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We are still developing and optimizing this protocol

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Dispersed Pancreas

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ABSTRACT

Dispersed pancreas protocols

Culture Media

1 **Human islets:**

-Dulbecco's Modification of Eagle's Medium (DMEM, 4.5 g/L glucose, L-glutamine, sodium pyruvate)

-Fetal Bovine Serum (FBS) at 10%

-Penicillin-Streptomycin at 1%

-GlutaMAX™ supplement at 1%

Human exocrine tissues (ducts and acinar):

-Dulbecco's Modification of Eagle's Medium (DMEM, 4.5 g/L glucose, L-glutamine, sodium pyruvate)

-Fetal Bone Serum (FBS) at 5%

-Dexamethasone at 1 ug/mL

-Soya Trypsin Inhibator at 0.1 mg/mL

-Insulin at 100 nM

Incubation

-37°C Cell Culture Incubator in an atmosphere of >95% humidity

Dithizone (DTZ) Staining and Islet Equivalent

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Materials and Reagents:

-Dithizone (Sigma-Aldrich), cat # D-5130

-Dimethyl sulfoxide (DMSO)

-1mL Syringe

-30mm Syringe filter 0.46um nylon membrane

-Microscope with a micrometer in the scope to measure the diameter of the islets

Procedure:

Dissolve 10 mg of DTZ in 4 mL of DMSO. Add 36mL of HBSS (or any medium without serum). You can aliquot this solution and keep it frozen. Before using, filter and add a few drops to the medium where your islets are. Keep for up to 1 week at room temperature.

To count islet equivalence:

-Resuspend the islets in a known volume (25-50mL).

-Take three or more samples of 50 or 100uL and put them on a dish (make sure to mix the islets well before sampling and sample quickly, as they fall to the bottom of the tube fast).

-Count all the islets in the sample and separate the islets by size ranges by their diameter (50-100um, 100-150um, 200-250um, etc.). Write them on the provided sheet.

-Convert each size range with the conversion factor. Add up the numbers and multiply by the dilution factor to get a total number. This is based on volume and converting all the islets to how many there would be if they were all 150um in diameter. This means that you need six 50um islets to equal one 150um islet when you remember that volume is $4/3\pi r^3$.

Disperse Islets, Ducts and Acinar Into Single Cells

3 **Materials and Reagents:**

-Phosphate-Buffered Saline (PBS) without calcium and magnesium

-TrypLE™ Express [-] Phenol Red

-Water bath at 37°C

-Vortex

-Centrifuge

Procedure:

-Collect islets, ducts, or acinar + medium from dish to an individual 15mL tube. Centrifuge at 1.5 rpm for 2 minutes.

-Aspirate medium and add 10mL of PBS. Centrifuge at 1.5 rpm for 2 minutes.

-Aspirate PBS and add 10mL of PBS. Centrifuge at 1.5 rpm for 2 minutes.

-Aspirate PBS and add an appropriate amount of TrypLE™ (e.g., 750uL in about 1,000 islets. Acinar cells would need more. Add enough TrypLE™ to cover the tissue).

-Bring tubes to water bath at 37°C for 10 minutes. Vortex tubes in each 3 minutes for 10 seconds.

-Stop TrypLE™ reaction by adding cold media (complete the tube).

-EVERYTHING ON ICE

-Centrifuge at 2 rpm for 2 minutes. Aspirate the media and resuspend cell pellet with appropriate volume of pre-warmed medium.

-Incubate according to protocol.

B-Gal Activity While Excluding For CD45/CD11B Using APC a..

4 **Materials and Reagents:**

-Cellular Senescence Live Cell Analysis Assay Kit (Enzo) cat # ENZ-KIT 130-0010

-Phosphate-Buffered Saline (PBS) without calcium and magnesium

-Water bath at 37°C

-FACS Buffer: PBS with 2% FBS

-Normal Rat Serum

-Normal Goat Serum

-CD45

-CD11b

-Vortex

-Centrifuge

-37°C Cell Culture Incubator

-Flow cytometer equipped with 488 nm laser source

Procedure:

-Collect cells and media from dish to a 50mL tube. Centrifuge at 2 rpm for 2 minutes.

-Aspirate medium and add appropriate volume of pre-warmed medium to culture cells into 24-well plate (1mL per well).

-Add 1x Cell Pre-Treatment Solution. Incubate at 37°C for 2 hours.

-Collect aliquots of each sample to be the unstained (do not add SA-b-Gal Substrate).

-Add 1x SA-b-Gal Substrate directly to the cells. Incubate at 37°C for 1 hour.

-Collect cells to 1.5mL tube. Centrifuge at 2 rpm for 2 minutes.

-Wash the stained cells two times with PBS.

-Block cells for 45 minutes on ice with 100uL per sample of: 494uL FACS buffer, 2.5uL CD45 and 2.5uL CD11b.

-Centrifuge at 2 rpm for 2 minutes. Aspirate buffer.

-Wash the stained cells 2 times with FACS buffer.

-Add 400u: of FACS buffer and bring to Flow cytometer.

Glucose Stimulated Insulin Secretion (GSIS) In Human Panc...

5 **Materials and Reagents:**

-Krebs Ringer Bicarbonate Hepes (KRBH)

Mixed Salts Stock Solution:

	g/L
NaCL	35.4
KCL	1.78
KH2PO4	0.81
MgSO4.7H20	1.46
CaCL2.2H20	1.85

Sodium Bicarbonate Stock Solution

	g/L
NaCL	5.35
NaHCO3	2.63

Working KRBH

-20mL mixed salts stock solution

-16mL sodium bicarbonate stock solution

-64mL dH2O

-0.42g NaHEPES

-0.1g BSA

-pH 7.4

-45% Glucose Solution (CORNING) cat # 25-037-CI

-Phosphate-Buffered Saline (PBS) without calcium and magnesium

-10mL Syringe

-30mm Syringe filter 0.45um nylon membrane

-37°C Cell Culture Incubator

Procedure:

-Filter working KRBH into two 50mL tubes (one will be the low glucose and another one the high glucose solution)

-For low glucose (2.8mmol/L): add 56uL of glucose solution to 50mL KRBH. For high glucose (20.2mmol/L): add 404uL of glucose solution to 50mL KRBH.

-Wash islets two times in low glucose KRBH. Then put in 24-well plate in 1mL per well for 1 hour in 37°C Cell Culture Incubator.

-Remove 800uL and add 800uL of low glucose KRBH and incubate at 37°C for 1 hour.

-Collect 800uL for insulin. Then add 800uL of high glucose KRBH to give final glucose concentration of 16.8mmol/L. Incubate at 37°C for 1 hour.

-Collect 800uL for insulin.

-Remove remaining solution and add 500uL of PBS. Collect islets + PBS for DNA quantification.

-Freeze samples for insulin assay later.

Protocol for Human Islets

6 **Media:**

CMRL (Invitrogen 11530-037)

100X GlutaMAX (Gibco 35050-061).....1%

50X Penicillin Streptomycin Solution (Corning, 30-001-C1).....1%

FBS.....10%

Day 1:

1. Pool the shipped islets that are suspended (usually) in transplant media into 50mL conical tube.
2. Centrifuge the islets at 1000 rpm for 5 minutes.
3. Aspirate the media and add 20mL of islet culture into 15mm x 150mm ultra-low attachment culture dish and incubate 24hr/overnight 37°C incubator.

Day 2:

1. Transfer the overnight islet culture from 15mm x 150mm dish to 50mL conical tube.

Note: If not working with ultra-low attachment dish: carefully scrape the bottom of the tissue culture dish using cell scraper to detach the islets.

2. Centrifuge the cells at 1000 rpm for 5 minutes.
3. Aspirate the media and wash the pellet with 5mL of 1X PBS.
4. Centrifuge the cells at 1000 rpm for 5 minutes.
5. Aspirate PBS and select islets for Glucose-Stimulated-Insulin Secretion (GSIS) or proceed to dispersion.

GSIS of beta-cells

-Krebs-Ringer Bicarbonate HEPES

Mixed Salts Stock Solution:

NaCl.....35.4g/L
KCl.....1.78g/L
KH2PO4.....0.82g/L
MgSO4.7H2O.....1.46g/L
CaC12.2H2O.....1.85g/L

Sodium Bicarbonate Stock Solution:
NaC1.....5.35g/L
NaHCO3.....2.63g/L

Working KRBH:
-20 mL mixed salts
-16mL Sodium Bicarbonate
-64mL dH20
-100mL final volume
-0.42g NaHEPES
-0.1g BSA
-pH 7.4
-Filter
-Low glucose = 2.8mmol glucose/L
-High glucose = 20.2mmol glucose/L
-Wash islets in low glucose KRBH twice. Then put in 24 well plate in 1mL per well for 1 hour. Then remove 800uL and add 800uL more for further hour, collecting 800uL for insulin. Then add 800uL high glucose KRBH to give final glucose concentration of 16.8mmol/L and collect 800uL at end of hour. Remove 100uL from bottom of each tube before freezing samples for insulin assay later.
-Collect islets for DNA extraction by adding 300uL of PBS.

FACS sorting for beta-Gal positive cells:
Human islets are dispersed with TrypLE Express at 37°C for 10 minutes and β-Gal activity is measured using Enzo's cellular senescence live cell senescence assay (ENZ-KIT130-0010) following manufacturer's instruction with the exception of the substrate incubation time which is optimized to 1 h for mouse islets.

Using a BD FACS Aria Ilu and BD FACS Aria Special Order Research Product, cells are first gated according to forward scatter and autofluorescence to remove non-β-cell population; propidium iodide is used to exclude dead cells and β-cells are sorted based on acid β-Gal activity.

Protocol for Ki67 or HMGB1 Stain

Cells must be adhered to treated dish overnight before stain

DAY 1
1. Wash treated dish with 1x PBS-CMF once.
2. Add 10% formalin into well and let rest at room temp for 30 minutes.

3. Wash with 1x PBS 3 times.
4. Add 0.3% Triton-X and let rest for 15 minutes at room temperature.
5. Wash 3 times with 2% PBS+ lamb serum
6. 2% PBS + Lamb serum stock=1mL lamb serum+49mL 1x PBS. Can be kept at room temp.
7. Add 1:50 NDS (Normal Donkey Serum) and let rest for 45 minutes at room temp.
8. Make stock 1:50 NDS= 100uL NDS+4.9mL 1x PBS. Make 200uL aliquots.
9. Wash with 2% PBS + lamb serum once.
10. Incubate overnight at 4°C with: 1:500 Rabbit Anti-Ki67 or 1:400 Rabbit Anti-HMGB1

DAY 2

1. “Defrost” dish at room temperature for 1 hour.
2. Wash with 2% PBS + lamb serum 3 times.
3. Add 1:200 488 anti-Rabbit and incubate at room temp for 1 hr.
4. Wash with 2% PBS + lamb serum 3 times.
5. Add 1:200 Guinea pig anti-Insulin and incubate for 2 hrs at room temp.
6. Wash with 2% PBS + lamb serum 3 times.
7. Add 1:200 594 anti-Guinea pig and incubate for 1 hr at room temp.
8. Wash with 1x PBS 3 times.
9. Mount circular cover class with DAPI mounting media, no bubbles, and use cotton swabs to wipe excess media.
10. Use nail polish to seal all around the cover glass.

Attachment and Staining Protocol for Human Pancreatic Tis..

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1. Glass bottom dishes were treated with 1X PEI for at least 24hrs in a 37°C incubator then left in the fridge prior to attachment.

2. Cells were dispersed according to protocol and an aliquot of 200-300uL of media with dispersed cells was attached to the treated dish and allowed to sit overnight in a 37°C incubator.

3. Next day, cells were washed with 1X PBS-Calcium/Magnesium Free one time.

4. Cells fixed with 10% Formalin for 30 min at room temperature.

5. Cells washed with 1X PBS three times.

6. Cells incubated with 0.3% Triton-X for 15 min at room temperature.

7. Cells washed with 1X PBS with 2% Lamb Serum three times.

8. Cells incubated with Normal Donkey Serum (1:50 in PBS) for 45 min at room temperature.

9. Cells washed with 1x PBS with 2% Lamb Serum one time.

10. Cells incubated with Rabbit anti-HMGB1 antibody (1:400 in PBS) overnight at 4°C.

11. Next day, cells taken out and allowed to “defrost” for 1 hour at room temperature.

12. Cells washed with PBS with 2% Lamb Serum three times.

13. Cells incubated with 488 anti-Rabbit (1:200 in PBS) for 1 hour at room temperature.

14. Cells washed with PBS with 2% Lamb Serum three times.

15. Cells incubated with Guinea Pig anti-Insulin (1:200 in PBS) for 2 hours at room temperature.

16. Cells washed with PBS with 2% Lamb Serum three times.

17. Cells incubated with 594 anti-Guinea Pig (1:200 in PBS) for 1 hour at room temperature.

18. Cells washed with 1X PBS three times.

19. Cells cover slipped with mounting media containing DAPI.