Version 2
Jun 11, 2020

Viability Estimation of Islets for Distribution Using Inclusion and Exclusion Fluorescent Dyes (FDA/PI) V.2

Integrated Islet Distribution Program[1]
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1 Works for me dx.doi.org/10.17504/protocols.io.bhdmj246

Integrated Islet Distribution Program
Integrated Islet Distribution Program, City of Hope

ABSTRACT

This Standard Operating Procedure is adapted from the work of the '*National Institutes of Health-Sponsored Clinical Islet Transplantation Consortium Phase 3 Trial: Manufacture of a Complex Cellular Product at Eight Processing Facilities"*following the SOP cited in the document *'Purified Human Pancreatic Islet - Viability Estimation of Islet Using Fluorescent Dyes (FDA/PI): Standard Operating Procedure of the NIH Clinical Islet Transplantation Consortium'*

This SOP defines the procedure for assessment of viability of human isolated islet preparations, which include endocrine and exocrine tissue, for use in the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) sponsored research in the Integrated Islet Distribution Program (IIDP). This protocol is written to assist the participating islet isolation centers and investigators who are part of this program.

Integrated Islet Distribution Program (IIDP) (RRID:SCR_014387)

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.

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KEYWORDS
Inclusion and Exclusion Fluorescent Dyes, Fluorescein Diacetate, Propidium Iodide, Viability Assay, Human Islets, Human Acinar Tissue, Viability

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GUIDELINES

Background information:

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.

- 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

- FDA is a nonpolar ester, which passes through plasma membranes and is hydrolyzed by intracellular esterase(s) 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.

- PI 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.

- Both of the fluorescent dyes used in this assay are light sensitive and must be kept in the dark. Covered with aluminum foil is a good option.

- The fluorescent dyes are temperature sensitive and must be stored as follows:
  - ♦ FDA: ≤ -20°C
  - ♦ PI: 2 - 8°C

Responsibilities:

- It is the responsibility of the IIDP 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 relevant personnel from each of the participating centers.

- It is the responsibility of each IIDP center to follow the procedures listed in this SOP and to work to the best of their ability to follow all requirements.

Definitions:

- ***Integrated Islet Distribution Program (IIDP) (RRID:SCR_014387):*** The IIDP is a contracted 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, the Project Officer (PO), the External Evaluation Committee (EEC) 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. is the Principal Investigator for the IIDP CC and leads staff from the Department of Research Information Sciences at COH to coordinate the activities of the IIDP and assist the participating centers and investigators in the distribution of human islets.

- ***Islet Equivalent (IEQ)***: An islet that is 150 µm diameter by mathematically compensating for their volumes.

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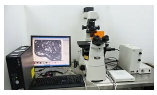
MATERIALS

NAME	CATALOG #	VENDOR
Fluorescein Diacetate	F7378	Sigma Aldrich
Propidium Iodide	P4170	Sigma Aldrich
Acetone	179124	Sigma Aldrich
Corning® Dulbecco's Phosphate-Buffered Saline 1X without calcium and magnesium	21-031-CM	Corning

MATERIALS TEXT

Fluorescence Microscope, Nikon EPI-Fluorescence Attachment for ECLIPSE TS100/TS100-F
Fluorescence Microscope or equivalent

Nikon NA



Nikon COOLPIX 950 Digital Camera
Digital Camera or equivalent

Nikon NA

Computer or calculator or equivalent
Computer software (e.g. Excel) with the mean and standard deviation functions

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Fisherbrand™ Elite™ Adjustable-Volume Pipetter or equivalent
Adjustable Pipettor

Fisherbrand™ Elite™ FBE00050
5 to 50µL Volume Range

Gilson™ PIPETMAN Classic™ Pipets or equivalent
Adjustable pipettor

Gilson F123602
200-1000 uL pipettor or equivalent

Fisherbrand™ Large-Orifice Pipet Tips, 1 to 200µL or equivalent
Genomic/Wide Orifice Pipet Tips

Fisherbrand 02-707-134
1 to 200µL

Thermo Scientific™ Nunc™ or equivalent
Cell Culture/Petri Dishes

Thermo Scientific™ Nunc™ Cell Culture/Petri Dishes
35x10mm Dish, Nunclon™ Delta, gridded

12-565-92

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Snap Cap Microcentrifuge Tubes or equivalent
2 mL polypropylene
Corning Costar Snap Cap Microcentrifuge Tube
2 mL snap cap polypropylene micro tube

07200210

SAFETY WARNINGS

Always wear gloves and observe standard chemical procedures:

Fluorescein Diacetate: FDA MSDSAction.pdf

- Protect from light. Avoid contact and inhalation. Nitrile gloves are recommended in the MSDS when handling FDA.

Propidium Iodide: PI MSDSAction.pdf

- Use personal protective equipment. Product may be toxic if inhaled, swallowed, or splashed on skin. Avoid dust formation. Avoid breathing vapors, mist, or gas. Ensure adequate ventilation. Wear gloves and observe Safety Data Sheet. Suspected of causing genetic defects.

Acetone: AcetoneMSDSAction.pdf

- Solvent/Flammable. Keep away from heat, spark, and open flame. Keep container tightly closed. Use with adequate ventilation. Avoid contact with eyes. Avoid prolonged or repeated breathing of vapor. Handle with gloves. Gloves must be inspected prior to use. Use proper glove removal technique (without touching glove's outer surface) to avoid skin contact with this product. Dispose of contaminated gloves after use in accordance with applicable laws and good laboratory practices. Wash and dry hands. Store aliquots in tightly sealed in glass or polypropylene tubes with polypropylene or polyethylene closure.

BEFORE STARTING

Prepare Fluorescein Diacetate (FDA) and Propidium Iodide (PI) Stock Solution according to Attachment 1 and store according to the SOP.

Attachment 1: Preparation of Fluorescein Diacetate and Propidium Iodide Stock Solutions (FDA/PI)
by Carol Swanson

PREVIEW RUN

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Islet Assessment Preparation

1 Assemble all items described in the “Assay Set Up” section.

2 Prepare for Fluorescent Microscopy and Image Recording: Refer to the Microscope and camera using manufacture instructions and settings.

3 Prepare [IIDP Islet Viability Worksheet] for computer tally in Step 8 with calculations using pre-formatted programming.

Preparation of Working Dye Solutions

4 ⚠

- Obtain Stock Solutions prepared previously using the link to Attachment 1:

  Attachment 1: Preparation of Fluorescein Diacetate and Propidium Iodide Stock Solutions (FDA/PI)

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

- Remove a 4 mL aliquot of [M]**24 Micromolar (µM)** FDA stock solution from the freezer (≤ - 🌡 **-20 °C**)
- Verify that the expiration date for the FDA stock solution is not expired. The expiration date is six months from the date of preparation.
- Record lot number and Expiration Date of the [M]**24 Micromolar (µM)** FDA stock solution.

  Stock Solution Lot#_____

  Stock Solution Expiration Date _____

5
- Remove a 0.5 mL aliquot of [M]**750 Micromolar (µM)** PI stock solution from the refrigerator (🌡 **2 °C** to 🌡 **8 °C**).
- Verify that the expiration date for the PI stock solution is not expired. The expiration date is six months from the date of preparation.
- Record lot number and Expiration Date of the [M]**750 Micromolar (µM)** PI stock solution.

  Stock Solution Lot#_____

  Stock Solution Expiration Date _____

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Preparation of Islets for Viability Staining

6 Prepare islets for viability assessment. Obtain islet aliquot for viability assessment.

6.1 Add 460 µL DPBS to the culture dish in which islets will be dyed and counted.

This volume will adjust the final working concentrations of FDA to 0.46 Micromolar (µM) and PI to 14.34 Micromolar (µM).

6.2 Mix islet flask well and using a large orifice pipet tip, take an aliquot that would equal roughly 100 to 200 IEQ before islets settle and place it into a separate, 2 mL Snap Cap Microcentrifuge Tube.

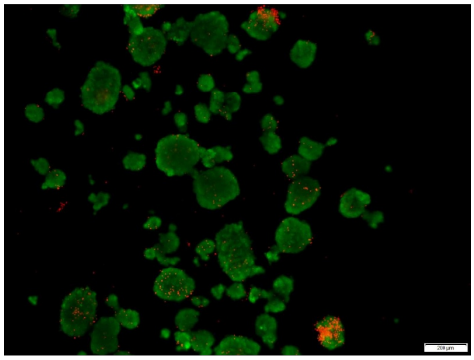
6.3 Allow islets from the aliquot to settle for 2 to 3 minutes and, using a large orifice pipet tip, take 43 µL of settled islets from the bottom of the tube and add to the culture dish containing the 460 µL DPBS.

6.4 Quickly add first 10 µL of Stock PI and then 10 µL of Stock FDA to the islet suspension. Gently swirl to mix. Turn off the lights in the room.

Estimation and Calculation of Viability

7

Assess the preparation immediately using the fluorescent microscope using either a green/red filter set or a single emission filter determined by which microscope is used.



- FDA is a cell permeable esterase substrate. The fluorescein substrate readily diffuses into cells where intracellular

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esterases hydrolyze the dye to produce fluorescein-A negatively charged molecule, which diffuses out of intact cells very slowly. Fluorescein emits light energy at a wave length of 517 nm.

- PI is poorly permeable to living cells, but readily permeable to dead cells and binds to the DNA. When bound to the DNA, PI emits light energy at a wavelength of 617 nm
- Some microscopes use green/red filter set to detect each color under the appropriate filter, and superimpose the images to get the final image. Note, however, filters allow one to observe the fluorescing as red alone, or red and green together, as the green fluorescence is much brighter than the red and can overshadow one's interpretation of the viability.
- Some microscopes use only a single emission filter to detect both green and red fluorescence so that both colors can be seen at the same time.

7.1

00:15:00 The assessment must take place as quickly as possible once the dye is added to the islets. 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.

7.2 Estimate the percentage of viable tissue vs. total tissue as follows:

Note: *FDA produces bright green fluorescence (viable cells) cells, while PI produces red Fluorescence (dead or dying cells). Islets are aggregates of cells, therefore, it is expected that some cells within an individual islet will stain green and some will stain red. The objective of the test is to assess, as accurately as possible, the percentage of islet cells that are viable.*

7.3 Evaluate the viability of 50 consecutive tissue particles.

7.4 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.

- Few or no cells are green; Average viability = non-viable
- Less than 33% of islet cells are green; Average viability = 25%
- Between 33 and 66 % of islet cells are green; Average viability = 50%
- Greater than 66% of the islet cells are green; Average viability = 75%
- Few or no cells are red; Average viability = 100%

8 After assessment, swirl contents of the petri dish to the center of the dish and capture a fluorescent image of the preparation and document with Isolation Number, Date, RRID, Sample Type (Post-Purification, Post-Culture, or Pre-shipping) as well as Purity Layer (High, Middle, or Low).

9

Tally results and record all data and calculations on the attached [IIDP Viability Worksheet] in this step. Auto calculate the percent viability mean and standard deviation of the 50 observations made.

IIDP Viability Worksheet.xlsx

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Counted by _____ Date _____

Approved by _____ Date _____

9.1 Discard unused stain, do not freeze.

Interpretation of Results

10 

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.

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

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