Dec 18, 2019

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

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1 Works for me dx.doi.org/10.17504/protocols.io.5bgg2jw

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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.

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.

GUIDELINES

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
- 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.
- 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.
- 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

MATERIALS

NAME	CATALOG #	VENDOR
Fluorescein Diacetate	F7378	Sigma Aldrich

NAME	CATALOG #	VENDOR
Propidium Iodide	P4170	Sigma Aldrich
Acetone	179124	Sigma Aldrich
Thermo Scientific™ Nunc™ Cell Culture/Petri Dishes	174926	Fisher Scientific
Corning® Dulbecco's Phosphate-Buffered Saline 1X without calcium and magnesium	21-031-CM	Corning

MATERIALS TEXT

OHAUS™ Explorer™ Semi-Micro Balance or equivalent
Semi-Micro Balance

Ohaus 01-919-370
0.01 mg to 52 gm capacity

Snap Cap Microcentrifuge Tube or equivalent
Polypropylene Microcentrifuge Tube

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

07200210

5 mL Transport Tubes
5 mL Transport Tubes

Globe Scientific Self-Standing Transport Tubes or
5 mL Polypropylene tube with Polyethylene Screw cap

22-010-1227

Fisherbrand™ Pipette-Specific Tips
Pipette Tips, 100 to 1000uL or equivalent

Pipet Tips 02-681-182

100 to 1000uL

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.

Preparation of Stock Fluorescein Diacetate (FDA) Solution

1 Prepare [M]**24 Micromolar (µM)** Fluorescein Diacetate (FDA) according to the formulation in sub-steps below.

> *The expiration date, for both PI and FDA stains, is six months from the date of preparation.*
>
> *Both of the fluorescent dyes used in this assay are light sensitive and must be kept in the dark, covered with aluminum foil.*

1.1 Calculation:

FDA FW = 416.4
Stock Concentration = 24 µM
Volume required = 200 mL

Formula: FW X Concentration X Volume = (416.4) x (24 x 10^{-6}) X (200 x 10^{-3}) = 0.00199 g FDA

Sigma Aldrich has a Mass Molarity Calculator that can be used to determine smaller quantities:
https://www.sigmaaldrich.com/chemistry/stockroom-reagents/learning-center/technical-library/mass-molarity-calculator.html

1.2 Dissolve 0.00199 g of FDA in 200 mL of acetone in a glass bottle and cover with aluminum foil.

0.00199 g FDA

- FDA Supplier_____
- FDA Lot # _____
- Expiration Date _____

200 ml Acetone

- Acetone Supplier_____
- Acetone Lot # _____
- Expiration Date _____

1.3
- Store Stock FDA solution tightly sealed in 4 mL or smaller aliquots in polypropylene tubes or glass tubes at ≤ **-20 °C** , **protected from light**, for up to six months.
- Record the following on each aliquot tube:

  FDA (24 µM) Stock Solution Assigned Lot number:_____
  Expiration Date: _____
  Prepared By/Date: _____
  Reviewed By/Date: _____

Preparation of Stock Propidium Iodide (PI)

2 Prepare [M]**750 Micromolar (µM)** Propidium Iodide (PI) according to the formulation in sub-steps below.

2.1 Calculation:
PI FW = 668.4
Stock Concentration = 750 µM
Volume required = 25 mL

FW X Concentration X Volume = (668.4) x (750 x 10^{-6}) X (25 x 10^{-3}) = 0.0125 g PI

2.2 Dissolve 0.0125 g of PI in 25 mL of DPBS and cover with aluminum foil.

0.0125 g Propidium Iodide

- PI Supplier_____
- PI Lot # _____
- Expiration Date _____

25 ml DPBS

- PBS Supplier_____
- PBS Lot # _____
- Expiration Date _____

2.3
- Store Stock PI solution tightly sealed in 0.5 mL or smaller aliquots in polypropylene Microcentrifuge Snap Cap tubes or glass tubes at **2 °C** to **8 °C** , **protected from light**, for up to six months.

- Record the following on each aliquot tube:

PI (750 µM) Stock Solution Assigned Lot number:_____
Expiration Date: _____
Prepared By/Date: _____
Reviewed By/Date: _____