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Qualitative & Quantitative Assessment of Human Islets for Distribution Using Dithizone (DTZ)

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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: Qualitative and Quantitative Assessment of Islets Using Dithizone (DTZ) – Standard Operating Procedure of the NIH Clinical Islet Transplantation Consortium'

This SOP defines the assay method for quantitative and qualitative determination in the identification 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.

Dithizone (diphenyl thiocarbazon; 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.

GUIDELINES

- **Percent Purity:** the percentage of islets compared to all tissue present in the islet preparation (islets, acinar and ductal cells) which is determined by visual inspection of a representative sample of the islet preparation. Islets are distinguished from non-islet tissue by using Dithizone (DTZ) to stain red the zinc granules in the beta cells.
- **Actual Islets (AI) or Islet Particle Number (IPN):** The actual number of islets or islet particles counted. For this SOP, the nomenclature of Actual Islets (AI) will be used.
- **Islet Equivalent (IEQ):** A conversion factor of the size of an actual islet to an equivalent size of an islet which is 150 μ m diameter by mathematically compensating for the volume. This is mathematical compensation for islets of varying diameters normalizes the counts for comparison within and between islet preparations.
- **Islet Quality Grade:** A qualitative designation given to the islet preparation after microscopic evaluation based on the parameters of shape, border, integrity, number of single cells, and overall islet diameter.
- **Equations for Total Equivalent (Total IEQ) and Total Actual Islet (AI):**
 1. **Total IEQ** = Dilution Factor x

$$[(AI \text{ of diameter } 50 - 100 \mu\text{m} \times 0.167) +$$

$$(AI \text{ of diameter } 101 - 150 \mu\text{m} \times 0.667) +$$

$$(AI \text{ of diameter } 151 - 200 \mu\text{m} \times 1.685) +$$

$$(AI \text{ of diameter } 201 - 250 \mu\text{m} \times 3.500) +$$

$$(AI \text{ of diameter } 251 - 300 \mu\text{m} \times 6.315) +$$

$$(AI \text{ of diameter } 301 - 350 \mu\text{m} \times 10.352) +$$

$$(AI \text{ of diameter } > 350 \mu\text{m} \times 15.833)]$$

2. **Total AI** = Dilution Factor x \sum AI of each diameter

- **Islet Index (II)**: A quantitative designation given to the islet preparation after microscopic evaluation determined by dividing the IEQ by the AI. This designation determines the overall size distribution of the islets being shipped. If the majority of AI is around 150 μ m (1-IEQ) then the II will equal 1.0. If the majority of AI is larger than 150 μ m, then the II will be > 1.0. If the majority of the AI is smaller than 150 μ m, then the II will be < 1.0.

References:



Ricordi, C. (1992). Pancreatic Islet Cell Transplantation. Austin: R.G. Landes Company.



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MATERIALS

NAME 	CATALOG # 	VENDOR 
Dithizone (Diphenylthiocarbazone)	D5130	Millipore Sigma
Gibco DPBS without Calcium and Magnesium	14190136 or equivalent	Fisher Scientific
Dimethyl sulfoxide	D8779 or equivalent	Sigma Aldrich

MATERIALS TEXT



Gilson™ PIPETMAN Classic™ Pipets-
F123601 or equivalent
Adjustable pipettor -P200

Gilson F123601G [↗](#)
50 to 200µL, ±0.5, ±1µL



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

Fisherbrand 02-707-134 [↗](#)
1 to 200µL



Fisherbrand™ Petri Dishes with Clear Lid or
equivalent
Petri Dishes

Fisherbrand FB0875713A [↗](#)
Round, Raised Ridge, 60mm, 15mm



Thermo Scientific™ Nalgene™ Rapid-Flow™
Sterile Disposable Bottle Top Filters with
PES Membrane or equivalent
0.45 µm Bottle Top filter

Thermo Scientific 09-740-62C [↗](#)
0.45µm nylon Filter





Invitrogen™ EVOS™ XL Core Imaging System or equivalent
Inverted Microscope
Light Microscope AMEX1000 [↗](#)



Lab inverted light microscope
Eyepiece with Calibrated reticle, 1mm Microscope specific, lab SOP



Bal Supply Cell Counter or equivalent
Cell Counter (Manual or Electronic)
Bal Supply 02-670-14 [↗](#)



Drummond™ Fixed-Volume Microdispensers or equivalent
Drummond 3000385
Drummond™ 21176F [↗](#)
Volumetric Range 100/200UL with borosilicate glass bores



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

SAFETY WARNINGS

Dimethyl sulfoxide (DMSO) [DMSO_MSDSAction.pdf](#)

- Hazard statement(s): Combustible liquid.
- Precautionary statement(s): Keep away from heat/sparks/open flames/hot surfaces. No smoking. Wear protective gloves/ protective clothing/ eye protection/ face protection.
- DMSO itself is not toxic but it can be a carrier of chemicals, viruses, etc. into the skin


Dithizone (DTZ) [Dithizone_MSDSAction.pdf](#)

- Hazard Statement(s): Causes skin irritation. Causes serious eye irritation.
- Precautionary statement(s): Wash skin thoroughly after handling. Wear protective gloves/ eye protection/ face protection.

Preparation of Working Dithizone

- 1 Assemble all items described in the Materials section
- 2 Prepare DTZ stain as described below. Observe all safety precautions when working with DMSO.
 - 2.1 Dissolve **50 mg** dithizone in **10 ml** DMSO.
 - 2.2 Add DPBS to bring the total volume to **50 ml**.
 - 2.3 Filter the combined solution using a 0.45 μ m nylon filter.
 - 2.4 Place solution in a 50 mL conical tube and label "Dithizone Stain or DTZ" with:
 - Preparation Date and Time
 - **Expiration Date and Time (24 hours after preparation)**
 - Initials of person preparing solution

Preparation of Islets for Counting

- 3 Samples for islet quantitation and purity should be taken for islet broadcast either after the isolation or post culture. Sampling must be repeated prior to packaging for shipment in order to ensure that samples after culture are representative of what is being distributed to the investigators.
 - 3.1 

Mix the final islets suspension very gently but thoroughly by inverting the islet prep in a conical 2-3 times before quickly taking a sample. (Do not swirl.) As islets settle rapidly, care must be taken to ensure a representative sample is taken from the middle of the suspension.

3.2 

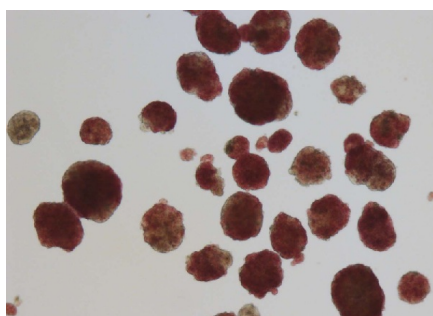
Take replicate 100-200 µl sample volumes from 100 mL final prep using a wide bore tip of pipettor. Two duplicate counts should be performed by two separate technicians or 2 separate counts by one technician.

3.3 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 ½ the height of the dish. Count the islets under the microscope following the steps below.

Islet Quantification

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Identify AI, IEQ, and % purity.



Dithizone (diphenyl thiocarbazon; 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.*

- Islets are counted by size of the Actual Islets (AI), tabulated, and mathematically calculated to determine the Total Islet Equivalents (IEQ).
- 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.

- 4.1 There are different types of reticles that can be used but all should be certified in the eyepiece to the correct specification by the microscope company representative. Once calibrated, the eyepiece of the microscope is used to determine the size of each islet.

Using the table below as a guide, Regardless of which type of reticle is used, count (tally) the number of Actual Islets (AI) in each diameter group using the manual or electronic cell counter. Do not count islets smaller than 50 μm because their contribution is not significant.

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

- 4.2 Calculate the dilution factor as follows:
Total volume of preparation that sample taken from (mL) X (1000) = Dilution Factor
Volume of sample taken (μL)

4.3

Record and calculate the results using Excel Spreadsheet in Attachment 1.

Example for a 100 μ L sample from a 100 mL total volume:

Islet Diameter Range (μ m)	Actual Islet Number (AI)	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	0.000
>350	1	x 15.833	15.833
	Σ AI	Σ IEQ=	165.771
Dilution Factor [(mL total volume / μ L sample volume) X 1000]			1000
Total AI = Σ AI X Dilution Factor			98,000
Total IEQ = Σ IEQ X Dilution Factor			165,771

- Calculate the Total Actual Islets (Σ AI), and the Total Islet Equivalents (Σ IEQ) by inputting the data from the tally, in step 4.1, into the Excel spreadsheet in Attachment 1 for Most Pure Shipment and Least Pure Shipment if applicable.
- Calculate the total number of IEQ present by entering the total Actual Islets (AI) in each range which will be converted using the conversion factor, to IEQ per each range. This conversion normalizes the AI to 150 μ m sized islet.
- Both the AI and IEQ will be totaled and multiplied by the dilution Factor for a Final AI count and Final IEQ Count of the preparation.
- The Islet Index (II), a calculation made by dividing the IEQ by the AI, is calculated automatically in Attachment 1. This designation determines the overall size distribution of the islets being shipped.
- Repeat process, as needed, for a Least Pure Shipment.

 **Attachment 1-Islet Tabulation Counting Sheet (v1).xlsx**

Islet Quality Grade by Islet Ranking

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Determine the Islet Quality Grade based on the chart below and Islet Ranking Guide (Attachment 2) and record and calculate the results on Attachment 1 for Most Pure Shipment and Least Pure Shipment, if applicable, and on broadcast website.

 [Attachment 2-Islet Ranking Guide.pdf](#)

Parameter	0 Points	1 Point	2 Points
Shape (3D)	flat/planar	in between	spherical
Border (2D)	irregular	in between	well-rounded
Integrity	fragmented	in between	Solid/compact
Single Cells	many	a few	almost none
Diameter	all<100µm	a few>200 µm	>10%>200 µm

Islet Purity

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Calculate the percent purity of the islets to the nearest 5% by estimating the portion of dithizone (red stained islets) to all tissue (islets, acinar, ductal cells). Record the purity of each sample on Attachment 2. The average will be calculated automatically.

Photographic Documentation


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Take digital images and upload (when applicable) for IIDP Islet Broadcast of the count sample.

- 7.1 Gently swirl the count sample in order to center the islets in the dish. This will give a representative photographic image of the purity and size distribution of the islet preparation. If possible, take a well focused digital photo using center's SOP for its specific microscope and camera set-up so the final image size is a 10x-40x magnification. Label and save image with RRID#, date, time when photo was taken (prior to broadcast), and magnification.
- 7.2 With the use of the digital imaging techniques on the IIDP website, upload images and match the background grid lines per the on-line instructions in the broadcast section (when applicable).

Subsequent Counting, Grading, Purity, and Viability

- 8 Repeat all counting, grading, purity, and viability estimates and digital image uploads for islets prior to packaging for distribution if more than 6 hours post original broadcast evaluations. Update broadcast records under the "Confirm Islet Information" section of the broadcast and repeat these procedures for rebroadcasts and shipments with every subsequent 24 rebroadcast/shipment.

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