**Document Title:**

# PURIFIED HUMAN PANCREATIC ISLETS, QUALITATIVE & QUANTITATIVE ASSESSMENT OF ISLETS USING DITHIZONE (DTZ)

**PURPOSE:** To be a model for site-specific SOPs that define the assay method for quantitative and qualitative determination of the Purified Human Pancreatic Islet product manufactured for use in the DAIT-sponsored clinical studies in the CIT consortium.

**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

Dithizone (diphenyl thiocarbazone, DTZ) is an organic chemical that chelates the zinc in the insulin granules present in the beta cells of the pancreatic islets. The islet cells are stained red while the acinar cells remain unstained.

DTZ staining is used as a lot release and as an in-process assay:

(i) Lot release testing: DTZ staining is used to identify islets and to determine the quantity and quality of the final islet product. Islet quantity is expressed as the number of islet equivalents (IEQ), which is calculated based on the number and diameter of the islets present in the preparation, mathematically corrected for islet volume.

(ii) In-process testing: DTZ staining is used to identify islets and to assess the effectiveness of the digestion, isolation and purification processes. The quality of the preparations is expressed as percent islet purity, and percent trapped islets. Islet quantity (IEQ) is also assessed.

## II. DEFINITIONS

**A.** **Percent Purity**: the percentage of islets compared to all tissue present in the islet preparation (islets, acinar and ductal cells), determined by visual inspection of a representative sample of the islet preparation.

**B.** **Percent Trapped**: the percentage of islets that are embedded or trapped in acinar tissue (at least 25% of the border attached to acinar tissue) compared to all islets (free and trapped), determined by visual inspection of a representative sample of the islet preparation.

C. **Islet Particle Number (IPN):** The number of islets counted.

D. **Islet Equivalent (IEQ):** an islet that is 150 μm in diameter. Islets of varying diameters are normalized to a number of Islet Equivalents of 150 μm diameter by mathematically compensating for their volumes.

E. **Equations for Total Islet Equivalent (Total IEQ) and Total Islet Particle Number (Total IPN):**

1. Total IEQ = Dilution Factor X
[(IPN of diameter 50 – 100 μm X 0.167) +
(IPN of diameter 101 – 150 μm X 0.667) +
(IPN of diameter 151 – 200 μm X 1.685) +
(IPN of diameter 201 – 250 μm X 3.500) +
(IPN of diameter 251 – 300 μm X 6.315) +
(IPN of diameter 301 – 350 μm X 10.352) +
(IPN of diameter > 350 μm X 15.833)]

2. Total IPN = Dilution Factor X $\sum$IPN of each diameter

## III. MATERIALS

A. Equipment
- Light Microscope
- Eyepiece with calibrated reticle, 1 mm
- Computer with Excel Counting Worksheet or equivalent
- Manual or Electronic Cell Counter

B. Supplies and Materials
- Positive displacement pipette and associated tips
- 0.45 μm nylon filter
- Sterile 10 x 35 mm counting dishes with grid marks
- Dithizone (DTZ) (Sigma Cat. #D5130)
- Dulbecco's Phosphate Buffered Saline (DPBS), Mediatech Part #99-597 or equivalent
- Dimethyl sulfoxide, DMSO (Sigma Cat. #D8779 or equivalent)

C. Attachment
- "Islet Counting Worksheet"

## IV. PROCEDURE

A. Assay set up
- Assemble all items described in the Section III, "Materials."
- Prepare DTZ stain as described below.

DTZ Solution Preparation

a. Dissolve: 50 mg dithizone in10 mL DMSO.
b. qs to 50 mL with DPBS.
c. Filter the combined solution using a 0.45 µm nylon filter.
d. Place solution in a 50 mL conical tube and label it
   - "Dithizone Stain"
   - Preparation Date and Time
   - Expiration Date and Time (24 hours after preparation)
   - Initials of person preparing solution

B. Islet Cell Quantitation

1. Mix the final islets suspension very gently but thoroughly before taking a sample. As islets settle rapidly, care must be taken to ensure a representative sample is taken.

2. Take sample volumes and replicates according to the Table, below. Place each sample in a 10 x 35 mm counting dish with grid lines.

**Table: Sampling and replicates for islets evaluation by DTZ**

| | Sample | No. of replicates, Sample Volume | Assay |
|---|---|---|---|
| In-Process or Intermediate Islet Product | Digest | Multiple, 1-2 mL from digest | Identity, Digest Progress |
| | Pre-purification | Duplicate, 100 µL from 200 g | Identity, Count |
| | Continuous Purification Fractions | Single, 500 µL from 250 mL | Identity, % Purity |
| | Discontinuous Purification Fractions | 500 µL, of each fraction | Identity, % Purity |
| | Post-purification | Duplicate, 100 µL from 100 g | Identity, Count |
| Final Product | | Duplicate, 100 µL from 100 mL | Identity, Count |

3. Add 3 drops (30 µL) of the DTZ solution to the islets sample and allow staining for 1 – 2 minutes at room temperature. Cover the bottom of the counting dish with DPBS to approximately 1/2 the height of the dish. Count the islets under the microscope following the steps below.

4. Examine the islets sample (stained islets will appear red) using the 10X eyepiece and the 4X objective to give a total magnification of 40X. Using the grid lines on the counting dish as a guide, methodically scroll through the dish from side to side, and top to bottom, examining each islet. Count islets within the perimeter of the grid's squares, including only islets touching the top and right lines (not the bottom and left lines), to avoid counting the same islet twice.

5. Use a reticle certified to a correction factor of 0.98 to 1.02 in the eyepiece of the light microscope to determine the size of each islet. The distance across two spaces on the calibrated reticle in the eyepiece equals 50 µm. Do not count islets smaller than 50 µm because their contribution is not significant. Using the table below as a guide, place each islet into one of the diameter groups.

| Number of Spaces Spanned | Diameter of Islet (μm) |
|---|---|
| < 2 | < 50 |
| 2 – 4 | 50 – 100 |
| 4 – 6 | 101 – 150 |
| 6 – 8 | 151 – 200 |
| 8 – 10 | 201 – 250 |
| 10 – 12 | 251 – 300 |
| 12 – 14 | 301 – 350 |
| > 14 | > 350 |

6. Count the number of islets in each diameter group using the manual or electronic cell counter. If there is a print-out, attach it to the Production Batch Record.

7. Calculate the dilution factor as follows:

$$\frac{\text{Total volume of preparation that sample taken from (mL) X (1000)}}{\text{Volume of sample taken (μL)}} = \text{Dilution Factor}$$

8. Calculate the Total Islet Particle Number (Total IPN), and the Total Islet Equivalents (Total IEQ) using the formulas provided in Section II, E, above, and record the results in the Table in the Production Batch Record.

Example for a 100 μL sample from a 100 mL total volume:

| Islet Diameter Range (μm) | Islet Particle Number (IPN) | IEQ Conversion Factor | IEQ per Range |
|---|---|---|---|
| **50 – 100** | 11 | **X 0.167** | 1.837 |
| **101 – 150** | 42 | **X 0.648** | 27.216 |
| **151 – 200** | 26 | **X 1.685** | 43.810 |
| **201 – 250** | 13 | **X 3.500** | 45.500 |
| **251 – 300** | 5 | **X 6.315** | 31.575 |
| **301 – 350** | 0 | **X 10.352** | 0 |
| **> 350** | 1 | **X 15.833** | 15.833 |
| **Σ IPN** | **98** | **Σ IEQ** | **165.771** |
| Dilution Factor [(mL total volume / μL sample volume) X 1000] | | | 1000 |
| Total IPN = Σ IPN X Dilution Factor | | | 98,000 |
| Total IEQ= Σ IEQ X Dilution Factor | | | 165,771 |

C. Percent Free Islets & Percent Trapped Islets Determination

1. Methodically examine at least 50 islets to determine if each is free or trapped. Free islets have less than 25% of their border attached to acinar tissue. Trapped islets have 25% or more of their border attached to acinar tissue.

2. Record these quantities in the Table in the Production Batch Record.

3. Calculate to 1% the percent free and the percent trapped by dividing the number free islets and the number trapped islets, respectively, by total number of islets counted.

Example:

| | | | | | |
|---|---|---|---|---|---|
| # of free islets | 32 | ÷ total # of islets X 100 | 54 | = % free islets | 59% |
| # of trapped islets | 22 | ÷ total # of islets X 100 | 54 | = % trapped islets | 41% |

4. Record the results in the Table in the Production Batch Record.

D. Percent Purity

1. Determine the percent purity to the nearest 5% by estimating the proportion of red-stained islets to all the tissue (islets, acinar, ductal cells) across several fields.

2. Record the result in the Table in the Production Batch Record.

## V. RECORD REVIEW

Records will be reviewed as defined by the site-specific SOPs. At a minimum the operator supervisor and/or QA personnel should review the records.

## VI. 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.

## VII. REFERENCES


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

Ricordi, C. (Ed). Methods in Cell Transplantation. Austin: R.G. Landes Company, 1995:Section G.