ANALYSIS OF ISLET FUNCTION IN DYNAMIC CELL PERIFUSION SYSTEM

I. DEFINITION

1. **Actual Islets (AI):** The actual number of islets counted.
2. **Islet Equivalent (IEQ):** An islet with a diameter of 150 µm determined mathematically by compensating for islet shape.
3. **Islet Perifusion Assay:** A functional assay that acquires dynamic hormone secretory profiles simultaneously from islet cell types such as β and α cells in response to their respective secretagogues. Insulin and glucagon are detected in perifusion fractions by radioimmunoassay. Islet hormone secretory profile is generated by graphing hormone concentration over time with respect to islet volume and/or hormone content.
4. **Secretagogue:** A substance that elicits another substance to be secreted.
5. **Radioimmunoassay (RIA):** A very sensitive in vitro assay technique used to measure concentrations of antigens such as hormone levels by use of antibodies.

II. EQUIPMENT AND MATERIALS

1. Equipment
    - Biological Safety Cabinet (BSC)
    - 37°C CO_2 Incubator
    - Analytical Balance
    - Micropipettes (10-100 µL, 20-200 µL, and 100-1000 µL ranges)
    - Pipette Aid automatic pipettor or equivalent
    - pH Meter
    - Light Microscope
    - Eyepiece with Calibrated reticle, 1 mm
    - Computer with Excel Counting Worksheet or equivalent
    - Manual or Electronic Cell Counter
    - Fraction Collector
2. Supplies/Materials – Quantitative/qualitative assessment of human islets
    - Phosphate buffered saline (PBS) without Ca/Mg (Invitrogen 14190-144)
    - 1.5 mL Centrifuge Tube (Fisher Scientific 05-408-129)
    - $NaHCO_3$ (Sigma S6014-500G)
    - L-Glutamine (Sigma G8540-100G)
    - Sodium Pyruvate (Sigma P2256-25G)
    - HEPES (Sigma H7523-50G)
    - DMEM (Sigma D503010X1L)
    - RIA-grade BSA (Sigma A7888)
    - 0.5% phenol red (Sigma P-0290)
    - Glucose (Sigma D16)
    - Isobutylmethylxanthine (IBMX, Sigma 1395)
    - KCl (Sigma BP366-500)

- Epinephrine HCl (Sigma E4642)
- Ascorbate (Sigma A5960)
- 200-µL (P-200 ART) and 1000-µL pipette tips (P-1250 ART)
- 0.22 µM Vacuum Filtration System (Millipore SCGPU05RE)
- 5-Luer Caps (Western Analytical BC-125)
- 25 µM Frits, Polyethylene (Western Analytical Products 006FR-10-25-PE)
- 13 X 100 mm Polyethylene Tubes (Fisher Scientific 149567A)
- Caps for 13 X 100 mm Tubes (Fisher Scientific 2681204)
- 12 X 75 mm Polyethylene Tubes (Fisher Scientific 0334222A)
- Glucometer (Bayer 9545C)
- Glucose Strips (Bayer 7097C)
- Pressurized Gas, 95% O_2, 5% CO_2
- Insulin RIA Kit (Millipore RI-13K)
- Glucagon RIA Kit, (Millipore GL-32K)

3. Supplies/Materials – Human islet culture

- CMRL 2066 (Cellgro 15-110-CV)
- L-Glutamine (Invitrogen 25030-081)
- Penicillin/Streptomycin (Invitrogen 15140-122)
- Fetal Bovine Serum (FBS Millipore TMS-013-B)
- P-1000 Pipet Tips, Sterile (Fisher Scientific 2079-HR)
- 10 cm Petri Dishes, SureGrip (Sarstedt 83.3902.500)
- 6 cm Petri Dishes, SureGrip (Sarstedt 83.3901.500)

III. PROCEDURES

1. General Perifusion Startup

- Fill water bath with deionized water to about 1 inch from the top, and turn the water bath to 37°C.
- Label perifusion tubes with date, islet type, and any other identifying information, position the fraction collector trays for perifusion, and load the tubes.
- Rinse the tubing with deionized water at max pump speed for 15 minutes, then place new frit into the chamber to be used.

2. Preparation of Base Perifusion Media and Secretagogues

- Prepare Base Perifusion Media by combining compounds below in a 1-liter Erlenmeyer flask. Add 1L of deionized water and mix for at least 15 minutes until dissolved.
  - 3.2g g $NaHCO_3$
  - 0.58 L-Glutamine
  - 0.11 g Sodium Pyruvate
  - 1.11 g HEPES
  - 1 bottle DMEM for 1 L of media
  - 1 g RIA-Grade BSA
- Check the pH of the solution and adjust to 7.3 to 7.5 using either 1N NaOH or 1N HCl as necessary.

- Add 70 mg Ascorbate, Sigma A5960 (Only used when Epinephrine is a secretagogue).
- Use a vacuum-filtration system to filter media, transfer to a side-arm flask, and de-gas at 37°C for at least 30 minutes.
- Reserve some baseline media in a 50 mL conical tube for islet loading and unloading.
- Prepare 1.7 mM Glucose Media (for use with Epinephrine)
    - Add 0.0613 g glucose to 200 mL Base perifusion media to 250 mL bottle and mix until dissolved.
    - Wait 30 min and check glucose levels using a glucose meter.
- Prepare 5.6 mM Low Glucose Media
    - Add 0.5549 g glucose to 550 mL Base perifusion media to 500 mL bottle and mix until dissolved.
    - Wait 30 min and check glucose levels using a glucose meter.
- Prepare 16.7 mM High Glucose Media
    - Add 0.7522 g glucose to 250 mL Base perifusion media to 250 mL bottle and mix until dissolved.
    - Wait 30 min and check glucose levels using a glucose meter.
- Prepare 1.7 mM Glucose plus 1 µM Epinephrine/HCl (Store at -20° C)
    - Prepare 200 mM Epi stock by adding 0.043934 g Epinephrine in 1mL 1.7 mM Glucose Solution
    - Prepare 0.4 mM Intermediate Epi Dilution (1:500): Dissolve 20 µL 200 mM Epi stock into 10mL 1.7 mM Glucose Solution
    - 1 µM Final Epi Concentration (1:400). Dissolve 250 µL Intermediate Epi Dilution into 100mL 1.7mM Glucose
- Prepare 5.6 mM Glucose plus 20 mM KCl (Store at room temperature)
    - Add 0.149g KCl to 100 mL 5.6 mM Glucose Solution
- Prepare 16.7 mM Glucose Media with 100 µM IBMX
    - Weigh out around 10 mg IBMX
    - Make 100 mM stock IBM by dividing the IBMX weight in mg by 22.22 and add “X” mL of Dimethyl Sulfoxide (DMSO). Caution: Wear gloves and avoid spilling any DMSO on skin.
    - Make 100 µM Final Concentration (1:1000) by adding 100 µL of 100 mM stock into 100 mL of 16.7 mM Glucose.

3. Setup of Secretagogues in Perifusion Water Bath

- Place the bottles of media in the water bath to warm up for at least 10 minutes before beginning the perifusion.
- Replace Pyrex orange caps with 4-Luer (+1) caps on every bottle of media to be used, and tape over the holes so that gas cannot escape.
- Turn on gas (95% O_2, 5% CO_2), and place one gas catheter into each of the bottles with the new caps. Put tape over the other holes on the caps. Make sure that gas catheter is suspended above the media, and not inside the liquid.

- Place intake catheters into baseline media bottle, making sure that they reach to the bottom of the bottle. Run the media through the chambers for about 10 minutes while islets are being aliquoted for perifusion, *disposing of media*.

4. Preparing Islets for Perifusion

   - *On the day of islet receipt*, plate a half of islet shipment (1000 - 5000 IEQs) in 10-cm non-tissue culture treated Petri dish and culture in CMRL-1066 plus 10% FBS media at 37°C/5% CO2 for *2 hours prior to perifusion.*
   - Label a 1.5 mL clear Eppendorf tube for islets.
   - Place petri dish with cultured islets on the stage of inverted microscope and view the islets under 4x objective, and use the micrometer in the 10x eyepiece to size the islets.
   - Under microscope guidance hand-pick 267 – 300 IEQs using the chart below and record islet size and count in *Islet Cell Calculation Excel Worksheet*.

Number of Tick marks	Diameter of Islet (µm)	Islet Size Category
>4 and ≤6	>100 and ≤150	Small
>6 and ≤8	>150 and ≤200	Medium
>8 and ≤10	>200 and ≤250	Large
>10 and ≤12	>250 and ≤300	Extra Large

5. Islet Loading into the Perifusion Chamber

   - Turn off the pump
   - Turn the stopcock on the air bubble uptake lines so that the waste pathway is open, and close the stopcock on the outlet line at the fraction collector.
   - Remove the chamber from its mounting, turn it upside down, and remove the red end piece (inlet).
   - Remove and discard two thirds of the media from the chamber.
   - Using a 1 mL pipettor set to 700 µL transfer the slurry of islets from the Eppendorf tube to the perifusion chamber. Rinse the tube at least 3 times with 700 µL of baseline medium, and transfer to the chamber.
   - Place the chamber back onto the mounting, and fill it up with baseline medium until there is a convex meniscus. Tap the sides of the column lightly to dislodge air bubbles from the walls, and collect and discard any bubbles from the top of the meniscus.
   - When all the bubbles have been removed, carefully replace the inlet plunger. (NOTE- make sure that no bubbles are introduced into the chamber during this process; if bubbles get in, remove the plunger, and repeat step 20.
   - Turn the chamber right side up, and put it back on the mounting rack.

- Open the outlet line at the fraction collector and close the waste line on the air bubble uptake line.
- Turn on the pump, set the fraction collector to 3 minutes and flip the collector arm so that it is over the first collection tube. Press “Start” on the collector to start the timer.
- Tighten column end pieces, and end fittings, and make sure there are no leaks.
- Lower the column mounting rack into the water bath, and tighten the clamp to prevent wobbling.

6. Fraction Collector Startup and Islet Wash Period
    - Collect 10 preliminary fractions to synchronize pump speed to deliver 3 mL 3-minute fractions, and to rinse the islets.
    - Record each pump speed on perifusion worksheet in the perifusion logbook.
7. Collection of Perifusate Fractions
    - Begin to collect fractions.
    - Change secretagogues at predetermined fractions.
    - As soon as the fraction collector moves, switch off the pump.
    - Move the needles from one secretagogue to the other, making sure to not tangle the tubing, and inserting the needle all the way to the bottom of the bottle.
    - When the needles have been moved, restart the pump.
8. Recovery of Islets from Chamber and Perifusion System Cleanup
    - At the end of the perifusion raise the mounting rack from the water.
    - Stop pumps and close all outlets. Wait about 2 minutes, so that the islets can drift down to the bottom of the chamber.
    - Carefully remove the blue end piece (outlet) from the first chamber.
    - Pipette the medium from the chamber into a 60 mm untreated dish, and rinse out the chamber and the blue column end piece with 1 mL of baseline medium 5 times each into the dish.
    - Rinse the chamber with deionized water, and remove frit with a frit removal stage and tool.
    - Put the column back together, and run 10% bleach through them at maximum pump speed for about 15 minutes. Make sure that all the tubing gets bleached, including all the waste lines. After the bleach, run deionized water through the entire system at maximum speed for 1.5 hours.
    - After the system cleanup, turn off the pump using the **master power switch**, turn off and drain the water bath, and log the perifusion in the notebook.
    - Perform any scheduled maintenance.

9. Islet Hormone Extraction
    - After retrieving islets from the perifusion chamber, size and count retrieved islets (step 6.4.4) to determine IEQ and *record islet size and count in Islet Cell Calculation Excel Worksheet.* Transfer the islets to a 1.5-mL Eppendorf tube. Note: The average expected retrieval rate is approximately 93%. Lower islet recovery has been observed for islets showing hallmarks of disintegration.
    - Centrifuge the tubes for 1 minute at 1000 RPM, and pipet off the supernatant, being careful not to disturb the islet pellet. Wash with 1 mL ice-cold PBS.
    - Repeat step 2 twice more, and spin again after the last wash.
    - Remove as much supernatant as possible from the tube, using a 1 mL tip, followed by a 200-µL tip and set islets on ice.
    - Freshly prepare acid alcohol for hormone extraction by adding 50 µL of concentrated HCl to 5.5 mL of 95% ethanol.
    - Add 200 µL of acid alcohol to tube containing islets.
    - Incubate sample at 4°C for 24 hours.
    - Spin samples down at 4°C for 5 minutes at 4400 RPM, and transfer three 50 µL supernatant aliquots into pre-labeled 1.5-mL Eppendorf tubes and store at -80°C.
10. Hormone Assay and Data
    - Determine insulin and glucagon concentration in perifusion samples and islet extracts by radioimmunoassay (insulin, RI-13K; glucagon, GL-32K; Millipore). Radioimmunoassays for analysis of insulin and glucagon secretion are performed with internal standards and have intra- and inter-assay variation of less than 10%.
    - Normalize insulin and glucagon concentration in perifusion fractions per islet volume (IEQs) and islet insulin and glucagon content, respectively.
    - Calculate insulin results and express as [ng/100 IEQs/min] and as [% content/min]. Likewise, calculate glucagon results and express as [pg/100 IEQs/min] and as [% content/min].
    - Values for two control samples corresponding to lower and upper assay detection limits must be within their established ranges.
        - Standard values must be within ±15% of their nominal values.
        - Duplicates of standards, controls and samples must have Coefficients of Variation (CV) ≤ 20%.
        - Inspect data for any errors and verify that appropriate assay procedures are used. Record lot number of RIA.

IV. DATA STORAGE AND REPORTING

1. Example of human islet perifusion is shown in **Figure 1**. In addition to providing insulin and glucagon secretory profiles, other parameters including stimulation index (SI) and area under curve (AUC) are calculated for each secretagogue.

- **Stimulation index (SI) -** calculated as ratio of maximum response to a given stimulus relative to baseline.
- **Area under curve (AUC) -** calculated by integrating islet secretory response to a given stimulus over time.

2. Store data in the appropriate server location(s).
3. Annotated images may be uploaded to the HPAP database.
4. Document any deviations from this protocol that occurred.

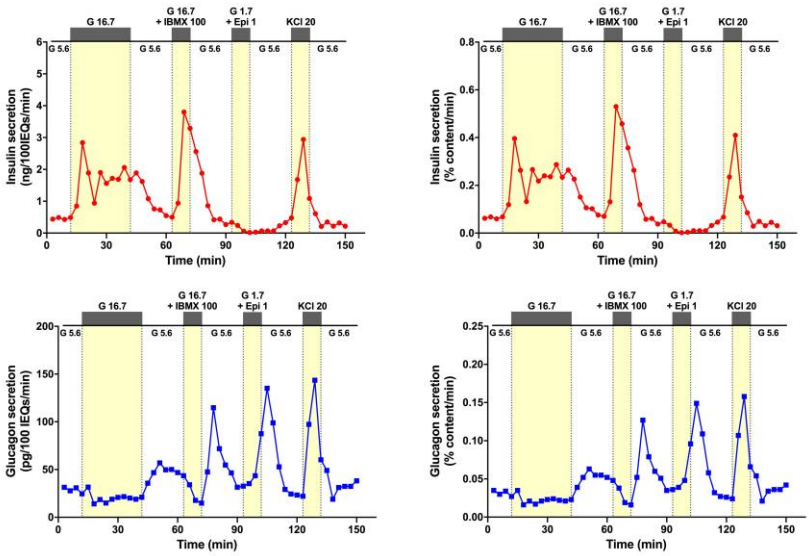

Figure 1. Protocol for analysis of human islet function by the Vanderbilt HIPP. Islets are challenged with 16.7 mM glucose for 30 minutes to resolve biphasic insulin secretory response, followed by 15-minute stimulations with 16.7 mM glucose + 50 μM IBMX, 1.7 mM glucose + 1 μM epinephrine, and 20 mM KCl. Insulin secretory response to these stimuli is shown in red and glucagon secretion is displayed in blue.

REFERENCES

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