QR-QA-010-02: Guide for Mixing and Sampling at the Isolation Centers

1) Objective

To provide direction to IIDP Islet Isolation Centers (IIC) staff on a standardized procedure for mixing the islet preparation prior to sampling for islet counts and for islet sampling prior to shipment.

(*Note: The Human Islet Phenotyping Program, (HIPP) was also present for the IIDP Virtual Workshop 2020 and was involved in the standardization discussions but the sample sizes with which HIPP works requires different sizes of mixing tubes, sample sizes, and pipette, however similar standardization was discussed. This is reflected in QR-QA-011-01: Protocol for Mixing and Sampling at the HIPP*)

2) Prerequisites

a) Representatives of all IICs and the HIPP participated in a Virtual Counting Workshop over the summer of 2020 in order to try to standardize the islet counting procedure. During the discussions, it was agreed upon that the major cause of dissimilar counts within a laboratory and probably among laboratories was the preparation mixing and the means of sampling the islets.

b) Videos of the mixing and sampling procedures were supplied by some of the centers for better understanding of the variety of techniques.

c) Information was gathered from each of the IICs and the HIPP and this Standard Operating Procedure (SOP) represents the culmination of center cooperation and standardization of the mixing and sampling procedure conducted pre-broadcast count and pre-sampling for distribution.

3) Procedures

a) Consolidation of Tissue, Transfer of Flasks

i) Settle islets for 5-10 minutes; transfer settled islet pellets from up to 6 flasks to one **250 mL** conical using a **25 mL pipette (2.8 mm tip)**

(1) check to make sure all islets have been transferred; if viable islets remain in the unsettled supernatant, it may be spun at 200 g/1 min and pellet can be transferred to the consolidation conical, serial rinse.

ii) Top conical to **100 mL** with Transport Media Complete

b) Preparation Mixing and Sampling Technique for Count Samples

i) Prepare two 60 mm gridded counting dish by adding 2 mL of transport media.

ii) Two people are ideal for mixing and taking samples; 1st person gently shakes (vibrates) the conical containing the settled pellet to ensure there is no clumping, then inverts the 250 mL conical 2-3 times.

iii) 1st person quickly removes the cap and then 2nd person takes a 100 µL (0.1 mL) (for preparations of 100,000 – 300,000 IEQ; 200 µL (0.2 mL) for preparations <1000,00 IEQ) sample using preset 200 µL (2.2 mm bore) Drummond Microcapillary pipettor or two samples using the 100 µL setting, from estimated middle of cell suspension. Gently and slowly release the plunger to allow a consistent sample to enter the pipette.

iv) Disperse entire amount of sample into counting dish, gently rinsing the pipette two times with the clean media from the dish.

v) Dispose of the first pipette tip and repeat the mixing and sampling technique for a duplicate sample.

vi) Add 3 drops (~30 µL) of prepared dithizone (DTZ) to the counting dish and wait 1-2 minutes for islets to fully stain. (See IIDP SOP for DTZ recipe from links at the end of this protocol.)

c) Counting of Islet Sample

i) Two duplicate samples should always be counted, ideally by 2 different technicians.

ii) Using a Counting Mechanism such as an image analysis system or machine can be used as a backup, but duplicate samples should always be taken.

iii) Examine the islet sample *(stained islets will appear red*) using the 10X eyepiece and the 4X objective to give a total magnification of 40X. Sizing grid is specific for your microscope and ocular type and should be calculated as such.

iv) 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 without disturbing their positions in the dish. 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.

i) A calibrated reticle in the eyepiece of the microscope should be used for determining the size of each Actual Islet (AI), and islet sizes should be sorted in the following categories:
Note: Do not count islets smaller than 50µm because their contribution is not significant to total volume or yield.

Diameter of Islet (µm)
< 50
50 – 100
101 – 150
151 – 200
201 – 250
251 – 300
301 – 350
> 350

ii) Determine the total number of AI counted in each diameter category. Record.

iii) Calculated the total Islet Equivalents (IEQ) for each category by multiplying the total number of AI by its appropriate conversion factor below:

Islet Diameter Range (µm)	IEQ Conversion Factor
50 – 100	0.167
101 – 150	0.648
151 – 200	1.685
201 – 250	3.5
251 – 300	6.315
301 – 350	10.352
>350	15.833

Note: If IEQ counts are more than 10% different, a third sample should be taken, counted, and an average of three should be calculated as final.

iv) Calculate the dilution sample of the sample taken using the following equation:
Total Volume of Preparation (mL) / Sample Volume (mL) = Dilution Factor
Note: For this protocol: 100 mL / 0.1 mL = 1000 Dilution Factor (or 500 when counting <100,000 IEQs and taking a 0.2 mL sample.)

v) Record and calculate Total IA and IEQ and record on IIDP Calculation Sheets. (*Attachment 1-printed version or Attachment 3-Excel file from SOP*.)

vi) Islet Index (II) designates the overall size distribution of the islets in the preparation and is calculated by dividing the Total IEQ by the Total IA.

vii) Calculate the percent purity of the islets to the nearest 5% by estimating the portion of red stained islets to all tissue (islets, acinar, ductal cells). Record on Worksheet.

viii) Islet Quality Grade is determined by ranking the overall islet preparation using the 5 parameters listed below and ranking each between 0-2 (*with 0 being the worst and 2 being the best for each parameter*) and adding the total number of points. The final Total Quality Grade will be between 0 and 10. (*10 being the best able to be achieved*)

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

b) Sampling for Distribution

i) Use similar protocol for mixing and sampling as stated above to take samples for distribution. Mix preparation before each distribution sample to ensure consistent and proper distribution of islets.

2) Attachments:

a) QA 001 Attachment 1 v4 – Printed Counting Sheet
b) QA 001 Attachment 2 – Islet Quality Ranking Guide
c) QA 001 Attachment 3 v2 - Excel File Counting Sheet
d) QA 010 Attachment 4 – Workshop Summary Table

3) References:

a) Ricordi, C., Gray, D.W.R., Hering, B.J. *et al.* Islet isolation assessment in man and large animals. *Acta diabet. lat* **27,** 185–195 (1990). https://doi.org/10.1007/BF02581331

b) Kin T: Islet Isolation for Clinical Transplantation. The Islets of Langerhans Advances in Experimental Medicine and Biology. Dordrecht: Springer: 2010. pp. 683-710.

c) Integrated Islet Distribution Program. Qualitative & Quantitative Assessment of Human Islets for Distribution Using Dithizone (DTZ) V4. Sept 2020 2020 June. Available at: https://www.protocols.io/edit/qualitative-quantitative-assessment-of-human-islet-bk5vky66.

d) Committee NCCCMCM, Consortium NC. Purified Human Pancreatic Islet: Qualitative and Quantitative Assessment of Islets Using Dithizone (DTZ): Standard Operating Procedure of the NIH Clinical Islet Transplantation Consortium. CellR4 Repair Replace Regen Reprogram 2015; 3.