Analysis of Islet Function in Dynamic Cell Perifusion System V.1

IIDP-HIPP [1]

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IIDP-HIPP : Integrated Islet Distribution Program and Human Islet Phenotyping Program

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Integrated Islet Distribution Program and Human Islet Phenotyping Program
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IIDP-HIPP

This Standard Operating Procedure (SOP) is based on the Vanderbilt Human Islet Phenotyping Program (HIPP) Islet Functional Analysis. This SOP provides HIPP procedure for dynamic perifusion and hormone secretion measurement to assess islet function.

This SOP defines the assay method used by the Human Islet Phenotyping Program (HIPP) for qualitative determination of the Purified Human Pancreatic Islet product, post-shipment, manufactured for use in the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK)-sponsored research in the Integrated Islet Distribution Program (IIDP).

The goal of this SOP is to define the method for quantitative determination of insulin released after glucose stimulation for proving the potency of the human islet preparation shipped by the IIDP.

This Standard Operating Procedure (SOP) #: HIPP-05-v03

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IIDP-HIPP, Islet Function, Dynamic Cell Perifusion System, insulin, glucose

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- ***Integrated Islet Distribution Program (IIDP) (RRID:SCR_014387)*:** The IIDP is a grant funded program commissioned and funded by the NIDDK to provide quality human islets to the diabetes research community to advance scientific discoveries and translational medicine. The IIDP consists of the NIDDK Project Scientist and Program Official, the External Evaluation Committee and the CC at City of Hope (COH). The IIDP CC integrates an interactive group of academic laboratories including the subcontracted IIDP centers.

- ***IIDP Coordinating Center (CC)*:** Joyce Niland, Ph.D. and Carmella Evans-Molina, M.D., Ph.D. serve as Co-Principal Investigators (Co-PIs) for the IIDP Program located within the Department of Diabetes and Cancer Discovery Science at COH to coordinate the activities of the IIDP and Human Islet Phenotyping Program (HIPP). Dr. Niland, contact PI, oversees the daily activity of the IIDP staff, provides informatics/ biostatistical input, and subcontracts with the Islet Isolation Centers (IICs) to ensure the delivery of the highest quality human islets to IIDP-approved investigators. Dr. Evans-Molina serves as the liaison to the HIPP, interacting closely to ensure that extensive, high quality phenotypic data are collected on islets distributed by the IICs. She also facilitates the delivery of this information to both the IICs and the IIDP-approved investigators, while responding to questions, issues, or suggestions for further HIPP enhancements.

- ***Human Islet Phenotyping Program (HIPP)*:** The HIPP is a subcontracted entity of the IIDP through the COH and Vanderbilt University. The HIPP is directed by Marcela Brissova, Ph.D. and is responsible for performing specific standardized phenotyping assays agreed upon by both the IIDP and the HIPP, in order to provide enhanced, quality data on the human islets post-shipment, to the IIDP. The results of these assays will be approved by the CC and posted on the IIDP website for both the centers and the approved investigators.

- ***Actual Islets (AI)*:** The actual number of islets counted.

- ***Islet Equivalent (IEQ)*:** An islet with a diameter of 150 µm determined mathematically by compensating for islet shape.

- ***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 hormone assay. The islet hormone secretory profile is generated by graphing hormone concentration over time with respect to islet volume and/or hormone content.

- ***Secretagogue*:** A substance that elicits another substance to be secreted.

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References:

Dai C, Brissova M, Hang Y, Thompson C, Poffenberger G, Shostak A, et al. Isletenriched gene expression and glucose-induced insulin secretion in human and mouse islets. Diabetologia. 2012 Mar;55(3):707–18. PMCID: PMC3268985.
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Kayton NS, Poffenberger G, Henske J, Dai C, Thompson C, Aramandla R, et al. Human islet preparations distributed for research exhibit a variety of insulin-secretory profiles. Am J Physiol Endocrinol Metab. 2015 Apr 1;308(7):E592–602. PMCID: PMC4385877.
https://pubmed.ncbi.nlm.nih.gov/25648831/

1. The following equipment is necessary to assess human islet function by perifusion.

1.1 Biological Safety Cabinet (BSC)
1.2 **37 °C** CO_2 Incubator
1.3 Analytical Balance
1.4 Micropipettes (10-100 µL, 20-200 µL, and 100-1000 µL ranges)
1.5 Drummond Pipette Aid automatic pipettor or equivalent
1.6 pH Meter
1.7 Light Microscope
1.8 Eyepiece with Calibrated reticle, 1 mm
1.9 Computer with Excel Counting Worksheet or equivalent
1.10 Manual or Electronic Cell Counter
1.11 Fraction Collector
1.12 Olympus SZX12 stereomicroscope equipped with an Olympus DP-80 high-resolution digital camera
1.13 Olympus cellSens™ image acquisition and analysis software

2. The following supplies and materials are necessary to perform quantitative and qualitative assessment on human islets.

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2.1

Phosphate buffered saline (PBS) without Ca/Mg **Thermo Fisher Scientific Catalog #14190144**

1.5 mL Centrifuge Tube **Fisher**

2.2 **Scientific Catalog #05-408-129**

NaHCO3 **Sigma**

2.3 **Aldrich Catalog #S6014-500G**

L-Glutamine **Sigma**

2.4 **Aldrich Catalog #G8540-100G**

Sodium Pyruvate **Sigma**

2.5 **Aldrich Catalog #P2256-25G**

HEPES **Sigma**

2.6 **Aldrich Catalog #H7523-50G**

DMEM **Sigma**

2.7 **Aldrich Catalog #D5030-10X1L**

RIA-grade BSA **Sigma**

2.8 **Aldrich Catalog #A7888**

Glucose **Fisher**

2.9 **Scientific Catalog #D1610**

Isobutylmethylxanthine

2.10 (IBMX) **Sigma Catalog #I5879**

2.11 KCl **Sigma Catalog #BP366-500**

Epinephrine HCl **Sigma**

2.12 **Aldrich Catalog #E4642**

Ascorbate (L-Ascorbic

2.13 acid) **Sigma Catalog #A5960**

2.14 **200 µL** (P-200 ART) and **1000 µL** pipette tips (P-1250 ART)

0.22 µM Vacuum Filtration

2.15 System **Millipore Catalog #SCGPU05RE/S2GPU05RE**

5-Luer Caps **Thomas**

2.16 **Scientific Catalog #Western Analytical BC-125**

25 µM Frits Polyethylene **Cole-**

2.17 **Parmer Catalog #11945-04** Mfr # 006FR-10-25-PE

13 X 100 mm Polyethylene Tubes **Fisher**

2.18 **Scientific Catalog #149567A**

Caps for 13 X 100 mm Tubes **Fisher**

2.19 **Scientific Catalog #02681204**

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12 X 75 mm Polyethylene Tubes **Fisher**

2.20 **Scientific Catalog #0334222A**

2.21 Glucometer **ASCENSIA Catalog #Bayer 9545C**

Glucose

2.22 Strips **ASCENSIA Catalog #Bayer 7097C**

2.23 Pressurized Gas, 95% O2, 5% CO2 **Contributed by users**

3. The following supplies and materials are necessary to culture human islets.

CMRL

3.1 1066 **Corning Catalog #15-110-CV**

L-Glutamine **Thermo Fisher**

3.2 **Scientific Catalog #Invitrogen 25030-081**

Penicillin/Streptomycin **Thermo Fisher**

3.3 **Scientific Catalog #Invitrogen 15140-122**

Fetal Bovine Serum **Millipore**

3.4 **Sigma Catalog #TMS-013-B**

P-1000 Pipet Tips Sterile **Thermo Fisher**

3.5 **Scientific Catalog #2079-HR**

3.6

10 cm Petri Dishes SureGrip **Sycamore Life Sciences Catalog #Sarstedt 83.3902.500**

6 cm Petri Dishes SureGrip **Sycamore Life**

3.7 **Sciences Catalog #Sarstedt 83.3901.500**

37° C CO2 Incubator

Thermo Fisher N/A

37° C CO2 Incubator

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Analytical Balances

Mettler Toledo **N/A**

Analytical Balances

Micropipettes

10-100 µL, 20-200 µL, and 100-1000 µL

Eppendorf **N/A**

Drummond Pipette Aid automatic pipettor

Drummond **N/A**

pH Meter

Mettler Toledo **N/A**

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Light Microscope

Leica Microsystems **N/A**

Fraction Collector

Thermo Scientific **N/A**

Fraction Collector

Olympus SZX12 stereomicroscope
SZX12

Olympus **OLYMPUS-SZX12**

Olympus DP-80 high resolution digital camera
DP80

Olympus **N/A**

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Epinephrine HCl (Sigma E4642)

Epinephrine HCl (Sigma E4642) .pdf

Procedures

1 General Perifusion Startup

1.1 Fill water bath with deionized water to about 1 inch from the top, and set the temperature to **37 °C** .

1.2 Label perifusion tubes with date, islet type, and any other identifying information, position the fraction collector trays for perifusion, and load the tubes.

1.3 Rinse the tubing with deionized water at max pump speed for 15 minutes, then place new frit into the islet chamber.

2 Preparation of Base Perifusion Medium and Secretagogues

2.1 Prepare Base Perifusion Medium by combining compounds below in a 1-liter Erlenmeyer flask. Add 1L of deionized water and mix for at least 15 minutes until dissolved.

2.1.1 **3.2 g** $NaHCO_3$

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2.1.2 **0.58 L** -Glutamine

2.1.3 **0.11 g** Sodium Pyruvate

2.1.4 **1.11 g** HEPES

2.1.5 1 bottle DMEM for **1 L** of media

2.1.6 **1 g** RIA-Grade BSA

2.2 Check the pH of the solution and adjust to 7.3 to 7.5 using either 1N NaOH or 1N HCl as necessary.

2.3 Add **70 mg** Ascorbate, Sigma A5960

2.4 Use a vacuum-filtration system to filter medium, transfer to a side-arm flask, and de-gas at **37 °C** for at least 30 minutes.

2.5 Prepare 1.7 mM Low Glucose Medium (for use with Epinephrine)

2.5.1 Add **0.0613 g** glucose to **200 mL** Base perifusion medium to **250 mL** bottle and mix until dissolved.

2.5.2 Wait 30 min and check glucose levels using a glucose meter.

2.6 Prepare 5.6 mM Glucose Medium

2.6.1 Add **0.5549 g** glucose to **550 mL** Base perifusion medium to **500 mL** bottle and mix until dissolved.

2.6.2 Wait 30 min and check glucose levels using a glucose meter.

2.6.3 Reserve some 5.6mM medium in a **50 mL** conical tube for islet loading and unloading.

2.7 Prepare 16.7 mM High Glucose Medium

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2.7.1 Add **0.7522 g** glucose to **250 mL** Base perifusion medium to **250 mL** bottle and mix until dissolved.

2.7.2 Wait 30 min and check glucose levels using a glucose meter.

2.8 Prepare 1.7 mM Glucose plus 1µM Epinephrine/HCl (Store at **-20 °C**)

2.8.1 Prepare 200mM Epi Stock by adding **0.043934 g** Epinephrine in **1 mL** 1.7 mM Glucose medium.

2.8.2 Prepare 0.4 mM Intermediate Epi Dilution (1:500): Dissolve **20 µL** 200 mM Epi stock into **10 mL** 1.7 mM Glucose medium.

2.8.3 Prepare 1µM Final Epi Concentration (1:400). Dissolve **250 µL** Intermediate Epi Dilution into **100 mL** 1.7mM Glucose medium.

2.9 Prepare 5.6 mM Glucose plus 20mM KCl

2.9.1 Add **0.149 g** KCl to **100 mL** 5.6 mM Glucose medium.

2.10 Prepare16.7 mM Glucose Medium with 100 µM IBMX

2.10.1 Weigh out around **10 mg** IBMX

2.10.2 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.

2.10.3 Make 100 µM Final Concentration (1:1000) by adding **100 µL** of Stock into **100 mL** of 16.7 mM Glucose medium.

3 Setup of Secretagogues in Perifusion Water Bath

3.1 Place the bottles of media in the water bath to warm up for at least 10 minutes before beginning the perifusion.

3.2 Replace Pyrex orange caps with 4-Luer (+1) caps on every bottle of media to be used,

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and tape over the holes so that gas cannot escape.

3.3 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.

3.4 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

4.1 *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% CO_2 for *2 hours prior to perifusion*.

4.2 Label a **1.5 mL** clear Eppendorf tube for islets.

4.3 Place petri dish with cultured islets on the stage of inverted microscope and view the islets under 4x objective. Use the micrometer in the 10x eyepiece to size the islets.

4.4 Under microscope guidance handpick 267–300 IEQs using the chart below and record islet size and count in the *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

4.5 Place the dish containing islets on the stage of a stereomicroscope equipped with a high-resolution camera and swirl until all islets are in the camera field of view at 10x magnification. Capture brightfield images at approximately 12-ms exposure and darkfield images at approximately 1.2-s exposure, each at 10x magnification. Ensure all islets are present in image. Save all image files.

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4.6 Transfer all islets from the center of the dish to a labeled **1.5 mL** clear Eppendorf tube for loading into perifusion chamber.

4.7 Open the darkfield image in the cellSens software. Using the manual HSV threshold function, segment the islet tissue channel.

4.8 Use the custom Count and Measure algorithm to determine islet count and mean islet diameter. Split adjacent but discrete islets using the Manually Split Objects tool to get an accurate islet count and mean diameter measurements.

> *Note: Perifusion IEQ analysis by cellSens began 06/19/2018.*

4.9 Use the mean diameter measurements to assign islets to a diameter group using the chart provided in 4.4 above.

5 Islet Loading into the Perifusion Chamber

5.1 Turn off the pump.

5.2 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.

5.3 Remove the chamber from its mounting, turn it upside down, and remove the red end piece (inlet).

5.4 Remove and discard two thirds of the media from the chamber.

5.5 Using a **1 mL** pipette set to **700 µL** transfer the slurry of islets from the

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Eppendorf tube to the perifusion chamber. Rinse the tube at least 3 times with **700 µL** of baseline medium, and transfer to the chamber.

5.6 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.

5.7 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 5.6.

5.8 Turn the chamber right side up, and put it back on the mounting rack.

5.9 Open the outlet line at the fraction collector and close the waste line on the air bubble uptake line.

5.10 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.

5.11 Tighten column end pieces, and end fittings, and make sure there are no leaks.

5.12 Lower the column mounting rack into the water bath, and tighten the clamp to prevent wobbling.

6 Fraction Collector Startup and Islet Wash Period

6.1 Collect 10 preliminary fractions to synchronize pump speed to deliver **3 mL** 3-minute fractions, and to rinse the islets.

6.2 Record each pump speed on the perifusion worksheet in the perifusion logbook.

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7 Collection of Perifusate Fractions

7.1 Begin to collect fractions.

7.2 Change secretagogues at predetermined fractions.

7.3 As soon as the fraction collector moves, switch off the pump.

7.4 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.

7.5 When the needles have been moved, restart the pump.

8 Recovery of Islets from Chamber and Perifusion System Cleanup

8.1 At the end of the perifusion raise the mounting rack from the water.

8.2 Stop pumps and close all outlets. Wait about 2 minutes, so that the islets can drift down to the bottom of the chamber.

8.3 Carefully remove the blue end piece (outlet) from the first chamber.

8.4 Pour 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.

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8.5 Rinse the chamber with deionized water, and remove frit with a frit removal stage and tool.

8.6 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.

8.7 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.

8.8 Perform any scheduled maintenance.

9 Islet Hormone Extraction

9.1 After retrieving islets from the perifusion chamber, size and count retrieved islets (step 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.

9.2 Centrifuge the tubes for 3 minutes at 200 rcf and aspirate the supernatant using a pipette, being careful not to disturb the islet pellet.

9.3 Remove as much supernatant as possible from the tube, using a **1 mL** tip, followed by a **200 µL** tip and place tube on ice.

9.4 Prepare fresh acid alcohol for hormone extraction by adding **50 µL** of concentrated HCl to **5.5 mL** of 95% ethanol.

9.5 Add **200 µL** of acid alcohol to tube containing islets.

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9.6 Incubate sample at 4 °C for 24 hours.

9.7 Spin samples down for 5 minutes at 3000 rcf, and transfer three 50 µL supernatant aliquots into pre-labeled 2 mL screwcap tubes and store at -80 °C .

Data Storage and Reporting

10 Data Storage and Reporting

10.1 To facilitate data management and ensure data security, the Vanderbilt HIPP uses an institutional server-based platform for data storage and analysis.

Deviations and Resolutions

11 Deviations and Resolutions

11.1 Document any deviations that occurred during this protocol that affect the final results and report with the analysis of the assay.

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