Human Islet Phenotyping Program of the IIDP

(Based on the Viability SOP from the Clinical Islet Transplant Consortium Protocol and the IIDP for Distribution Centers)

STANDARD OPERATING PROCEDURE (SOP)

Qualitative Assessment of Islet Viability by Staining with Fluorescein Diacetate (FDA) and Propidium Iodide (PI) Dyes

Version: HIPP-03-v02

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1.0 Objective

1.1 To define the assay method used by the Human Islet Phenotyping Program (HIPP) for quantitative and 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).

2.0 Scope and Applicability

2.1 This SOP applies to the HIPP using funds from the IIDP through a subcontract mechanism to assess purified human pancreatic islets shipped for basic research studies to IIDP approved investigators.

3.0 Responsibilities

3.1 It is the responsibility of each staff member of the HIPP to follow the procedures listed in this SOP and to work to the best of their ability to follow all requirements.

3.2 The HIPP Director is responsible for assuring that all technicians are properly trained in the correct procedures for this SOP and that equipment and facilities are in good working order.

3.3 The HIPP Director and all HIPP laboratory personnel are responsible for reading and understanding the SOP, for performing the tasks in accordance with this SOP, and signing each SOP worksheet when used for each assay to verify that the procedures have been followed.

3.4 It is the responsibility of the IIDP Coordinating Center (CC) to both follow and ensure adherence to the procedures outlined in this SOP. In order to accomplish this, the IIDP CC will interact with the HIPP Director and relevant personnel, as needed.

4.0 Definitions

4.1 Integrated Islet Distribution Program (IIDP): 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 Scientific Panel and the CC at City of Hope (COH). The IIDP CC integrates an interactive group of academic laboratories including the subcontracted IIDP centers.

4.2 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.

4.3 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 quality control 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.

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

4.5 Islet Equivalent (IEQ): An islet is quantified as 150 μm diameter by mathematically compensating for the volume of the islet.

4.6 Fluorescein Diacetate/Propidium Iodide (FDA)/(PI) Viability Assay is a rapid fluorometric method to test the integrity of the plasma membrane simultaneously using inclusion and exclusion dyes; the assay differentiates between viable and nonviable cells and is, consequently, used for determination of viability of islet preparations.

4.6.1 The inclusion dye is fluorescein diacetate (FDA) and the exclusion dye is propidium iodide (PI). The final concentrations are as follows:

FDA: 0.46 µM
PI: 14.34 µM

4.6.2 Fluorescein diacetate is a nonpolar ester, which passes through plasma membranes and is hydrolyzed by intracellular esterases to produce free fluorescein. The polar fluorescein is confined within cells with an intact plasma membrane and can be

observed under appropriate excitation conditions. FDA functions as an inclusion dye, i.e., viable cells will appear bright green fluorescent using FDA.

4.6.3 Propidium iodide functions as an exclusion dye that cannot penetrate living cells but readily enters dead or dying cells. Once PI penetrates through the cell membrane, it binds to nucleic acids and causes them to fluoresce bright orange/red. PI absorbs in green light and fluoresces orange/red.

5.0 Materials

5.1 Equipment

5.1.1 Fluorescent Microscope

5.1.2 Calculator or computer software (e.g. Excel) with the mean and standard deviation functions

5.2 Supplies and Materials:

5.2.1 Fluorescein diacetate, stock solution 24 µM (9.9 µg/mL in acetone), Sigma, Cat. #F-7378, or equivalent

5.2.2 Propidium iodide, stock solution 750 µM (0.5 mg/mL in DPBS, pH approximately 7.4), Sigma, Cat. #P-4170, or equivalent

5.2.3 Acetone, Sigma, Cat. #179124, or equivalent

5.2.4 Sterile 10 x 35 mm cell culture dishes, Nunc Cat. #174926, or equivalent

5.2.5 DPBS (Dulbecco's Phosphate Buffered Saline) without calcium or magnesium, Mediatech, Part #21-031, or equivalent

5.2.6 Pipettes: 200 and 1000 µL with associated tips (P-200 ART and P-1250 ART)

6.0 Procedures

6.1 Limitations

6.1.1 Once the dye is added to the islets, the assessment must take place as quickly as possible. If there is a delay of more than 15 minutes, the accuracy of the assessment will be diminished as the islets lose their viability with time.

6.1.2 Both of the fluorescent dyes used in this assay are light sensitive and must be kept in the dark, covered with aluminum foil.

6.1.3 The fluorescent dyes are temperature sensitive and must be stored as follows:

- FDA: ≤ -20°C
- PI: 2-8°C

6.2 Assay Set Up

6.2.1 Assemble all items described in the "Supplies and Materials" section.

6.2.2 Prepare Fluorescent dyes: Fluorescein Diacetate (FDA) and Propidium Iodide (PI), if required.

6.3 Preparation of Fluorescein Diacetate and Propidium Iodide

6.3.1 Remove FDA from the freezer and PI from the refrigerator. Weigh the required amount of the reagent on an analytical balance.

6.3.2 Dissolve 0.00199 g of FDA in 200 mL of acetone in a glass bottle and cover with aluminum foil. Store in 10 mL aliquots at -20°C.

6.3.3 Dissolve 0.0125 g of PI in 25 mL of DPBS and cover with aluminum foil. Store in 5 mL aliquots at 2-8ºC.

6.3.4 Discard used stain.

6.3.5 Record the expiration date on each aliquot tube. The expiration date, for both PI and FDA stains, is six months from the date of preparation.

6.3 Staining and Estimation of Viability

6.4.1 Add two 400 ul replicates of well-mixed total islet suspension to the culture dish.

6.4.2 Quickly add first 10 µL of PI and then 10 µL of FDA to the islet suspension. Gently swirl to mix.

6.4.3 Turn off the lights in the room.

6.4.4 Incubate plate in the dark for 15 minutes.

6.4.5 Assess the preparation immediately using the fluorescent microscope. Image islets using an Olympus SZX12 stereomicroscope system. Capture multiple fields ensuring that FDA/PI staining is visualized in 50 – 100 islets per preparation.

Note: *FDA produces bright green fluorescence in viable cells, while PI produces red fluorescence in dead or dying cells.*

6.5 Interpretation of Results

6.5.1 FDA freely passes through the cell membrane of live cells. Viable cells appear bright fluorescent green when stained with FDA. In a live cell, FDA is hydrolyzed to the polar free fluorescein, and it is trapped within the intact membranes of the viable cells present in islets.

6.5.2 PI stains the nuclei of dead/non-viable cells only. Dead cells appear bright fluorescent red/orange. PI does not cross the membrane of viable cells.

6.5.3 FDA/PI staining captures viable and dead cells in both islets and residual exocrine tissue. This staining provides a rapid qualitative viability assessment of the human islet preparation; however, the 3-D nature of islets and exocrine tissue remnants precludes accurate cell counting. The Vanderbilt HIPP will supplement viability assessment with counts of live and dead cells in a single cell suspension using Countess II technology (Life Technologies AMQAX1000) (see HIPP Protocol-04-v02 Quantitative Assessment of Islet Viability by Trypan Blue Staining).

7.0 Data Storage and Reporting

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

7.2 Annotated images containing metadata (***Figure 1***) will be uploaded to the IIDP-HIPP database and immediately disseminated to IIDP-affiliated investigators and islet isolation centers.

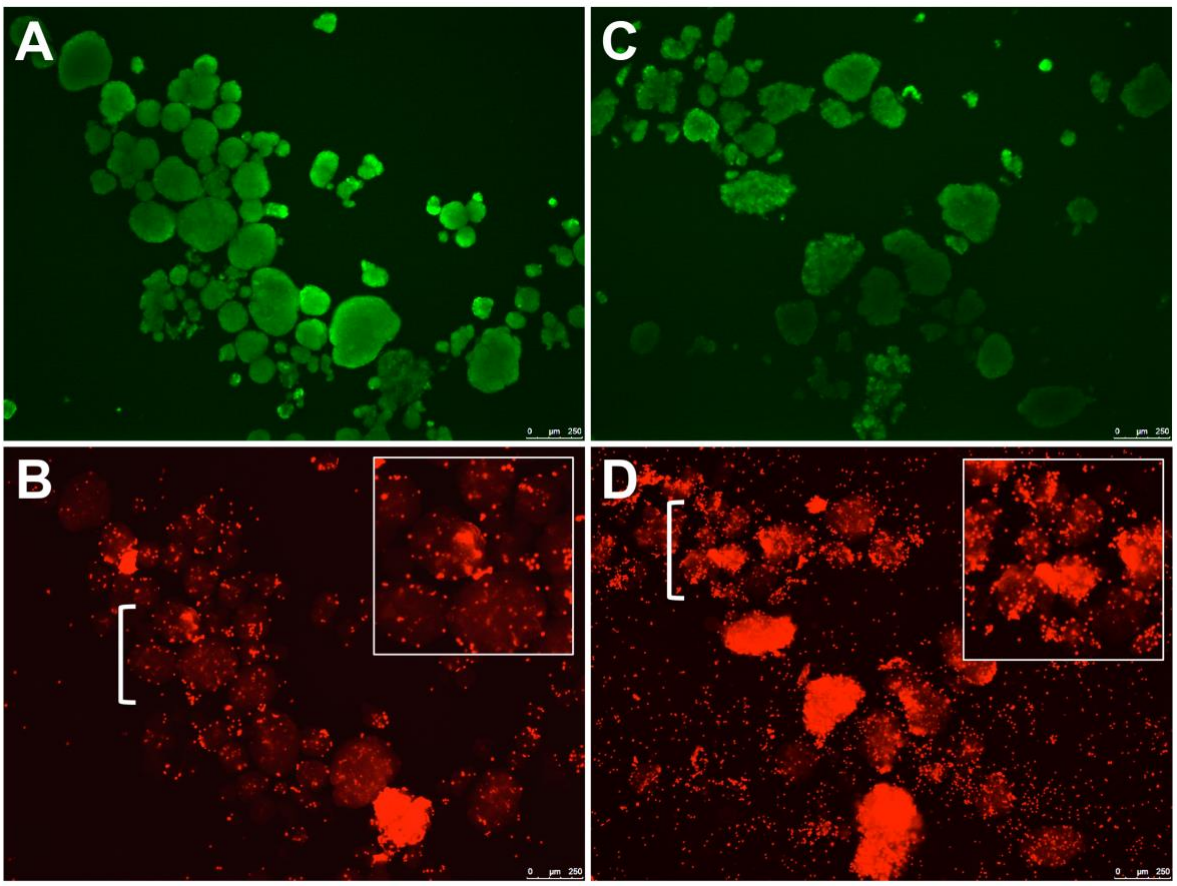

Figure 1. Qualitative viability assessment of human islet preparations by FDA/PI staining. Two human islet preparations; preparation #1 (**A**, **B**) and preparation #2 (**C**, **D**) were stained side-by-side with FDA and PI dyes. FDA staining (green) appeared to be weaker in preparation #2 compared to preparation #1. Preparation #1 had less PI labeling (red) in cell clusters and fewer PI+ single cells compared to preparation #2. Brackets in panels **B** and **C** indicate the region magnified in respective insets.

8.0 Deviations and Resolutions:

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

9.0 References

9.1 Bank, HL (1987). Assessment of Islet Cell Viability Using Fluorescent Dyes. Diabetologia, 30:812-816. Bank, HL (1988).

9.2 Rapid Assessment of Islet Viability with Acridine Orange and Propidium Iodide. Invitro Cellular & Developmental Biology, 24:4, pp. 266-273.

9.3 Ricordi, C. Pancreatic Islet Cell Transplantation. Austin: R.G. Landes Company, 1992:137-138.