Document Title:

PURIFIED HUMAN PANCREATIC ISLETS, VIABILITY ESTIMATION OF ISLETS USING FLUORESCENT DYES (FDA/PI)

PURPOSE: To be a model for site-specific SOPs that define the procedure for assessment of viability of human isolated islet preparations, which include endocrine and exocrine tissue.

RESPONSIBILITY: It is the responsibility of the Islet Cell Processing Principal Investigator or designee to:

- establish a site-specific SOP based on this document,
- train the site personnel in the execution of the site-specific procedure,
- validate the site-specific procedure,
- assure that the site-specific procedure is executed, and
- maintain records of the execution of the site-specific procedure.

SCOPE: This SOP applies to trained personnel participating in the CIT consortium manufacturing the Purified Human Pancreatic Islets product for use in DAIT-sponsored clinical studies.

I. INTRODUCTION

- FDA/PI 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.
- 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

- 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.[1] FDA functions as an inclusion dye, i.e., viable cells will appear bright green fluorescent using FDA.
- 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.[2] PI absorbs in green light and fluoresces orange/red.

II. EQUIPMENT & MATERIALS

A. Equipment

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

B. Supplies and Materials:

- Fluorecein diacetate, stock solution 24 µM (9.9 µg/mL in acetone), Sigma, Cat. #F-7378, or equivalent
- Propidium iodide, stock solution 750 µM (0.5 mg/mL in DPBS, pH approximately 7.4), Sigma, Cat. #P-4170, or equivalent
- Acetone, Sigma, Cat. #179124, or equivalent
- Sterile 10 x 35 mm cell culture dishes, Nunc Cat. #174926, or equivalent
- DPBS (Dulbecco's Phosphate Buffered Saline) without calcium or magnesium, Mediatech, Part #21-031, or equivalent
- Pipets: 200-1000 µL and 1-50 µL (1 µL increment in volume) with associated tips.

C. Attachments

- Attachment I, "Preparation of Fluorecein Diacetate and Propidium Iodide Solutions"
- Attachment II, "Islet Viability Worksheet"

III. LIMITATIONS

- 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.
- Both of the fluorescent dyes used in this assay are light sensitive and must be kept in the dark, covered with aluminum foil.
- The fluorescent dyes are temperature sensitive and must be stored as follows:
  - FDA: ≤ -20°C
  - PI: 2-8°C.

IV. PROCEDURE

A. Assay Set Up

- Assemble all items described in the "Supplies and Materials" section.
- Prepare fluorescent dyes: Fluorecein Diacetate (FDA) and Propidium Iodide (PI) according to Attachment I of this SOP, if required.

B. Preparation of Fluorescein Diacetate and Propidium Iodide

- Check the required amount of each dye necessary to make up indicated stock solutions according to Attachment I of this SOP. Remove FDA from the freezer (≤ - 20°C) and PI from the refrigerator (2°C -8°C). Weigh the required amount of the reagent on an analytical balance.
- 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.
- Dissolve 0.0125 g of PI in 25 mL of DPBS and cover with aluminum foil. Store in 5 mL aliquots at 2-8 °C.
- Discard unused stain, do not freeze.

- 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.
- Record the lot numbers of all the reagents used in the preparation of dyes on Attachment I of this SOP.
- Fluorescent Microscopy: Refer to the site-specific SOPs.

C. Staining and Estimation of Viability

1. Add 460 µL DPBS to the culture dish (this volume will adjust the final concentrations of FDA and PI to 0.46 µM and 14.34 µM, respectively).
2. Mix product well and take a 100 µL sample before islets settle and place it into a separate, empty tube.
3. Allow islets to settle for 2 to 3 minutes and take 43 µL of settled islets from the bottom of the tube and add to the culture dish containing the 460 µL DPBS.
4. Quickly add first 10 µL of PI and then 10 µL of FDA to the islet suspension. Gently swirl to mix.
5. Turn off the lights in the room.
6. Assess the preparation immediately using the fluorescent microscope.

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

7. Estimate the percentage of viable tissue vs. total tissue as follows:
   a. Evaluate the viability of 50 consecutive tissue particles.
   b. Determine the percent viability for each tissue particle by estimating to the nearest 1% the viable (green fluorescence) tissue quantity vs. the total (viable plus non-viable, green and red fluorescence) tissue quantity.
   c. Calculate the percent viability mean and standard deviation of the 50 observations made.
8. Record all data and calculations on Attachment II of this SOP.
9. If using a calculator, perform each calculation independently. Copy the results from the calculator screen onto Attachment II of this SOP.
10. Attach all print-outs and both of the completed SOP Attachments to the Production Batch Record.

V. INTERPRETATION OF RESULTS

The 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.

The 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.

VI. RECORD REVIEW

The facility manager or his/her designee, and the facility QA are responsible for initial and final records review, respectively, according to site-specific SOP. In addition, records will be review by the study sponsor (DAIT/NIAID/NIH), at a minimum, during site monitoring visits and annual audits.

VII. RECORD RETENTION

Records will be maintained by the manufacturing facility following the time period specified in the site-specific SOP describing Record Retention and Record Archival System. Do not destroy any records without consulting previously with DAIT, NIAID, NIH.

VIII. REFERENCES

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

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

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

4. Minnesota Molecular and Cellular Therapeutics Program, SOP MCT3-911.