Version 2

Nov 13, 2020

Qualitative & Quantitative Assessment of Human Islets Using Dithizone (DTZ) V.2

Human Islet Phenotyping Program (HIPP) of the IIDP[1]

[1]Integrated Islet Distribution Program and Human Islet Phenotyping Program

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

Integrated Islet Distribution Program and Human Islet Phenotyping Program
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IIDP-HIPP

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 used by the Human Islet Phenotyping Program (HIPP) for quantitative and qualitative determination of the Purified Human Pancreatic Islet product, post-shipment, manufactured for use in the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK)-sponsored research in the Integrated Islet Distribution Program (IIDP).

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

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.

DOI

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KEYWORDS

Human Islets, dithizone, IIDP, HIPP

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GUIDELINES

- ***Integrated Islet Distribution Program (IIDP) (RRID:SCR_014387):*** The IIDP is a funded 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. and Carmella Evans-Molina, M.D., Ph.D. serve as Co-Principal Investigators (Co-PIs) for the IIDP Program located within the Department of Diabetes and Cancer Discovery Science at COH to coordinate the activities of the IIDP and Human Islet Phenotyping Program (HIPP). Dr. Niland, contact PI, oversees the daily activity of the IIDP staff, provides informatics/ biostatistical input, and subcontracts with the Islet Isolation Centers (IICs) to ensure the delivery of the highest quality human islets to IIDP-approved investigators. Dr. Evans-Molina serves as the liaison to the HIPP, interacting closely to ensure that extensive, high quality phenotypic data are collected on islets distributed by the IICs. She also facilitates the delivery of this information to both the IICs and the IIDP-approved investigators, while responding to questions, issues, or suggestions for further HIPP enhancements."

- ***Human Islet Phenotyping Program (HIPP):*** The HIPP is a subcontracted entity of the IIDP through the COH and Vanderbilt University. The HIPP is directed by Marcela Brissova, Ph.D. and is responsible for performing specific standardized quality control assays agreed upon by both the IIDP and the HIPP, in order to provide enhanced, quality data on the human islets post-shipment, to the IIDP. The results of these assays will be approved by the CC and posted on the IIDP website for both the centers and the approved investigators to access.

- ***Percent Purity:*** The percentage of islets compared to all tissue present in the islet preparation (islets, acinar and ductal cells), determined by quantitative analysis 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):*** The actual number of islets counted.

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- ***Islet Equivalent(IEQ):*** An islet is quantified as 150µm diameter by mathematically compensating for the volume of the islet.

- ***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. (See step 4 for specifics.)

- ***Equations for Total Equivalent (Total IEQ) and Total Actual Islet(AI):***
  1. Total IEQ = Dilution Factor x
     [(AI of diameter 50 – 100 µm x 0.167) +
     (AI of diameter 101 – 150 µm x 0.667) +
     (AI of diameter 151 – 200 µm x 1.685) +
     (AI of diameter 201 – 250 µm x 3.500) +
     (AI of diameter 251 – 300 µm x 6.315) +
     (AI of diameter 301 – 350 µm x 10.352) +
     (AI of diameter > 350 µm x 15.833)]
  2. Total AI = Dilution Factor x ∑ AI of each diameter

- ***Islet Index (II):*** A quantitative designation given to the islet preparation after software-guided islet size and number analysis 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 150µm (1-IEQ) then the II will equal 1.0. If the majority of AI is larger than 150µm, then the II will be > 1.0. If the majority of the AI is smaller than 150µm, then the II will be <1.0.

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.

MATERIALS TEXT

MATERIALS

Dithizone (Diphenylthiocarbazone) **Millipore**

Sigma Catalog #D5130

Dimethyl sulfoxide DMSO **Sigma**

Aldrich Catalog #D8779 or equivalent

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Dulbecco's Phosphate Buffered Saline (DPBS) **Fisher Scientific Catalog #Mediatech Part 99-597 or equival**

The following *equipment* is necessary to perform quantitative and qualitative assessment on human islets.

1. Light Microscope

2. Eyepiece with Calibrated reticle, 1 mm

3. Computer with Excel Counting Worksheet or equivalent

4. Manual or Electronic Cell Counter

5. Olympus SZX12 stereomicroscope equipped with an Olympus DP-80 high-resolution digital camera

6. Olympus cellSens™image acquisition and analysis software

The following *supplies and materials* are necessary to perform quantitative and qualitative assessment on human islets.

1. Positive displacement pipette and associated wide bore tips.

2. 0.45µm nylon filter

3. 10 x 35 mm dishes without grid marks

4. Dithizone (DTZ) (Sigma Cat. #D5130)

5. Dulbecco's Phosphate Buffered Saline (DPBS), Mediatech Part #99-597 or equivalent

6. Dimethyl sulfoxide, DMSO (Sigma Cat. #D8779 or equivalent)

Stereo Microscope
SZX-ZB12
Olympus **OLYMPUS-SZX12**

High-resolution digital camera
DP-80
Olympus **OLY7-D780**

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Imaging software

Olympus cellSensTM **None**

Positive Displacement Pipette

1 µL to 1000 µl

Gilson **None**

Positive Displacement Tips

702852

BrandTech Scientific **100-500uL**

Nylon Syringe Filters

0.45 um

Tisch **SF13828**

Cell Culture/Petri Dishes or equivalent

10X35 mm

Nunc™ **150318**

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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.

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Preparation of Working Dithizone

1 Assemble all items described in Materials section.

2 Prepare DTZ stain as described below. Observe all safety precautions when working with DMSO.

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2.1 Wearing gloves, dissolve **50 mg** dithizone in **10 mL** DMSO.

2.2 Top up to **50 mL** with DPBS.

2.3 Filter the combined solution using a 0.45µm nylon filter.

2.4 Place solution in a **50 mL** conical tube and label "Dithizone Stain" with:

- Preparation Date and Time
- Expiration Date and Time (24 hours after preparation)
- Initials of person preparing solution

3 Triplicate samples for islet quantitation, grading, and purity should be obtained upon receipt of the islet shipment. Triplicate samples must be taken for each count and purity assessment. Islet grading should be performed by three certified personnel, but may be performed by two personnel if three are not available. Results will be averaged for final reporting on the IIDP website. If triplicate counts of IEQ are more than 30% different, then a fourth sample should be taken and the four averaged. Date should be documented on the worksheets and Excel files for each procedure.

3.1 Mix the final islet 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. It is best to have two staff perform this procedure together, one mixing and the second taking the sample.

3.2 Take replicate **320 µl** sample volumes from **32 mL** total volume of shipment plus **680 µl** CMRL medium in 35 mm dishes.

3.3 Add **50 µl** of the DTZ solution to the islet sample and allow staining for 1 – 2 minutes at room temperature. Add an additional **1 mL** CMRL medium to plate before imaging.

3.4 Place a dish containing islets on stand of stereomicroscope equipped with a high-resolution camera, and swirl until all tissue is in the camera field of view at 10x magnification. Capture brightfield images at approximately 12-ms exposure and darkfield images at approximately 1.2-s exposure, each at 10x magnification. Ensure all tissue is present in image. Save all image files. ***Examples of islet images are shown in Figure 1.***

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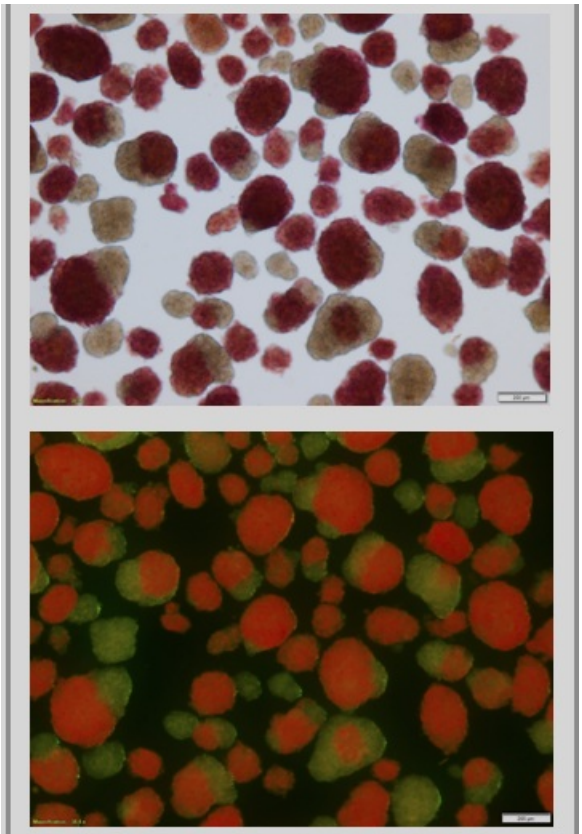

Figure 1. DTZ-stained human islet images captured in bright field (top) and dark (bottom). Scale bar is 200 µm.

3.5 Open the darkfield image in the cellSens software. Using the manual HSV threshold function, segment the islet tissue channel (stained red) and non-islet tissue (unstained).

3.6 Use the custom Count and Measure algorithm to determine islet count, mean islet diameter, and area of islet and non-islet tissue in the image. Split adjacent but discrete islets using the Manually Split Objects tool to get an accurate islet count (AI). For IEQ calculation, use the Object Filter to exclude islets <50µm in diameter. For Islet Purity calculation, turn off the Object Filter to include all tissue.

Note: Islet purity analysis by cellSens began 12/6/2016; islet count and IEQ analysis by cellSens began 10/24/2017.

3.7 Calculate the dilution factor as follows:

Dilution Factor = Total volume of islet preparation that sample aliquot was taken from (mL) x 1000 / Volume of sample aliquot (µL)

3.8 Use the mean diameter measurements to assign islets to a diameter group, then calculate the Total Actual Islet (Total AI) and the Total Islet Equivalents (Total IEQ) using the formulas provided in 3.7,

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above, and record the results on the *Islet Cell Calculation Excel Worksheet. (See Attachment 1)*

Example for a 100 µL sample from a 100 mL total volume:			
Islet Diameter Range (µm)	**Islet Particle Number (AI)**	**IEQ Conversion Factor**	**IEQ per Range**
50 – 100	11	**x 0.167**	1.837
>100 – 150	42	**x 0.667**	27.216
>150 – 200	26	**x 1.685**	43.810
>200 – 250	13	**x 3.500**	45.500
>250 – 300	5	**x 6.315**	31.575
>300 – 350	0	**x 10.352**	0
>350	1	**x 15.833**	15.833
∑ AI		**∑ IEQ**	**165.771**
Dilution Factor [(mL total volume / µL sample volume) x 1000]			1000
Total AI = ∑ AI x Dilution Factor			98,000
Total IEQ = ∑ IEQ x Dilution Factor			165,771

HIPP Protocol-07-IEQ_Counts_and_Islet_Ranking-Attachment_1_v3-2.xlsx

3.9 Using the islet and non-islet tissue area measurements, calculate Islet Purity using the following formula:

Islet Purity (%) = Total islet tissue area / (Total islet tissue area + Total non-islet tissue area) x 100

3.10 The Islet Index (II), a calculation made by dividing the IEQ by the AI, will be calculated by the system when these parameters are entered into the system. This designation determines the overall size distribution of the islets being shipped.

4 Determine the Islet Quality Grade based on the Islet Ranking chart below: *(See Attachment 2)*

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

HIPP Protocol-08-Islet_Ranking_Images-Attachment_2.pdf

4.1 Add up the Islet Ranking Points for each sample to obtain the Islet Quality Grade and record grade on Islet Cell Calculation Excel Worksheet. *(See Attachment 1)*

HIPP Protocol-07-IEQ_Counts_and_Islet_Ranking-Attachment_1_v3-2.xlsx

Deviations and Resolutions:

5 Document any deviations that occurred during this protocol that affect the final results and report with the analysis of the assay.

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Data Storage and Reporting

6 Data Storage and Reporting

6.1 To facilitate data management and ensure data security, the Vanderbilt HIPP uses an institutional server-based platform for data storage and analysis.

6.2 Upon completion of image acquisition and analysis, annotated images containing metadata, image analysis outputs, and documentation of any deviations and repetitions that are warranted will be uploaded to the IIDP-HIPP database and disseminated to IIDP-affiliated investigators and islet isolation centers.

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