Differentiating functional human islet-like aggregates from pluripotent stem cells

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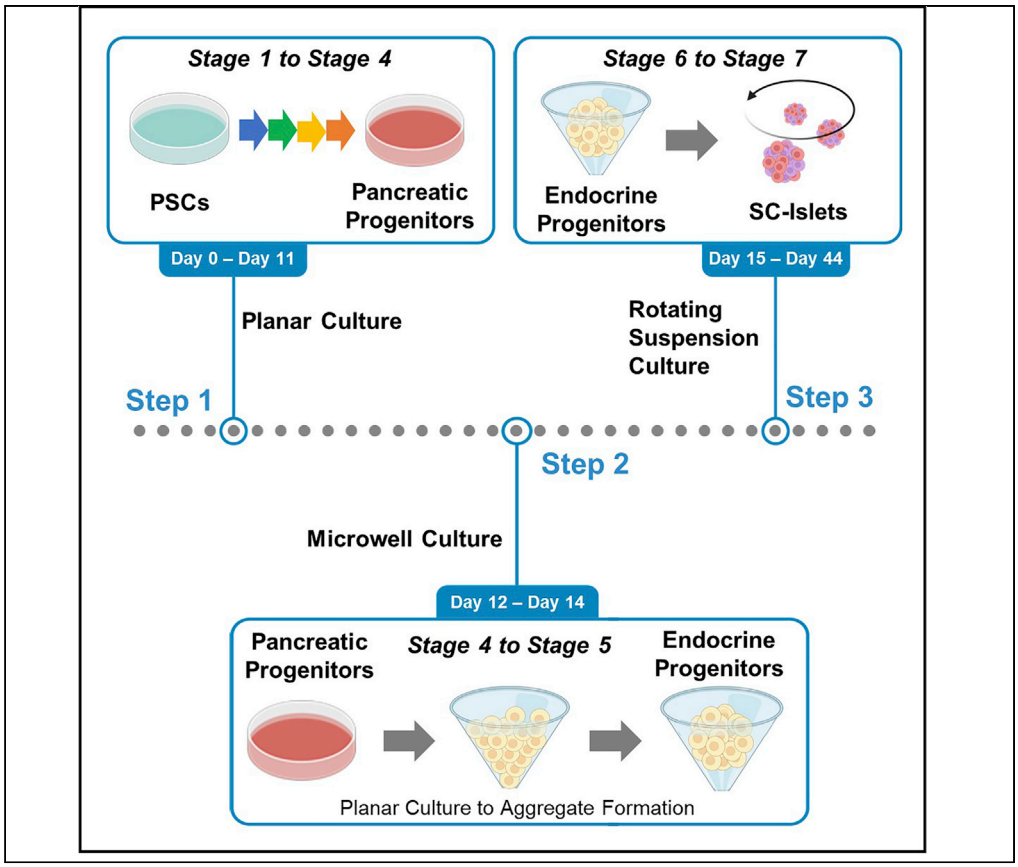

We present here a robust and reliable protocol by which to differentiate pancreatic islet-like aggregates (SC-islets) from human pluripotent stem cells. The 7-stage protocol mimics developmental patterning factors that induce endocrine lineage formation and spans monolayer, microwell, and aggregate suspension culture. The SC-islets demonstrate dynamic glucose-sensitive insulin secretion and an endocrine cell composition similar to those of primary human islets. SC-islets generated using this optimized protocol are suitable for *in vitro* modeling of islet cell pathophysiology and therapeutic applications.

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

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Highlights

7-stage stepwise differentiation of pluripotent stem cell-derived islets (SC-islets)

Monolayer-to-microwell formation of endocrine progenitor clusters

3D maturation of SC-islets in rotating suspension culture

SC-islets display glucose-sensitive insulin release and islet-like cell composition

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Protocol

Differentiating functional human islet-like aggregates from pluripotent stem cells

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SUMMARY

We present here a robust and reliable protocol by which to differentiate pancreatic islet-like aggregates (SC-islets) from human pluripotent stem cells. The 7-stage protocol mimics developmental patterning factors that induce endocrine lineage formation and spans monolayer, microwell, and aggregate suspension culture. The SC-islets demonstrate dynamic glucose-sensitive insulin secretion and an endocrine cell composition similar to those of primary human islets. SC-islets generated using this optimized protocol are suitable for *in vitro* modeling of islet cell pathophysiology and therapeutic applications.

For complete details on the use and execution of this protocol, please refer to Balboa et al. (2022).

BEFORE YOU BEGIN

The protocol below was optimized using the H1 human embryonic stem cell (hESC) line, however, it has also been used to generate SC-islets from numerous internally produced induced pluripotent stem cell (iPSC) lines. Some line-to-line variability may take place during the differentiation protocol, but we have outlined possible modifications that can be made to minimize this effect.

The development of this protocol was made possible through the adaptation and refinement of numerous studies of pancreatic islet differentiation (Pagliuca et al., 2014; Rezania et al., 2014; Nostro et al., 2015; Balboa et al., 2018; Velazco-Cruz et al., 2019; Veres et al., 2019). Therefore, presented here is the most robust and reliable method of SC-islet differentiation that we have developed to date.

Institutional permissions

This protocol has been developed for the generation of SC-islets from both embryonic- and induced-pluripotent stem cell lines. All research involving human PSCs should be done so in accordance with relevant institutional guidelines.

Preparation of basal media, reagents, and supplement stock solutions

Note: This protocol is contingent on the ready access to basic cell culture facilities and supplies. Necessary reagents, consumables and equipment specific to this protocol (that may not be readily available) are outlined in the key resources table.

1. We recommend the preparation of basal media and the reconstitution of all small molecules and recombinant proteins prior to each stage of the differentiation protocol.
   a. The reconstitution solvent for each reagent (and required stock concentrations) are outlined in the table below.
   b. To aid in the efficiency of media changes during the differentiation, we have also included tables of combinations of particular reagents that form differentiation stage-specific "supplements" (See materials and equipment).
   c. Basal media compositions are also outlined in the materials and equipment section below.

⚠ CRITICAL: Handle all reagents and small molecule additives in aseptic conditions whenever possible. Larger reconstitution volumes (such as with basal media) should be sterile filtered prior to use.

Reagent	Stock concentration	Solvent
Activin A	1 mg/mL	10 mM HCl
ALK5inhII	50 mM	DMSO
Betacellulin (BTC)	100 ng/μL	0.1% BSA in PBS
CHIR-99021	10 mM	DMSO
FGF-7	100 ng/μL	0.1% BSA in PBS
GC1	10 mM	DMSO
GSiXX	1 mM	DMSO
hEGF	100 μg/mL	0.1% BSA in PBS
Heparin sodium salt	10 mg/mL	ddH_2O
LDN-193189	1 mM	DMSO
N-acetylcysteine	100 mM	PBS
Nicotinamide	1 M	ddH_2O
Retinoic Acid	10 mM	DMSO
SANT1	2.5 mM	DMSO
TPB	2.5 mM	DMSO
Triiodothyronine (T3)	10 mM	1 N NaOH
Vitamin C	250 mM	ddH_2O
Y-27632	10 mM	ddH_2O
ZM-447439	5 mM	DMSO
$ZnSO_4$	10 mM	ddH2O

Store all small molecule and recombinant working aliquots at −20°C (−80°C for long-term storage), with the exception of Heparin and $ZnSO_4$, which can be stored at 4°C. Limit freeze-thaw cycles from stock volumes. Certain small molecules are light sensitive and should be handled and stored in conditions outlined in their relevant product documentation.

Human pluripotent stem cell (PSC) culture and expansion

Note: PSC lines should be expanded prior to the start of a differentiation to allow a sufficient yield of SC-islets. Individual research groups may culture and expand PSCs under differing conditions. The steps below outline the protocol used by us to serve as a general guide.

2. PSCs are cultured on Matrigel-coated 10 cm tissue culture plates (40–55 μg/mL Matrigel in DMEM/F12, 6 mL per plate) with daily media changes of 10–12 mL of Essential 8™ (E8) media.
3. The passaging of PSCs should occur at 80%–90% confluency:
   a. Aspirate the E8 media and wash each plate with 4–6 mL EDTA-PBS solution (0.5 mM).
   b. Incubate each plate in 4–6 mL EDTA-PBS (0.5 mM) at 37°C for 2–3 min.
   c. Aspirate the EDTA solution and replace with 6 mL of E8 medium.

d. Use a cell scraper or mildly fragment the colonies with a serological pipette.
e. Transfer a proportion of the cell colony suspension at a ratio of 1:3 to 1:6 to fresh Matrigel-coated 10 cm plates containing E8 media.
f. Return the plates to a cell culture incubator (37°C, 5% CO_2). Change E8 media daily until confluency threshold is reached for the next passage.

4. PSC culture plates at approximately 90% confluency are suitable for the dissociation and high confluency re-seeding step at the start of a differentiation (Day 0, see below).

⚠ CRITICAL: Ensure that PSC cultures show low degrees of spontaneous differentiation prior to starting the differentiation protocol. Long term PSC cultures should also be regularly assayed for karyotypic abnormalities and mycoplasma contamination.

⚠ CRITICAL: Do not use PSCs that have been freshly thawed or freshly passaged in the differentiation protocol. Allow at least 1 day from a passage (or 2–3 passages from a thaw) before starting the differentiation.

⚠ CRITICAL: Overly confluent PSC cultures (and/or overly large PSC colonies) may negatively affect the differentiation outcomes or cause differentiations to fail.

⚠ CRITICAL: In our experience, PSCs cultured in E8 FLEX medium prior to the start of a differentiation may also negatively affect cell survival during the initial stages.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-CXCR4-PE (1:20)	BD Biosciences	555974
Anti-PDX1-PE (1:80)	BD Biosciences	562161
Anti-NKX6.1-647 (1:80)	BD Biosciences	563338
Anti-INS-647 (1:80)	Cell Signaling Technology	9008
Anti-GCG (1:160)	Sigma-Aldrich	G2654
Chemicals, peptides, and recombinant proteins		
Activin A	Qkine	Qk001
ALK5inhII	Selleckchem	S7233
Betacellulin (BTC)	PeproTech	100-50
BSA	Sigma-Aldrich	A7030
CHIR-99021	Tocris	4423
CMRL 1066 Medium	Corning	15-110-CVR
DMEM/F12 Medium	Thermo Fisher Scientific	11320033
DMSO	Sigma-Aldrich	D8418
Essential 8 (E8) Medium	Thermo Fisher Scientific	A1517001
FGF-7	GenScript	Z03047
GC1	Tocris	4554
Glucose	Sigma-Aldrich	G8769
GlutaMAX	Life Technologies	35050038
GSiXX	Millipore	565789
hEGF	Peprotech	AF-100-15
Heparin Sodium Salt	Sigma-Aldrich	H3149
ITS-X	Thermo Fisher Scientific	51500056
LDN-193189	Selleckchem	S2618
Lipid Concentrate	Invitrogen	11905-031
Matrigel	Corning	354277

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
MCDB131 Medium	Life Technologies	10372-019
N-acetylcysteine (NAC)	Sigma-Aldrich	A9165
$NaHCO_3$	Sigma-Aldrich	S5761
Nicotinamide	Sigma-Aldrich	N0636
Pen/Strep	BioNordika	BN-ECB3001D
Retinoic Acid	Sigma-Aldrich	R2625
SANT1	Sigma-Aldrich	S4572
Sodium Pyruvate	Lonza	BE13-115E
TPB	Santa Cruz	sc-204424
Trace Elements A	Cellgro	25-021-CI
Trace Elements B	Cellgro	99-175-CI
Triiodothyronine (T3)	Sigma-Aldrich	T6397
TrypLE	Thermo Fisher Scientific	12563029
Vitamin C	Sigma-Aldrich	A4544
Y-27632	Selleckchem	S1049
ZM-447439	Selleckchem	S1103
$ZnSO_4$	Sigma-Aldrich	Z0251
Experimental models: Cell lines		
H1 ESC line	WiCell	WA01
Multiple iPSC and ESC lines	N/A	N/A
Other		
6-well AggreWell 400 Microwell plates	STEMCELL Technologies	34425
Anti-Adherence Rinsing Solution	STEMCELL Technologies	07010
Swinging Bucket Plate Centrifuge	N/A	N/A
Platform Rotator in humidified incubator (37°C, 5% CO_2)	N/A	N/A

MATERIALS AND EQUIPMENT

Basal 1 Medium (Stage 1–2)		
Reagent	Final concentration	Amount
MCDB131	N/A	500 mL
GlutaMAX (100×)	1×	5 mL
Glucose (2.5 M)	10 mM (incl. MCDB131 glucose content)	1 mL
$NaHCO_3$	1.5 g/l	750 mg
BSA	0.5%	2.5 g
Total	**N/A**	**506 mL**

Basal media should be prepared in aseptic conditions and sterile filtered after solid reagent additives have fully dissolved. Basal media can be stored at 4°C for 2–3 weeks.

Basal 2 Medium (Stage 3–4)		
Reagent	Final concentration	Amount
MCDB131	N/A	500 mL
GlutaMAX (100×)	1×	5 mL
Glucose (2.5 M)	10 mM (incl. MCDB131 glucose content)	1 mL
$NaHCO_3$	2.5 g/l	1,250 mg
BSA	2%	10 g
ITS-X	0.5×	2.5 mL
Total	**N/A**	**508.5 mL**

Basal media should be prepared in aseptic conditions and sterile filtered after solid reagent additives have fully dissolved. Basal media can be stored at 4°C for 2–3 weeks.

Basal 3 Medium (Stage 5–6)		
Reagent	Final concentration	Amount
MCDB131	N/A	500 mL
GlutaMAX (100×)	1×	5 mL
Glucose (2.5 M)	20 mM (incl. MCDB131 glucose content)	3 mL
$NaHCO_3$	1.5 g/l	750 mg
BSA	2%	10 g
ITS-X	0.5×	2.5 mL
$ZnSO_4$ (10 mM)	10 μM	0.5 mL
Heparin (10 mg/mL)	10 μg/mL	0.5 mL
Pen/Strep (100×)	1×	5 mL
Total	**N/A**	**516.5 mL**

Basal media should be prepared in aseptic conditions and sterile filtered after solid reagent additives have fully dissolved. Basal media can be stored at 4°C for 2–3 weeks.

Basal 4 Medium (Stage 7)		
Reagent	Final concentration	Amount
CMRL 1066	N/A	500 mL
GlutaMAX (100×)	1×	5 mL
Pen/Strep (100×)	1×	5 mL
ITS-X	0.5×	2.5 mL
Sodium Pyruvate (100 mM)	0.5 mM	2.5 mL
$ZnSO_4$ (10 mM)	10 μM	0.5 mL
Heparin (10 mg/mL)	10 μg/mL	0.5 mL
Lipid Concentrate (2000×)	1×	0.25 mL
Trace Elements A (2000×)	1×	0.25 mL
Trace Elements B (2000×)	1×	0.25 mL
BSA	2%	10 g
Total	**N/A**	**516.75 mL**

No extra glucose is added into the Basal 4 medium. Basal media should be prepared in aseptic conditions and sterile filtered after solid reagent additives have fully dissolved. Basal media can be stored at 4°C for 2–3 weeks.

Stage 3 Supplement (2500×)		
Reagent	Final concentration	Amount
SANT1 (2.5 mM)	0.625 mM	100 μL
Retinoic Acid (10 mM)	2.5 mM	100 μL
LDN-193189 (1 mM)	0.25 mM	100 μL
TPB (2.5 mM)	0.5 mM	80 μL
DMSO	N/A	20 μL
Total	**N/A**	**400 μL**

Store 25–50 μL aliquots at −20°C. Limit freeze-thaw cycles.

Stage 4 Supplement (2500×)		
Reagent	Final concentration	Amount
SANT1 (2.5 mM)	0.625 mM	100 μL
Retinoic Acid (10 mM)	0.25 mM	10 μL
LDN-193189 (1 mM)	0.5 mM	200 μL
TPB (2.5 mM)	0.25 mM	40 μL
DMSO	N/A	50 μL
Total	**N/A**	**400 μL**

Store 25–50 μL aliquots at −20°C. Limit freeze-thaw cycles.

Stage 5 Supplement (1500×)		
Reagent	Final concentration	Amount
SANT1 (2.5 mM)	0.375 mM	100 μL
Retinoic Acid (10 mM)	75 μM	5 μL
LDN-193189 (1 mM)	0.15 mM	100 μL
GC1 (10 mM)	1.5 mM	100 μL
GSiXX (1 mM)	0.15 mM	100 μL
ALK5inhII (50 mM)	15 mM	200 μL
DMSO	N/A	62 μL
Total	**N/A**	**667 μL**
Store 25–50 μL aliquots at −20°C. Limit freeze-thaw cycles.		

Stage 6 Supplement (2000×)		
Reagent	Final concentration	Amount
LDN-193189 (1 mM)	0.2 mM	100 μL
GC1 (10 mM)	2 mM	100 μL
GSiXX (1 mM)	0.2 mM	100 μL
Alk5inhII (50 mM)	20 mM	200 μL
Total	**N/A**	**500 μL**
Store 25–50 μL aliquots at −20°C. Limit freeze-thaw cycles.		

Alternatives: Although our protocol has been developed with the above reagents from specific suppliers, other sources of each small molecule can likely also be substituted.

STEP-BY-STEP METHOD DETAILS

The SC-islet differentiation protocol outlined below is divided into 7 stages, roughly corresponding with developmental stages of the pancreas. Each stage contains specific media compositions that sequentially guide PSCs into pancreatic endocrine lineages. Full media reagent lists will be outlined below, but the protocol itself will be broken into the key steps that modulate the success and efficiency of the differentiation. Namely, i) the initial seeding of PSCs in planar culture; ii) the efficient induction of definitive endoderm; iii) the formation of pancreatic progenitors and their generation into uniformly sized endocrine progenitor aggregates; and finally, iv) the maturation of the SC-islets in suspension culture. All steps in this protocol should take place within appropriate biosafety cabinets and under aseptic cell culture conditions.

This protocol will yield functional glucose-sensitive SC-islets after 44 days of culture. This functional state can also be maintained for a minimum of 3 weeks in the final Stage 7 culture conditions.

Although yields can vary, 2 fully confluent 10 cm plates of PSCs at the start of the differentiation (seeded on Day 0) should be able to generate enough pancreatic progenitors to fill each well of a 6-well AggreWell plate, allowing the formation of approximately 8,000–10,000 SC-islets at the end of the protocol. This can therefore be scaled up or down depending on the required yields.

High density seeding of pluripotent stem cells

Timing: Day 0, 1 h

This step ensures a **fully confluent** seeding of cultured PSCs that enables the highly efficient induction of definitive endoderm (DE). The planar steps of the protocol can take place in either Matrigel-coated 6-well or 10 cm tissue culture plates. For high yields and ease of media exchange we recommend running the differentiation in 10 cm plates. However, seeding a small number of wells in 6-well

plates in parallel may be useful for quality control steps throughout the protocol that require cell extraction (see **optional** steps throughout).

1. Allow Matrigel-coated plates to warm to room temperature (if stored at 4°C) and pre-warm E8 medium to room temperature.
2. Dissociate PSC colonies from 10 cm culture plates:
   a. Aspirate E8 media and wash with 4–6 mL mL EDTA-PBS (0.5 mM) per plate.
   b. Replace the wash solution with another 4–6 mL of EDTA-PBS.
   c. Incubate the PSC plates at 37°C for 8–10 min.
3. Gently knock each plate to ensure that PSC colonies are beginning to dissociate and fragment into single cells. Adjust the incubation time accordingly.
4. Add 4–6 mL E8 medium (or DMEM/F12 medium) to each plate. Gently triturate the colony fragments using a serological pipette to acquire a single cell suspension. Pool the PSC suspension from each plate to a sterile 50 mL tube.
5. Remove an aliquot of the cell suspension to perform a cell count. Centrifuge the remaining volume at 200 g for 3 min.
6. Remove the supernatant and gently resuspend the cell pellet in E8 medium to a density of 2×10^6 cells/mL.
7. Plate the dissociated cells at a density of $1.5–2 \times 10^6$ cells/well of a 6-well plate, or $12–14 \times 10^6$ cells/10 cm plate.
8. Add E8 medium to bring the total volume to 3 mL/well in 6-well plates and 12–14 mL in each 10 cm plate.
9. Add ROCK inhibitor (Y-27632, 10 mM) to each plate/well for a final concentration of 10 μM.
10. Evenly disperse the cells with a gentle criss-cross motion (avoid swirling the plate). Move plates to a humidified incubator and allow cells to attach overnight.

⚠ CRITICAL: High PSC confluency prior to the start of the induction of definitive endoderm is a key step in biasing the differentiation towards pancreatic lineage selection. The seeding densities here should act as a guide and may need optimizing depending on the viability and proliferative capacity of the PSC line being used.

Definitive endoderm induction (stage 1)

Timing: Days 1–3, 30 min per day

This stage of the protocol is necessary to generate a dense layer of definitive endoderm (DE) from which to derive posterior foregut and pancreatic lineage identities in future steps. We have found that this is a critical step of the protocol and can be a common point of failure of the differentiation.

⚠ CRITICAL: The PSCs seeded on Day 0 should be 100% confluent before continuing with the differentiation (see Figure 1). Low confluency, low cell viability or over-acidification of the media will result in poor differentiation efficiency. (See troubleshooting, problem 1).

11. **Day 1**: Prepare an aliquot of Stage 1-Day 1 media and warm to 37°C.
    a. 12 mL needed for each 10 cm plate.
    b. 2 mL needed for each well of a 6-well plate.

Stage 1-Day 1 Medium

Reagent	Final concentration	Amount
Basal 1 Medium	N/A	1 mL
Activin A (1 mg/mL)	100 ng/mL	0.1 μL
CHIR-99021 (10 mM)	3 μM	0.3 μL

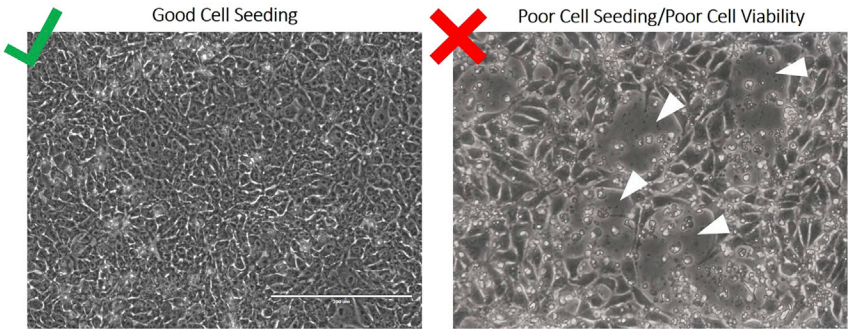

Figure 1. Examples of Good and Poor PSC seeding prior to the start of the differentiation

Before the start of DE induction PSCs should be at 100% confluency (left panel). Any gaps in the cell layer or drops in confluency due to excessive cell death or under-seeding will negatively affect the differentiation (right panel, white arrow heads). Scale bar represents 200 μm.

12. Gently remove the E8 media, rinse with 2 mL PBS per well (or 10 mL per 10 cm plate), and replace with the pre-warmed Stage 1-Day 1 media.
13. Return plates to an incubator overnight.
14. **Day 2**: Prepare an aliquot of Stage 1-Day 2 media and warm to 37°C.
    a. 12 mL needed for each 10 cm plate.
    b. 2 mL needed for each well of a 6-well plate.

Stage 1-Day 2 Medium		
Reagent	Final concentration	Amount
Basal 1 Medium	N/A	1 mL
Activin A (1 mg/mL)	100 ng/mL	0.1 μL
CHIR-99021 (10 mM)	0.3 μM	0.03 μL

15. Gently remove the previous media and replace with the pre-warmed Stage 1-Day 2 media.
16. Return plates to an incubator overnight.
17. **Day 3:** Prepare an aliquot of Stage 1-Day 3 media and warm to 37°C.
    a. 12 mL needed for each 10 cm plate.
    b. 2 mL needed for each well of a 6-well plate.

Stage 1-Day 3 Medium		
Reagent	Final concentration	Amount
Basal 1 Medium	N/A	1 mL
Activin A (1 mg/mL)	100 ng/mL	0.1 μL

18. Gently remove the previous media and replace with the pre-warmed Stage 1-Day 3 media.
19. Return plates to an incubator overnight.

Note: It is not uncommon to see a degree of cell death and detachment during DE induction. However, the proliferative nature of the cells at this stage should maintain a fully confluent cell layer.

⚠ CRITICAL: If a high rate of cell death or excessive detachment takes place during Stage 1 that results in gaps in the cell monolayer it is unlikely that the future steps of this protocol will be successful. To minimize the effect of harsh DE induction and excessive cell loss we recommend reducing the time in Stage 1 media slightly by initiating the change to Stage 2 media on Day 4 (below) as early as possible in the day. The media volume can also be

increased if over-acidification is resulting in high cell death (3 mL per well in 6-well plates/ 16 mL per 10 cm plate).

Pancreatic progenitor formation (stage 2 to stage 4)

Timing: Days 4–10, 30 min per day

This portion of the protocol also takes place in planar cell culture and includes patterning factors that trigger differentiation through primitive gut tube (Stage 2), posterior foregut (Stage 3) and into pancreatic progenitor (Stage 4) lineages. Media must be changed **each day** following the compositions outlined below.

20. **Day 4 – Day 6**: Prepare an aliquot of Stage 2 medium and warm to 37°C.
    a. 12 mL needed for each 10 cm plate.
    b. 2 mL needed for each well of a 6-well plate.

Stage 2 Medium		
Reagent	Final concentration	Amount
Basal 1 Medium	N/A	1 mL
Vitamin C (250 mM)	0.25 mM	1 µL
FGF-7 (100 ng/µL)	50 ng/mL	0.5 µL

21. Gently remove the previous media and replace with the pre-warmed Stage 2 media.

Optional: To ensure the efficient induction of DE in Stage 1, we recommend assaying the presence of the DE marker CXCR4 (CD184) through flow cytometry on Day 4. The dissociation of a well of Day 4 cells with EDTA-PBS followed by an antibody probe against CXCR4 should reveal an induction efficiency of >90% (Figure 2). For more detailed analyses of DE induction, it is also possible to co-stain for other DE markers (such as CD117, SOX17 and/or FOXA2).

22. **Day 7 – Day 8**: Prepare an aliquot of Stage 3 medium and warm to 37°C.
    a. 12 mL needed for each 10 cm plate.
    b. 2 mL needed for each well of a 6-well plate.

Stage 3 Medium		
Reagent	Final concentration	Amount
Basal 2 Medium	N/A	1 mL
Vitamin C (250 mM)	0.25 mM	1 µL
FGF-7 (100 ng/µL)	50 ng/mL	0.5 µL
Stage 3 Supplement (2500×)	1×	0.4 µL

23. Gently remove the previous media and replace with the pre-warmed Stage 3 media.
24. **Day 9 – Day 10**: Prepare an aliquot of Stage 4 medium and warm to 37°C.
    a. 12 mL needed for each 10 cm plate.
    b. 2 mL needed for each well of a 6-well plate.

Stage 4 Medium		
Reagent	Final concentration	Amount
Basal 2 Medium	N/A	1 mL
Vitamin C (250 mM)	0.25 mM	1 µL
FGF-7 (100 ng/µL)	2 ng/mL	0.02 µL

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Continued

Reagent	Final concentration	Amount
hEGF (100 ng/μL)	100 ng/mL	1 μL
Activin A (1 mg/mL)	10 ng/mL	0.01 μL
Y-27632 (10 mM)	10 μM	1 μL
Nicotinamide (1 M)	10 mM	10 μL
Stage 4 Supplement (2500×)	1×	0.4 μL

25. Gently remove the previous media and replace with the pre-warmed Stage 4 media.

Note: Throughout Stage 3 and 4 (Day 7 to Day 10) the cell layer should begin to noticeably thicken and retain a highly "crowded" appearance. Any break in the continuous cell layer or a "ribbon" pattern of raised cells is indicative of low pancreatic progenitor induction (Figure 3).

Aggregation of pancreatic/endocrine progenitors (stage 4 to stage 5)

Timing: Days 11–14: 2 h on Day 11 and 30 min other days

This step describes the critical transfer of pancreatic progenitors from planar culture into microwell plates to create uniformly sized aggregates. The formation of the aggregates serves two purposes. The first is to ensure that endocrine progenitor aggregates (formed throughout Stage 5) are of a regular size and composition to ensure the homogeneity of derived SC-islets at the end of the protocol. The second effect is that of an enrichment of pancreatic progenitors through the exclusion of non-pancreatic lineages, which tend not to re-aggregate following dissociation.

26. Day 11: Prepare AggreWell 400 6-well microwell plates (approximately 1 × microwell plate for every 2 × 10 cm differentiation plate).
    a. Add 2 mL anti-adherence rinsing solution per well and centrifuge at 1,300 g for 6 min.
    b. Check for air bubbles across the microwells under a microscope. Re-centrifuge as necessary to rid microwells of trapped air. Refer to Figure 4 as an example of trapped air within microwells.
    c. Aspirate the rinsing solution and add 2 mL DMEM/F12 per well.

Note: Appropriate pipette technique for coating and exchanging media in AggreWell plates should be adhered to, to ensure successful microwell seeding and aggregate maintenance (Figure 5).

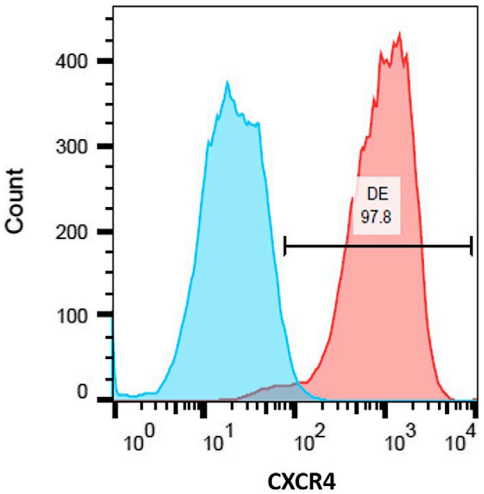

Figure 2. Estimating DE induction efficiency
A flow cytometry plot of CXCR4-labeling following a successful Stage 1 DE induction. CXCR4+ cells (red), negative control (blue).

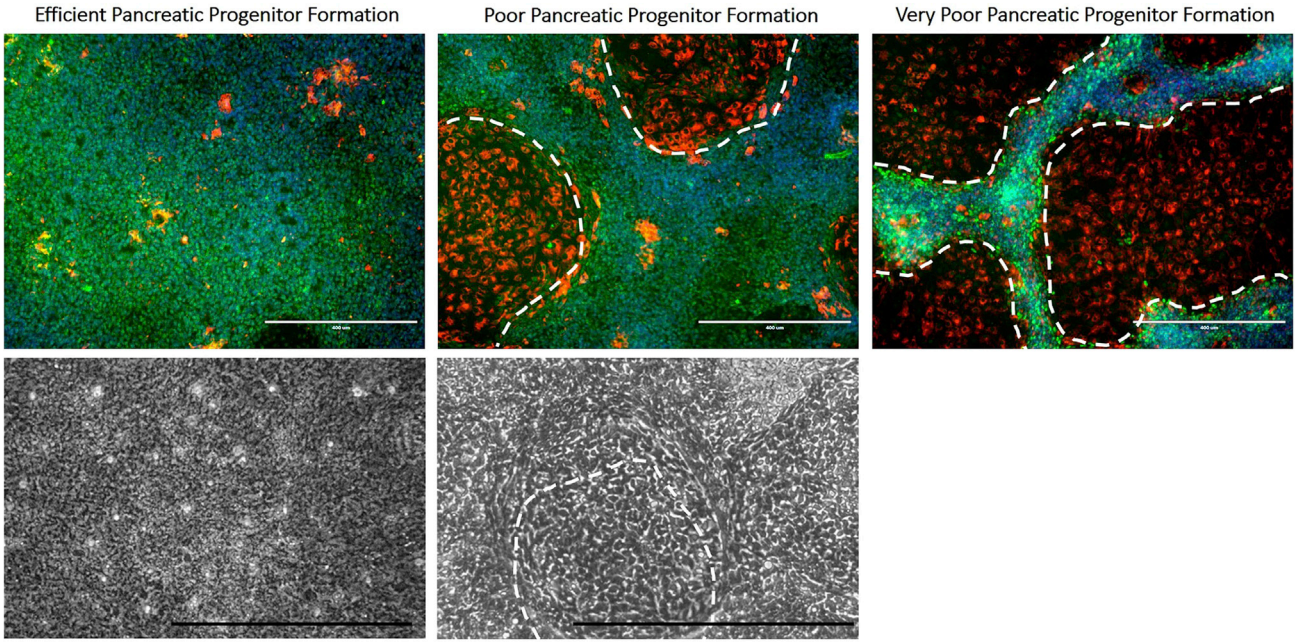

Figure 3. Morphological clues of pancreatic progenitor differentiation efficiency

Cell layer thickening during Stage 3 and Stage 4 of the protocol (Day 7 to Day 10) and maintenance of confluency is a strong indicator of pancreatic progenitor formation (left panels). A break in the cell layer or the formation of raised "ribbons" of cells is an indicator of poor pancreatic progenitor induction (middle and right panels, white dotted line areas). Pancreatic progenitor marker (PDX1, green), non-pancreatic marker (AFP, red). Scale bars represent 400 μm.

27. Dissociate pancreatic progenitors from the 10 cm culture plates:
    a. Gently wash each plate with 4–6 mL of EDTA-PBS (0.5 mM).
    b. Aspirate the EDTA-PBS and replace with 4–6 mL of TrypLE.
    c. Incubate for 8–12 min at 37°C.
    d. Mild tapping of the plate should cause noticeable cell detachment from the plate, keep at 37°C until this occurs.
    e. Add 4–6 mL of DMEM/F12 (or MCDB131) per plate and gently triturate with a wide-bore serological pipette.
    f. Pool the cell suspensions from each plate into a sterile 50 mL tube.

⚠ CRITICAL: Rough mechanical dissociation of the progenitor cells will drastically lower cell viability and cause clumping of cells in the suspension. If cells do not easily detach, we recommend extending the incubation time in TrypLE rather than relying on harsher physical detachment techniques. However, an overly long dissociation in TrypLE can also have a negative impact on cell viability and trigger the formation of cellular debris that will negatively impact aggregation. Monitoring of cell detachment throughout the incubation time will help minimize cell loss.

28. Take an aliquot of the cell suspension for a cell count estimation.
29. Centrifuge the cell suspension at 200 g for 3 min.
30. Gently aspirate the supernatant, preferably with a serological pipette to minimize the chance of losing the loose cell pellet.
31. Gently resuspend the pellet in pre-warmed Stage 4 media (composition outlined at step 24) to a cell density of 1.2×10^6 cells/mL.

⚠ CRITICAL: Limit the amount of pipetting of the cell pellet during resuspension, as these cells are very sensitive to over-handling and manipulation.

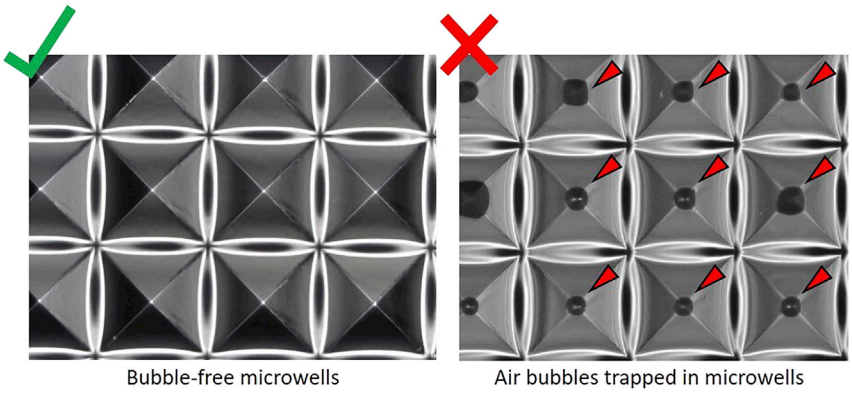

Figure 4. Representative images of microwell plates following anti-adherence treatment

Following anti-adherence treatment and centrifugation, microwells should be free of any trapped air/bubbles (left panel). However, some microwells may contain trapped air bubbles that would impede aggregate formation (right panel, red arrow heads) and require further centrifugation (step 26 b).

Note: An estimation of the volume of Stage 4 medium needed can be made during steps 28 and 29 of this protocol. Each well of the AggreWell plates will be seeded with 6 × 10^6 cells (5 mL of cell suspension, approximately 1,000 cells/microwell).

32. Aspirate the DMEM/F12 from each well of the microwell plate. Gently add 5 mL of the cell suspension to each well of the microwell plate (6 × 10^6 cells/well).
33. Centrifuge the plate at 100 g for 3 min to ensure an even deposition of cells into the microwells (Figure 6).
34. Gently move the AggreWell plates to a humidified incubator.
35. **Day 12**: Gently add 2 mL of pre-warmed Stage 4 media (see step 24) to each well of the AggreWell plate.

⚠ CRITICAL: Media changes in AggreWell plates should be done as gently and slowly as possible using the side of each well to minimize disturbing the aggregates, which may float into other microwells and fuse or be lost through media aspiration. Guidelines on the appropriate technique for exchanging media within AggreWell plates are outlined in Figure 5.

Note: In the days following AggreWell seeding, some tube-like cell aggregates or structures may form in each well. These can be gently removed and discarded without disturbing the aggregates underneath.

36. **Day 13 – Day 14**: Prepare an aliquot of Stage 5 medium and warm to 37°C.
    a. Exchange 4 mL of the previous media with 4 mL of Stage 5 media per well each day. Refer to Figure 5 for appropriate handling of seeded AggreWell plates.

Stage 5 Medium		
Reagent	Final concentration	Amount
Basal 3 Medium	N/A	1 mL
Betacellulin (100 ng/μL)	20 ng/mL	0.2 μL
Y-27632 (10 mM)	10 μM	1 μL
Stage 5 Supplement (1500×)	1×	0.67 μL

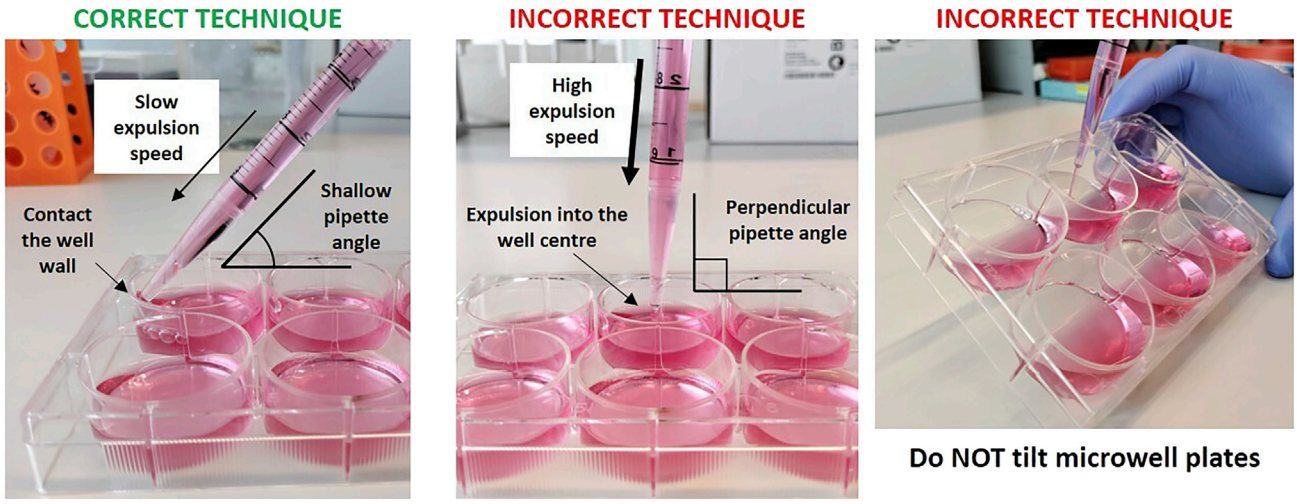

Figure 5. Overview of correct pipetting technique for media exchanges within AggreWell plates

Gentle handling of seeded microwell plates is integral for maintaining aggregate yield. Media exchanges should follow the key principles in the left most panel. Media aspiration also follows the same pattern with slow aspiration of media with a serological pipette. Poor handling of seeded microwells, such as overly fast media addition or aspiration, will cause aggregate loss during aspiration. AggreWell plates should never be tilted for media exchanges as this may also dislodge the seeded aggregates.

⚠ CRITICAL: Do not use vacuum-based aspiration to remove media from AggreWell plates. Instead use a serological pipette to aspirate the media using the technique outlined in Figure 5.

Note: During Days 12–14 the seeded progenitor cells should begin to aggregate and take on a spherical morphology (Figure 6). We recommend maintaining the aggregates in AggreWell plates until Day 14 even if aggregate formation occurs earlier, as early removal may lead to a high degree of aggregate fusion and loss.

Note: Some degree of aggregate loss is expected during the handling and media changes of AggreWell plates. However, careful handling of the plate and appropriate technique during media exchange should minimize the loss.

Optional: As another quality control step, an aliquot of cells from the planar detachment step (step 27 Day 11) or following aggregation (Day 12) can be used to quantify the degree of pancreatic progenitor formation. This is done through flow cytometric assessment of PDX1 and NKX6.1 positive cells (antibodies used are listed in the key resources table) (Figure 7). A yield of >60% double-positive cells is representative of an efficient pancreatic progenitor induction. Lower yields may result in fewer aggregates or aggregates of a reduced size.

Transfer of endocrine progenitor aggregates to suspension culture (stage 5)

Timing: Day 15, 1 h

Day 15: This step of the protocol transfers the endocrine progenitor (EP) aggregates formed through Stage 4 and 5 in the AggreWell microwell plates to a rotating suspension culture. These aggregates through further maturation steps will gain downstream cellular identities and form SC-islets. Each AggreWell well will be transferred into 1–1.5 wells of an ultra-low attachment (ULA) 6-well plate.

37. Remove approximately 2 mL of media from each well of the AggreWell plate and transfer the media to wells of a ULA 6-well plate (1–2 mL per well).

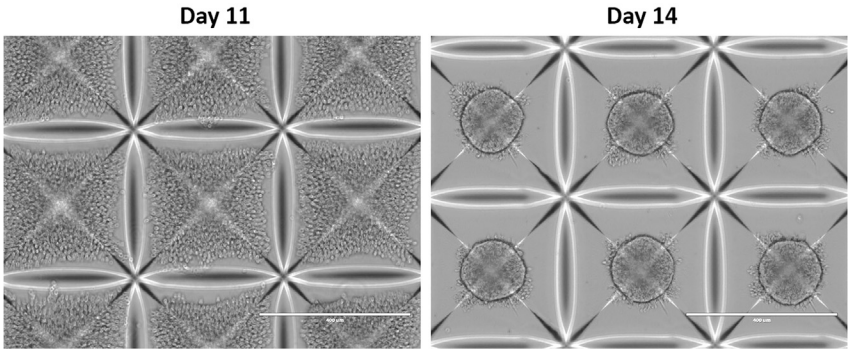

Figure 6. Example images of AggreWell seeding at Day 11 and aggregate formation by Day 14
Pancreatic progenitor seeding should appear evenly spread across all microwells and adequately fill each microwell (Day 11). In the following days (Stage 4 to Stage 5 transition) the progenitors should form spherical aggregates with low levels of cell debris. Scale bars represent 400 μm.

38. Agitate and swirl the AggreWell plate to gently dislodge aggregates from the microwells. A swirling action will pool the aggregates towards the center of the well and allow easy transfer into the ULA plate.
39. Use a serological pipette to transfer the aggregates into the wells of the ULA 6-well plate. Try to ensure an even distribution of aggregates into the ULA wells.
40. Use the remaining media in each of the AggreWells to **gently** dislodge any remaining aggregates from the microwells. Add the aggregates and remaining media to the ULA plate.
41. Transfer the ULA 6-well plate(s) to a platform rotator (95 RPM) housed in a humidified cell culture incubator (37°C, 5% CO_2) while preparing fresh Stage 5 media (step 42).

Note: The culture of aggregates on the platform rotator is to aid in the diffusion of oxygen and nutrients and reduce the fusion of aggregates in suspension.

⚠ CRITICAL: Absolute platform rotator speed may need to be altered depending on the particular model of rotator. A too high rate of rotation may result in the fragmentation and loss of EP aggregates and SC-islets. Conversely, a too low rate of rotation (and/or too large volumes of media per well (>5.5 mL)) will result in higher rates of aggregate fusion.

42. Prepare an aliquot of Stage 5 medium (see step 36) and warm to 37°C.
    a. 5 mL per well of aggregates in the ULA plate.
43. Transfer the ULA plate from the incubator, and swirl gently in a single direction to draw the aggregates to the center of each well.
44. Slowly remove the previous media using a serological pipette at the very edge of each well.
    a. Trace the edge of each well as media is aspirated to avoid drawing in aggregates to the pipette.
45. Add in 5 mL of fresh Stage 5 media to each well and return the plate to the platform rotator.
46. **Day 16**: Repeat steps 42–45 but only exchange 4 mL Stage 5 media (see step 36) per well.

Extended culture and maturation of SC-islets (stage 6 to stage 7)

Timing: Days 17–44, 30 min every 2–3 days

These final two stages of the protocol maintain and further develop the EP-aggregates into terminally differentiated endocrine lineages and act to stabilize and functionally mature the resultant

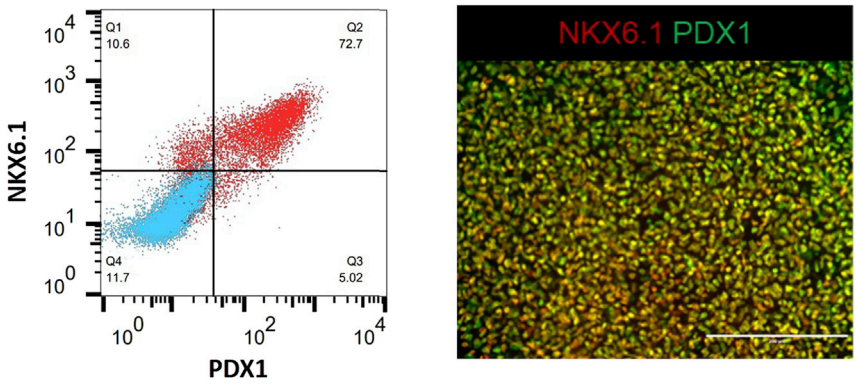

Figure 7. Flow cytometry and IHC assessment of pancreatic progenitors generated by Day 11 of this protocol

NKX6.1 and PDX1 double-positive cell proportion following a successful induction (left panel), negative control (blue) and antibody-probed cells (red). An even cell layer of double-positive cells can also be seen through IHC assessment of planar cultures prior to dissociation and aggregations (right panel). Scale bar represents 200 μm.

SC-islets. In contrast to Stage 1 to Stage 5, media changes in Stage 6 and 7 are done every 2–3 days, rather than daily media changes.

Note: For ease of culture during SC-islet formation and maturation phases (Day 17 to Day 44) we recommend three media changes per week (Monday, Wednesday, and Friday).

47. **Day 17 - Day 23**: Prepare an aliquot of Stage 6 medium.
    a. 4 mL of media needed per well of 6-well ULA plate.

Stage 6 Medium		
Reagent	Final concentration	Amount
Basal 3 Medium	N/A	1 mL
Stage 6 Supplement (2000×)	1×	0.5 μL

48. Transfer the ULA plate from the incubator, and swirl gently in a single direction to draw the aggregates to the center of each well.
49. Slowly remove 4 mL of the previous media using a serological pipette at the very edge of each well.
    a. Trace the edge of each well as media is aspirated to avoid drawing in aggregates to the pipette.
50. Add in 4 mL of fresh Stage 6 media to each well and return the plate to the platform rotator.
51. **Day 24 – Day 44+**: Prepare an aliquot of Stage 7 media.
    a. 4 mL of media needed per well of 6-well ULA plate.

Stage 7 Medium		
Reagent	Final concentration	Amount
Basal 4 Medium	N/A	1 mL
N-acetylcysteine (100 mM)	1 mM	10 μL
T3 (10 mM)	10 nM	0.001 μL
ZM-447439 (5 mM)	0.5 μM	0.1 μL

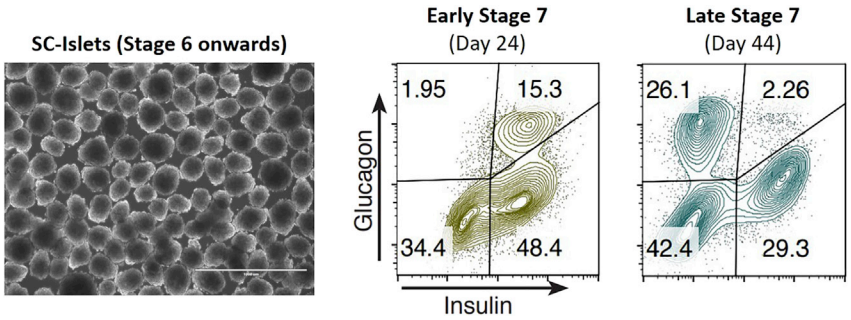

Figure 8. Morphology and predominant endocrine cell populations of SC-islets

The expected morphology of SC-islets throughout Stage 6 and Stage 7 (left panel). Flow cytometric analysis of insulin- and glucagon-positive cells from early and late Stage 7 SC-islets (right panels). Antibodies used are shown in the key resources table. Scale bar represents 1,000 μm.

Note: The T3 dilution from the 10 mM stock solution may be unfeasible for many aliquots of Stage 7 media. We recommend preparing a 1:1,000 dilution of T3 in PBS to form a working stock of 10 μM T3 from which to make Stage 7 media. This stock can be kept at 4°C for up to 1 week (used at 1 μL per mL of Stage 7 media).

52. Transfer the ULA plate from the incubator, and swirl gently in a single direction to draw the aggregates to the center of each well.
53. Slowly remove 4 mL of the previous media using a serological pipette at the very edge of each well.
    a. Trace the edge of each well as media is aspirated to avoid drawing in aggregates to the pipette.
54. Add in 4 mL of fresh Stage 7 media to each well and return the plate to the platform rotator.

Note: SC-islets formed in Stage 6 and Stage 7 do not require any form of passaging or splitting. The size of the aggregates should remain stable and low degrees of cell death and fragmentation should occur in these late maturation stages. Aggregates may darken slightly over time due to an accumulation of secretory granules but retain a rounded morphology (Figure 8).

Optional: Quantification of the endocrine subtypes present within the SC-islets can be assayed from Day 24 onwards. We have found that monohormonal insulin-positive beta-like cell numbers retain constant throughout Stage 7, whereas polyhormonal (insulin and glucagon double positive cells) will be prevalent early in Stage 7 but diminish during maturation, leading to an increasing pool of mono-hormonal glucagon-positive alpha-like cells (Figure 8).

EXPECTED OUTCOMES

By approximately Day 44 of this protocol (3 weeks of Stage 7 culture, S7w3) the derived SC-islets should display robust biphasic dynamic glucose-sensitive functionality, comparable to levels seen in primary islets. Prior to this, from S7w0 to S7w2, SC-islets will release insulin in response to receptor-mediated amplification and plasma membrane depolarization, but not to increased glucose concentrations. The degree of functionality seen at S7w3 can be maintained until at least 6 weeks of Stage 7 culture. Therefore, Day 44 of this protocol represents the earliest time point we recommend for starting function-based assays or interventions (some expected insulin secretion data is shown in Figure 9). Full functional analyses of SC-islets derived from this protocol (as well as transcriptomic, electrophysiological and metabolic profiling) and how they compare to primary islet samples can be

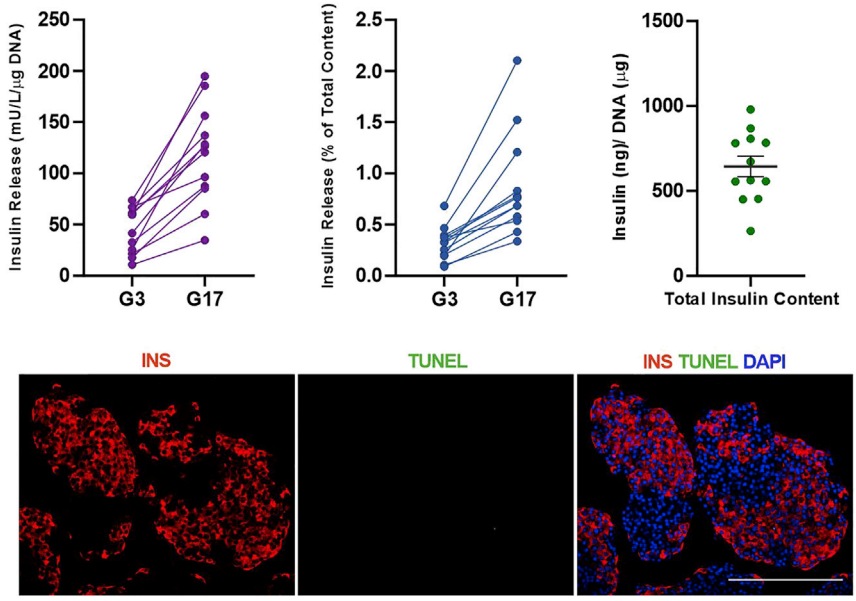

Figure 9. Expected insulin secretion and viability measurements of SC-islets

Normalized insulin secretion values of SC-islets in 3 mM glucose (G3) and 17 mM glucose (G17), together with expected total insulin content. Low TUNEL fluorescence indicates high cell viability within SC-islets. Scale bar represents 200 µm.

found in our recent open access research article (Balboa et al., 2022). We recommend referring to this study for in depth information about the functional outcomes of SC-islets generated from this protocol.

Endocrine cell compositions should be in the region of 30–50% beta-like cells, and 30%–50% alpha-like cells. A small proportion of delta-like cells (approx. 4%) are also generated, with < 5% of cells showing a polyhormonal (alpha/beta) identity. Overall, approximately 90% of SC-islet cells will have either an alpha-, a beta- or delta-like identity. Extended Stage 7 culture will tend to increase the proportion of alpha-like cells. A well-known byproduct of islet differentiation protocols is the aberrant generation of enterochromaffin-like cells which are still present at a frequency of approximately 6%. Full compositional profiles of SC-islets throughout stage 7 maturation can also be found in our recent study (Balboa et al., 2022). High cell viability is maintained throughout Stage 7 culture and we regularly see in situ assays of viability post-fixation showing >99% viability (Figure 9) in agreement with our previously published findings (Montaser et al., 2021). Viability post-dissociation is approximately 90% (week 0 to week 6 of Stage 7 SC-islets) (assayed by Acridine Orange/Propidium Iodide Stain, Logos Biosystems, F23001) if single cell analyses or manipulation of SC-islets is required.

There is inherent variability in the final yield of SC-islets that is dependent on the efficiency of the previous steps and the formation of endocrine progenitors. As a guideline, 2 fully confluent PSC-seeded 10 cm plates on Day 0 of the protocol should result in enough pancreatic progenitors to fill 1 × AggreWell microwell 6-well plate. This AggreWell plate should result in the formation of 8,000–10,000 SC-islets at the end of the maturation step.

An overview of the morphology of cells throughout the differentiation and moving from planar, to microwell, to suspension culture are shown in Figure 10. Key signaling molecules used at each step are also shown.

Overview of culture conditions and stage-specific media throughout the protocol

Day	Stage (*)	Culture format	Protocol steps	Culture media	Basal media	Supplement number	Additives
0	n/a	Planar	1–10	E8	N/A	N/A	N/A
1	1 (DE)	Planar	11–13	Stage 1-Day 1	1	N/A	ActA, CHIR-99021
2	1 (DE)	Planar	14–16	Stage 1-Day 2	1	N/A	ActA, CHIR-99021
3	1 (DE)	Planar	17–19	Stage 1-Day 3	1	N/A	ActA
4	2 (PG)	Planar	20–21	Stage 2	1	N/A	Vit C, FGF-7
5	2 (PG)	Planar		Stage 2	1	N/A	Vit C, FGF-7
6	2 (PG)	Planar		Stage 2	1	N/A	Vit C, FGF-7
7	3 (PF)	Planar	22–23	Stage 3	2	3	Vit C, FGF-7
8	3 (PF)	Planar		Stage 3	2	3	Vit C, FGF-7
9	4 (PP)	Planar	24–25	Stage 4	2	4	Vit C, FGF-7, hEGF, ActA, Y-27632, Nicotinamide
10	4 (PP)	Planar		Stage 4	2	4	Vit C, FGF-7, hEGF, ActA, Y-27632, Nicotinamide
11	4 (PP)	AggreWell	26–34	Stage 4	2	4	Vit C, FGF-7, hEGF, ActA, Y-27632, Nicotinamide
12	4 (PP)	AggreWell	35	Stage 4	2	4	Vit C, FGF-7, hEGF, ActA, Y-27632, Nicotinamide
13	5 (EP)	AggreWell	36	Stage 5	3	5	BTC, Y-27632
14	5 (EP)	AggreWell		Stage 5	3	5	BTC, Y-27632
15	5 (EP)	Rotating Suspension	37–45	Stage 5	3	5	BTC, Y-27632
16	5 (EP)	Rotating Suspension	46	Stage 5	3	5	BTC, Y-27632
17–23	6 (II)	Rotating Suspension	47–50	Stage 6	3	6	n/a
24–44+	7 (FI)	Rotating Suspension	51–54	Stage 7	4	N/A	T3, NAC, ZM-447439

Basal Media and Supplement formulations are outlined in the "before you begin" section of this protocol. We recommend that basal media preparation, supplement preparation, and additive stock solution reconstitution take place **before** starting a differentiation. Recommended reconstitution solvents and stock concentrations are also outlined in the "before you begin" section.

(*) DE definitive endoderm.

PG, primitive gut tube; PF, posterior foregut; PP, pancreatic progenitor; EP, endocrine progenitor; II, immature SC-islet; FI, functional SC-islet.

LIMITATIONS

This protocol has been used to robustly generate SC-islets from numerous genome-edited and wild type ESC and iPSC lines (some examples are shown in the table below) (De Franco et al., 2020; Lithovius et al., 2021; Montaser et al., 2021). However, it is known that different PSC lines show distinct propensities towards particular differentiation outcomes. It is therefore possible that certain PSC lines may intrinsically show enhanced or reduced SC-islet yield and purity.

Throughout this protocol we have tried to emphasize key steps (and modifications) that we have found to help maintain an efficient differentiation regardless of the PSC line used. Even so, culture conditions and the manipulation of the cells themselves may need to be altered ad hoc if differentiations consistently fail at a particular stage of the protocol. We also recommend examining another published streamlined SC-islet differentiation protocol that maintains planar culture as a mechanism of aiding the differentiation of "stubborn" PSC lines (Hogrebe et al., 2021).

It is also worth noting that although acute functionality is achieved with these SC-islets, in-depth profiling of transcriptomic and metabolic signatures show that SC-islets are still distinct from primary human islets (Balboa et al., 2022). This indicates that a certain degree of immaturity is still retained by SC-islets even after extended in vitro maturation. The current state-of-the-art SC-islet differentiation strategies we hope will act as a tool to further uncover the mechanisms and processes of islet maturation (Barsby and Otonkoski, 2022).

Cell line	DE (CXCR4+)	S4 (PDX1+ NKX6.1+)	S7 (INS+/C-PEP+)
HEL24.3	95–97%	65–67%	49–57%
HEL118.3	94–99%	85–92%	ND
HEL113	ND	77–96%	ND
H9	98%	69%	33%

Some example iPSC and ESC lines that have undergone this differentiation protocol. The proportion of cells displaying stage specific markers throughout the protocol are shown for each cell line. "ND" represents data that was not collected directly or evaluated under different assays.

TROUBLESHOOTING

Problem 1

PSC monolayer does not reach 100% confluency following seeding on Day 0 (pre-step 11, Figure 1).

Potential solution

If seeded PSCs look healthy and viable, then the seeded plate may be maintained until full confluency is achieved, preferably later the same day. We do not recommend keeping the seeded plates for an additional night prior to the start of the differentiation to increase cell confluency as it increases the likelihood of media acidification and cell death. For following differentiations, the seeding density should be increased.

If seeded PSCs show signs of cell death due to overly high seeding and/or high acidification of the growth media, then the differentiation should be aborted. Reduce the number of seeded PSCs for future differentiations.

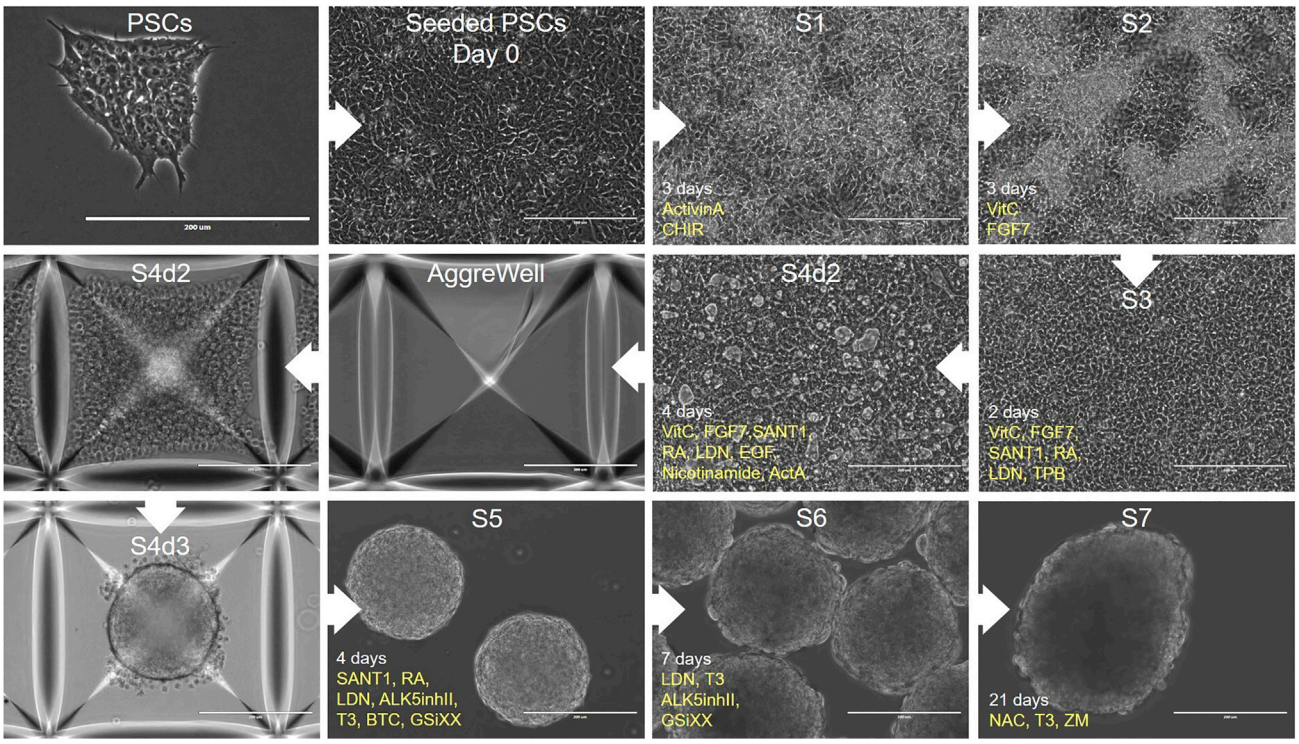

Figure 10. Expected cell culture morphology throughout the protocol

Each panel represents the expected cell confluency/structure at each Stage of the protocol (denoted by the S prefix). Key small molecule modulators of the differentiation step are highlighted in yellow. Approximate time of culture in each stage is also highlighted in white. All scale bars represent 200 μm.

Problem 2

High cell death/detachment during Stages 1 and 2 of the protocol (steps 11–21).

Potential solution

A portion of cell death during DE induction and early into Stage 2 can be expected with this protocol. However, the degree of cell death should never result in a loss of absolute confluency on the tissue culture plate. To minimize cell detachment during Stages 1 and 2 of the protocol:

- Exchange growth media carefully each day.
- Ensure Matrigel-coating of tissue culture plates is done appropriately, and coated plates are stored correctly.
- Reduce the time of DE induction on a line-to-line basis. Certain iPSC lines appear very sensitive to DE induction. Reduce the time in DE from 3 to 2 days (limit or omit step 17, Day 3 of the protocol). CXCR4+ cells should still be present at >90–95%.
- Increase the volume of media per well to 3 mL to avoid over-acidification and cell loss.

Note: Do not continue the differentiation if full confluency is lost in these early stages. Pancreatic progenitor induction efficiency will suffer and the rest of the protocol will fail.

Problem 3

Low formation of PDX1 and NKX6.1 pancreatic progenitors (PPs) by Stage 4 (Day 10–11).

Potential solution

The efficient generation of PPs can be somewhat assayed by the morphology of the cell layer throughout Stage 3 and 4. If a noticeable thickening of the cell layer does not occur, or there are breaks in the cell layer, then it is a sign that PP formation is low (Figure 3). Unfortunately, at this stage not much can be done to rescue the low induction of PPs. Ensuring high degrees of cell survival and confluency throughout DE induction and Stage 2 is the only way to ensure highly efficient PP formation.

A possible point of failure may be the use of degraded or improperly handled small molecule reagents. We recommend following the product guidelines on long term storage and reconstitution protocols. We also recommend forming tailored aliquot volumes that limit the number of freeze thaw cycles when planning ongoing differentiations.

Problem 4

Poor recovery of pancreatic progenitors (PPs) or poor aggregation in microwell culture (steps 26–36).

Potential solution

The harvesting of PPs from Stage 4 monolayer is a critical step in SC-islet differentiation. Low yields of viable PPs can result in low aggregate formation (See problem 3, Figure 11). However, harsh cell dissociation and handling at Stage 4 can also result in lowered PP viability and a poor aggregation step. To minimize this:

- Limit the handling and trituration of Stage 4 cells when detaching from planar culture. Use only wide-bore serological pipettes and minimize over-pipetting when dissociating. Rely on appropriate enzymatic (TrypLE) dissociation and detachment (see step 27). The incubation time may need to be altered on a line-to-line basis. Floating sticky strands of debris in the cell suspension may be indicative of "over handling".
- However, an overly-long enzymatic dissociation itself is also detrimental to PP viability and aggregation. For each differentiation, enzymatic detachment of planar Stage 4 should be done so with regular assessments of cell dissociation (e.g., mild tapping of the plate, inspection under the microscope).

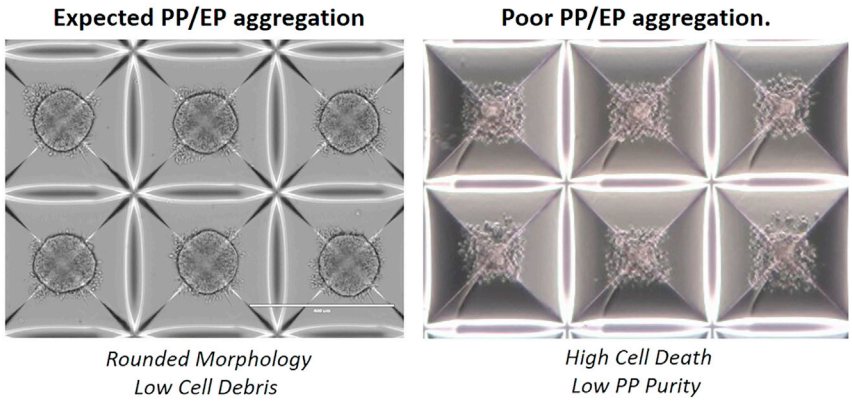

Figure 11. Examples of expected and poor microwell aggregation of PPs

Detached and reaggregated PPs of high purity and viability will quickly form rounded aggregates in microwell culture (left panel). Low PP formation or poor handling of Stage 4 cultures during the microwell plating process will result in poor aggregate formation and high cell death (right panel). Scale bar represents 400 μm.

Problem 5

Fragmentation or loss of aggregates in suspension culture (step 41 onward).

Potential solution

The rotation rate of the rotating platform may need to be lowered to avoid damaging SC-islets. Although we propose 95 RPM as an adequate speed for our particular platform model (Infors HT Celltron), this may need to be adjusted. If aggregates (or SC-islets) show hallmarks of fragmentation under the microscope (free floating cells, debris) we recommend lowering the rotation speed to avoid further damage.

Problem 6

Uneven SC-islet size and morphology (step 46 onwards).

Potential solution

Microwell aggregation aids in the initial formation of SC-islet size and morphology, however, extended periods in suspension culture can lead to fusion and mild re-shaping of aggregates. Unless large degrees of fragmentation occur (or high levels of fusion, which may necessitate higher rotation speeds) then mild differences in aggregate size and shape are tolerable and should not affect functional outcomes.

However, poor distribution when seeding into microwells can lead to over-loading or under-loading of microwells which can ultimately lead to aggregate loss when moved into suspension culture. Therefore, to improve SC-islet yields, we recommend ensuring an even distribution of PPs into microwells and centrifuging the microwell plate as soon as possible after loading of the cell suspensions.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Timo Otonkoski (timo.otonkoski@helsinki.fi).

Materials availability

This study did not generate any unique or new reagents. All reagents listed in this protocol are readily available from multiple suppliers.

Data and code availability

All current SC-islet profiling data using this protocol can be found published in (Balboa et al., 2022). An open access resource for the scRNA transcriptomic data of SC-islets that we generated can also be found at: https://singlecell.broadinstitute.org/single_cell/study/SCP1526.

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AUTHOR CONTRIBUTIONS

All authors contributed to the development and refinement of the protocol. T.B. wrote the manuscript with all authors contributing comments, figure panels, and refinements. T.O. supervised the work and provided funding support.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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