentiation media. If shed cells have low viability, Stage 1 media can be supplemented with ROCK Inhibitor (Y) to prevent excessive shedding due to cell death.

Problem 6

Over-flowering of 3D primitive gut tube clusters (Steps 7–9, Figure 10).

Potential solution

For smaller bioreactors, increase the size of 3D definitive endoderm clusters to prevent excessive folding due to excess surface area to volume ratio (Figure 8). This can be done by modifying the stirring rotation speed during differentiation, or by waiting longer than 72 h after seeding hPSC clusters before adding differentiation media.

Problem 7

Excessive shedding during SC-islet formation (Steps 24–26).

Potential solution

Decrease fluid shear stress to prevent shedding of peninsular buds containing endocrine cells. This can be achieved by lowering the RPM of the magnetic stir system, or by transferring SC-islets to low-attachment plates placed in orbital shakers. To avoid aspiration of endocrine buds during media change, collect clusters and pellet at 200 g for 2 min prior to media change (see SC-islet differentiation in 3D bioreactors).

Problem 8

Poor differentiation outcomes (Compare with Figure 7).

Potential solution

Maintain a strict 24 h feeding schedule, by changing the media within 23–25 h each day that requires a media change. Check quality controls at each differentiation stage to determine the time frame and conditions under which differentiation efficiency is being lost (QC sample collection).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Juan R. Alvarez-Dominguez (juan.alvarez@pennmedicine.upenn.edu).

Materials availability

This study did not generate unique or new reagents.

Data and code availability

No datasets or code was produced by this protocol.

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AUTHOR CONTRIBUTIONS

Conceptualization and design, S.D.P. and J.R.A.-D.; methodology, S.D.P., I.M.G.-S., M.L., and J.R.A.-D.; validation, S.D.P., I.M.G.-S., Z.L.G., and J.R.A.-D.; resources, S.D.P. and J.R.A.-D.; writing – review and editing, all authors; visualization, S.D.P., I.M.G.-S., and M.L.; supervision and funding acquisition, J.R.A.-D.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Alvarez-Dominguez, J.R., Donaghey, J., Rasouli, N., Kenty, J.H.R., Helman, A., Charlton, J., Straubhaar, J.R., Meissner, A., and Melton, D.A. (2020 Jan 2). Circadian Entrainment Triggers Maturation of Human In Vitro Islets. Cell Stem Cell *26*, 108–122.e10. https://doi.org/10.1016/j.stem.2019.11.011.

2. Leavens, K.F., Alvarez-Dominguez, J.R., Vo, L.T., Russ, H.A., and Parent, A.V. (2022). Stem cell-based multi-tissue platforms to model human autoimmune diabetes. Mol. Metab. *66*, 101610. https://doi.org/10.1016/j.molmet.2022.101610.

3. Melton, D. (2021). The promise of stem cell-derived islet replacement therapy. Diabetologia *64*, 1030–1036. https://doi.org/10.1007/s00125-020-05367-2.

4. Hogrebe, N.J., Ishahak, M., and Millman, J.R. (2023). Developments in stem cell-derived islet replacement therapy for treating type 1 diabetes. Cell Stem Cell *30*, 530–548. https://doi.org/10.1016/j.stem.2023.04.002.

5. Balboa, D., Iworima, D.G., and Kieffer, T.J. (2021). Human Pluripotent Stem Cells to Model Islet Defects in Diabetes. Front. Endocrinol. *12*, 642152. https://doi.org/10.3389/fendo.2021.642152.

6. Russ, H.A., Parent, A.V., Ringler, J.J., Hennings, T.G., Nair, G.G., Shveygert, M., Guo, T., Puri, S., Haataja, L., Cirulli, V., et al. (2015). Controlled induction of human pancreatic progenitors produces functional beta-like cells *in vitro*. EMBO J. *34*, 1759–1772. https://doi.org/10.15252/embj.201591058.

7. Sharon, N., Chawla, R., Mueller, J., Vanderhooft, J., Whitehorn, L.J., Rosenthal, B., Gürtler, M., Estanboulieh, R.R., Shvartsman, D., Gifford, D.K., et al. (2019). A Peninsular Structure Coordinates Asynchronous Differentiation with Morphogenesis to Generate Pancreatic Islets. Cell *176*, 790–804.e13. https://doi.org/10.1016/j.cell.2018.12.003.

8. Adams, M.T., and Blum, B. (2022). Determinants and dynamics of pancreatic islet architecture. Islets *14*, 82–100. https://doi.org/10.1080/19382014.2022.2030649.

9. Siehler, J., Blöchinger, A.K., Meier, M., and Lickert, H. (2021). Engineering islets from stem cells for advanced therapies of diabetes. Nat. Rev. Drug Discov. *20*, 920–940. https://doi.org/10.1038/s41573-021-00262-w.

10. Nair, G.G., Tzanakakis, E.S., and Hebrok, M. (2020). Emerging routes to the generation of functional beta-cells for diabetes mellitus cell therapy. Nat. Rev. Endocrinol. *16*, 506–518. https://doi.org/10.1038/s41574-020-0375-3.

11. Vining, K.H., and Mooney, D.J. (2017). Mechanical forces direct stem cell behaviour in development and regeneration. Nat. Rev. Mol. Cell Biol. *18*, 728–742. https://doi.org/10.1038/nrm.2017.108.

12. Pagliuca, F.W., Millman, J.R., Gürtler, M., Segel, M., Van Dervort, A., Ryu, J.H., Peterson, Q.P., Greiner, D., and Melton, D.A. (2014). Generation of functional human pancreatic beta cells *in vitro*. Cell *159*, 428–439. https://doi.org/10.1016/j.cell.2014.09.040.

13. Velazco-Cruz, L., Song, J., Maxwell, K.G., Goedegebuure, M.M., Augsornworawat, P., Hogrebe, N.J., and Millman, J.R. (2019). Acquisition of Dynamic Function in Human Stem Cell-Derived beta Cells. Stem Cell Rep. *12*, 351–365. https://doi.org/10.1016/j.stemcr.2018.12.012.

14. Veres, A., Faust, A.L., Bushnell, H.L., Engquist, E.N., Kenty, J.H.R., Harb, G., Poh, Y.C., Sintov, E., Gürtler, M., Pagliuca, F.W., et al. (2019). Charting cellular identity during human *in vitro* beta-cell differentiation. Nature *569*, 368–373. https://doi.org/10.1038/s41586-019-1168-5.

15. Farzaneh, M. (2021). Concise Review; Effects of Antibiotics and Antimycotics on the Biological Properties of Human Pluripotent and Multipotent Stem Cells. Curr. Stem Cell Res. Ther. *16*, 400–405. https://doi.org/10.2174/1574888x16999201203214425.

16. Osafune, K., Caron, L., Borowiak, M., Martinez, R.J., Fitz-Gerald, C.S., Sato, Y., Cowan, C.A., Chien, K.R., and Melton, D.A. (2008). Marked differences in differentiation propensity among human embryonic stem cell lines. Nat. Biotechnol. *26*, 313–315. https://doi.org/10.1038/nbt1383.

17. Hogrebe, N.J., Maxwell, K.G., Augsornworawat, P., and Millman, J.R. (2021). Generation of insulin-producing pancreatic beta cells from multiple human stem cell lines. Nat. Protoc. *16*, 4109–4143. https://doi.org/10.1038/s41596-021-00560-y.

18. Barsby, T., Ibrahim, H., Lithovius, V., Montaser, H., Balboa, D., Vähäkangas, E., Chandra, V., Saarimäki-Vire, J., and Otonkoski, T. (2022). Differentiating functional human islet-like aggregates from pluripotent stem cells. STAR Protoc. *3*, 101711. https://doi.org/10.1016/j.xpro.2022.101711.

19. Sui, L., Danzl, N., Campbell, S.R., Viola, R., Williams, D., Xing, Y., Wang, Y., Phillips, N., Poffenberger, G., Johannesson, B., et al. (2018). beta-Cell Replacement in Mice Using Human Type 1 Diabetes Nuclear Transfer Embryonic Stem Cells. Diabetes *67*, 26–35. https://doi.org/10.2337/db17-0120.

20. Leite, N.C., Sintov, E., Meissner, T.B., Brehm, M.A., Greiner, D.L., Harlan, D.M., and Melton, D.A. (2020). Modeling Type 1 Diabetes In Vitro Using Human Pluripotent Stem Cells. Cell Rep. *32*, 107894. https://doi.org/10.1016/j.celrep.2020.107894.

21. Sharon, N., Vanderhooft, J., Straubhaar, J., Mueller, J., Chawla, R., Zhou, Q., Engquist, E.N., Trapnell, C., Gifford, D.K., and Melton, D.A. (2019). Wnt Signaling Separates the Progenitor and Endocrine Compartments during Pancreas Development. Cell Rep. *27*, 2281–2291.e5. https://doi.org/10.1016/j.celrep.2019.04.083.

22. Rasouli, N., Melton, D.A., and Alvarez-Dominguez, J.R. (2020). Purification of Live Stem-Cell-Derived Islet Lineage Intermediates. Curr. Protoc. Stem Cell Biol. *53*, e111. https://doi.org/10.1002/cpsc.111.

23. Davis, J.C., Helman, A., Rivera-Feliciano, J., Langston, C.M., Engquist, E.N., and Melton, D.A. (2019). Live Cell Monitoring and Enrichment of Stem Cell-Derived beta Cells Using Intracellular Zinc Content as a Population Marker. Curr. Protoc. Stem Cell Biol. *51*, e99. https://doi.org/10.1002/cpsc.99.

24. Docherty, F.M., Riemondy, K.A., Castro-Gutierrez, R., Dwulet, J.M., Shilleh, A.H., Hansen, M.S., Williams, S.P.M., Armitage, L.H., Santostefano, K.E., Wallet, M.A., et al. (2021). ENTPD3 Marks Mature Stem Cell-Derived beta-Cells Formed by Self-Aggregation In Vitro. Diabetes *70*, 2554–2567. https://doi.org/10.2337/db20-0873.

25. Leavens, K.F., Liao, C.M., Gagne, A.L., Kishore, S., Cardenas-Diaz, F.L., French, D.L., and Gadue, P. (2020). Generation of a double insulin and somatostatin reporter line, SCSe001-A-3, for the advancement of stem cell-derived pancreatic islets. Stem Cell Res. *50*, 102112. https://doi.org/10.1016/j.scr.2020.102112.

26. Nair, G.G., Liu, J.S., Russ, H.A., Tran, S., Saxton, M.S., Chen, R., Juang, C., Li, M.L., Nguyen, V.Q., Giacometti, S., et al. (2019). Recapitulating endocrine cell clustering in culture promotes maturation of human stem-cell-derived beta cells. Nat. Cell Biol. *21*,

263–274. https://doi.org/10.1038/s41556-018-0271-4.

27. Parent, A.V., Ashe, S., Nair, G.G., Li, M.L., Chavez, J., Liu, J.S., Zhong, Y., Streeter, P.R., and Hebrok, M. (2022). Development of a scalable method to isolate subsets of stem cell-derived pancreatic islet cells. Stem Cell Rep. *17*, 979–992. https://doi.org/10.1016/j.stemcr.2022.02.001.

28. Balboa, D., Barsby, T., Lithovius, V., Saarimäki-Vire, J., Omar-Hmeadi, M., Dyachok, O., Montaser, H., Lund, P.E., Yang, M., Ibrahim, H., et al. (2022). Functional, metabolic and transcriptional maturation of human pancreatic islets derived from stem cells. Nat. Biotechnol. *40*, 1042–1055. https://doi.org/10.1038/s41587-022-01219-z.

29. Davis, J.C., Alves, T.C., Helman, A., Chen, J.C., Kenty, J.H., Cardone, R.L., Liu, D.R., Kibbey, R.G., and Melton, D.A. (2020). Glucose Response by Stem Cell-Derived beta Cells In Vitro Is Inhibited by a Bottleneck in Glycolysis. Cell Rep. *31*, 107623. https://doi.org/10.1016/j.celrep.2020.107623.

30. Barsby, T., and Otonkoski, T. (2022). Maturation of beta cells: lessons from in vivo and in vitro models. Diabetologia *65*, 917–930. https://doi.org/10.1007/s00125-022-05672-y.

31. Alvarez-Dominguez, J.R., and Melton, D.A. (2022). Cell maturation: Hallmarks, triggers, and manipulation. Cell *185*, 235–249. https://doi.org/10.1016/j.cell.2021.12.012.

32. Wortham, M., and Sander, M. (2021). Transcriptional mechanisms of pancreatic beta-cell maturation and functional adaptation. Trends Endocrinol. Metab. *32*, 474–487. https://doi.org/10.1016/j.tem.2021.04.011.

33. Velazco-Cruz, L., Goedegebuure, M.M., and Millman, J.R. (2020). Advances Toward Engineering Functionally Mature Human Pluripotent Stem Cell-Derived beta Cells. Front. Bioeng. Biotechnol. *8*, 786. https://doi.org/10.3389/fbioe.2020.00786.