QUALITATIVE & QUANTITATIVE ASSESSMENT OF HUMAN ISLETS USING DITHIZONE (DTZ)

I. Definitions

1. **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. Islets are distinguished from non-islet tissue by using Dithizone (DTZ) to stain red the zinc granules in the beta cells.

2. **Actual Islets (AI):** The actual number of islets counted.

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

4. **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 #4 for specifics)

5. **Equations for Total Equivalent (Total IEQ) and Total Actual Islet (AI)**

$$\text{Total IEQ} = \text{Dilution Factor} \times [(\text{AI of diameter } 50 – 100\ \mu\text{m} \times 0.167) + (\text{AI of diameter } 101 – 150\ \mu\text{m} \times 0.667) + (\text{AI of diameter } 151 – 200\ \mu\text{m} \times 1.685) + (\text{AI of diameter } 201 – 250\ \mu\text{m} \times 3.500) + (\text{AI of diameter } 251 – 300\ \mu\text{m} \times 6.315) + (\text{AI of diameter } 301 – 350\ \mu\text{m} \times 10.352) + (\text{AI of diameter} > 350\ \mu\text{m} \times 15.833)]$$

$$\text{Total AI} = \text{Dilution Factor} \times \sum \text{AI of each diameter}$$

6. **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 150µm (1 IEQ) then the II will equal 1.0. If the majority of AI is larger than 150 µm, then the II will be > 1.0. If the majority of the AI is smaller than 150 µm, then the II will be <1.0.

II. Equipment and Materials

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

    - Light Microscope

- Eyepiece with Calibrated reticle, 1 mm
- Computer with Excel Counting Worksheet or equivalent
- Manual or Electronic Cell Counter
- Stereo microscope equipped with high-resolution digital camera
- Olympus cellSens Dimension micro imaging software

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

- Positive displacement pipette and associated wide bore tips.
- 0.45 μm nylon filter
- 10 x 35 mm counting dishes with 2-mm grid marks
- 10 X 35 mm dishes without 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)

III. Procedures

1. Assemble all items described in II- Equipment and Materials.

2. Prepare DTZ stain as described below.

- Wearing gloves, dissolve 50 mg dithizone in 10 mL DMSO.
- Top up to 50 mL with DPBS.
- Filter the combined solution using a 0.45 µm nylon filter.
- 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. Duplicate samples for islet quantitation, grading, and purity should be obtained upon receipt of the islet shipment. Duplicate samples must be taken for each count but if two certified counters are not available, then a single counter may count both samples. Results will be averaged for final. If duplicate counts of AI are more than 30% different, then a third sample should be taken and the three averaged**.** Date should be documented on the worksheets and Excel files for each procedure.

- 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. It is

best to have two staff perform this procedure together, one mixing and the second taking the sample.

- Take replicate 400 µl sample volumes from 40 mL total volume of shipment plus 600 µl CMRL medium in 35 mm dishes (without grids.)

- 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.

- Using a high-resolution camera-equipped microscope, swivel until all tissue is in the camera field of view at 10x magnification. Capture brightfield images at approx. 12 ms exposure and darkfield images at approx. 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**.

- Open the darkfield image in the Olympus cellSens Dimension software. Specify the color range of the islet tissue channel (stained red) and the non-islet tissue channel (unstained) using the manual HSV threshold function.

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

Calculate the dilution factor as follows:

$$\frac{\text{Total volume of preparation that sample was taken from (mL) x (1000)}}{\text{Volume of sample taken } (\mu\text{L})} = \text{Dilution Factor}$$

- 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 6.3.7, above, and record the results on the *Islet Cell Calculation Excel Worksheet*

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
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
∑ 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

- Using the islet and non-islet area measurements, calculate Percent Purity using the following formula:

  (total islet area) / (total islet area + total non-islet area) * 100

- 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:

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<100 µm	a few>200 µm	>10%>200 µm

5. Add up the Islet Ranking Points for each sample to obtain the following Islet Quality Grades:
   - 9 to 10 points=A
   - 7 to 8 points=B
   - 4 to 6 points=C
   - 2 to 3 points=D
   - 0 to 1 point=F
6. Record Islet Rank and Quality Grade on Islet Cell Calculation Excel Worksheet.

IV. Deviations and Resolutions:

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

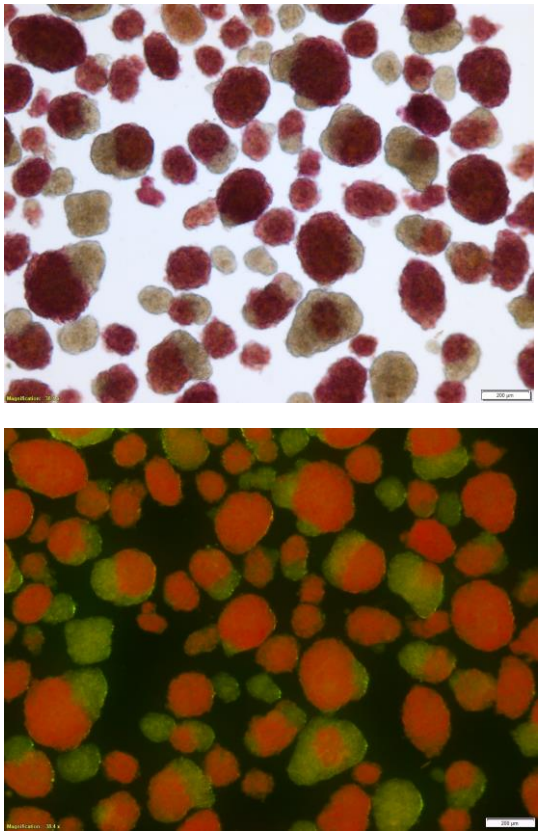

Figure 1. DTZ-stained human islet images captured in bright field (top) and dark (bottom). Scale bar is 200 μm.

References

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

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