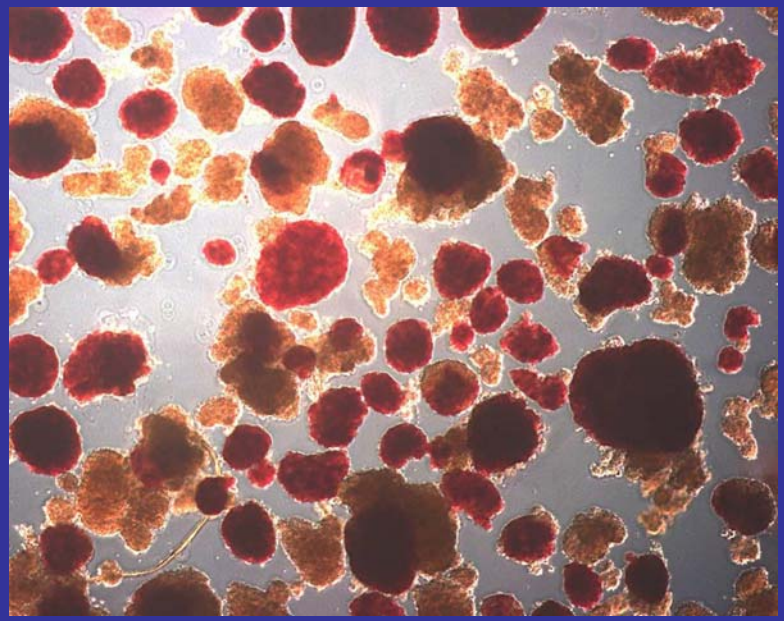
Islet Cell Resource Center (ICR) Consortium
4th Annual Islet Workshop

UW Extended Islet Quality Control Assessment



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Department of Surgery
Division of Transplantation
University of Wisconsin

FDA Product Release Requirements

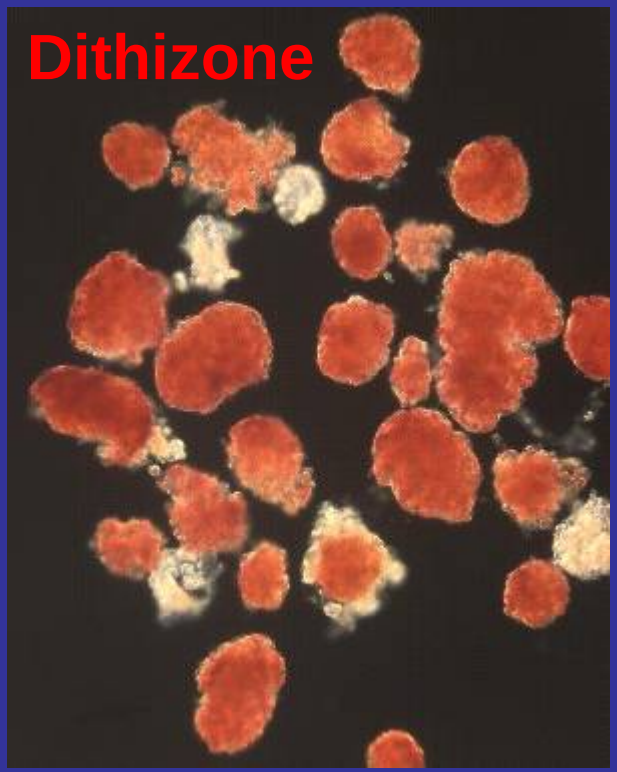
(21 CFR 610)

Manufacturing Safety

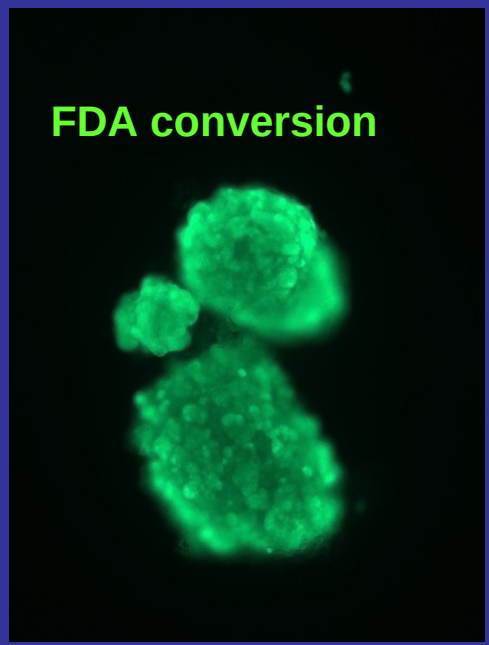
- Sterility (Gram stain negative)
- Endotoxin Free (<5 EU/kg/hr)
- Quantity (> 4000 IEQ/kg)
- Viability (≥ 80%)
- Purity (≥ 50%)
- Potency (Insulin secretion index > 1.2)

Is Islet Viability Assessment an Art or a Science?

Identity and Morphology



Viability



Necrosis

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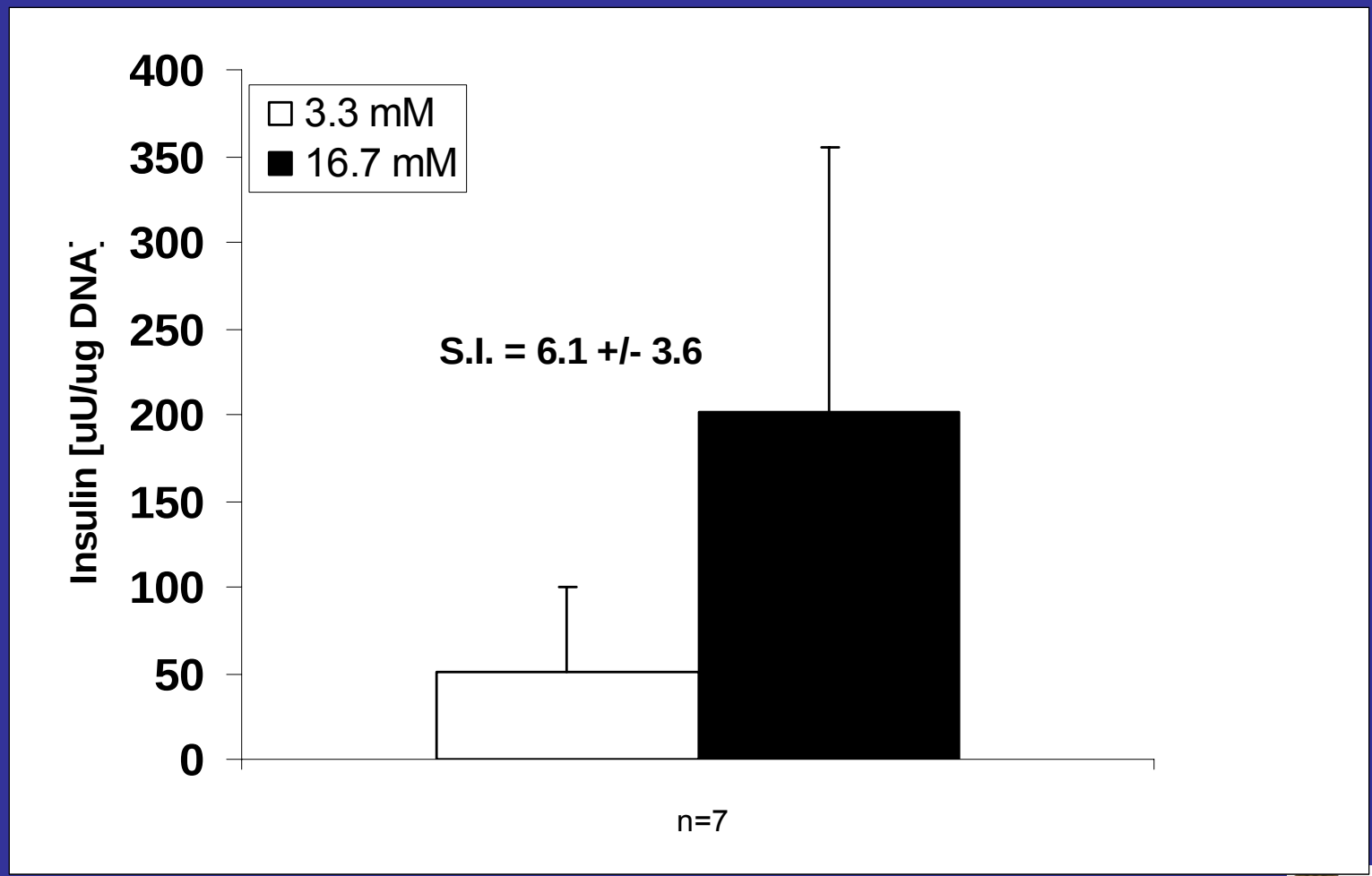
Islet Functional Potency Assessment by In Vitro Glucose Stimulated Insulin Secretion



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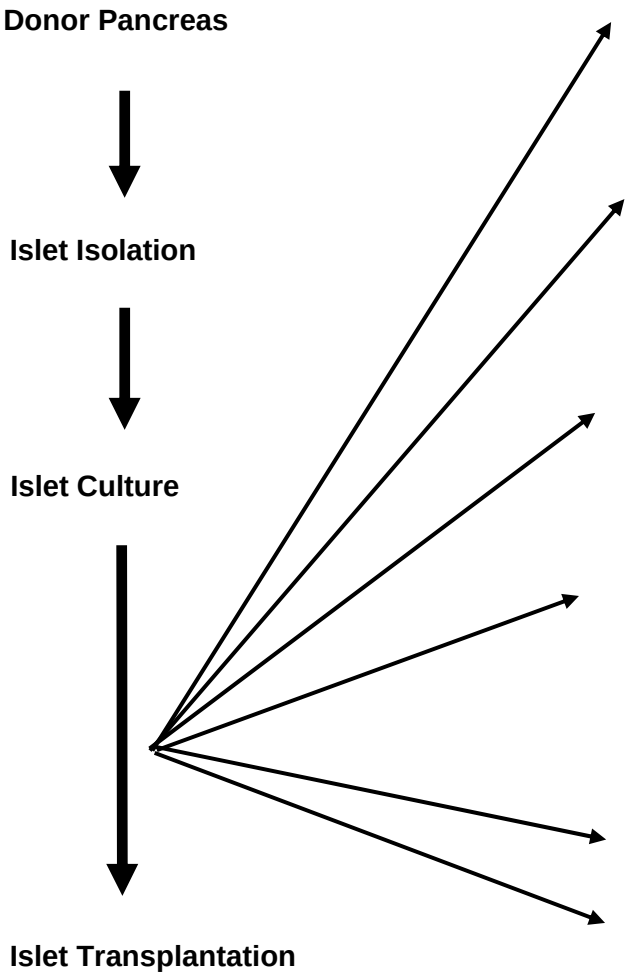
Objectives

Develop new methods of rapidly and accurately assessing islet:

- Viability
- Apoptosis
- Oxidative Stress
- Functional potency in response to glucose

Ultimate goal is to reduce the rate of primary graft non-function and diminished long term function.

UW-Madison Islet Transplantation
Comprehensive Quality Control Assessment Program



Quantification of Islet Yield and Purity
COPAS large particle flow cytometry

Cellular Integrity and Apoptosis
Flow Cytometry: ToPro3, Annexin V, VADFMK, JC-1, DHE
Immunohistochemistry: Active Caspase 3

Glucose Responsiveness
Flow Cytometry: Glucose induced metabolic flux (pyridine and flavin nucleotide reduction, intracellular calcium flux)
Static Incubation: Glucose stimulated insulin secretion

Basal Energy Status
HPLC: Quantification of adenine nucleotides

Oxidative Stress
Luminol-enhanced Chemiluminescence: ROS
Colorometric Assays: Lipidperoxidation (Malondialdehyde, MDA) and Glutathione-Peroxidase Activity (GPx)

Activation of Stress Pathways
Western blotting: Cell signaling (ERK, p38, MAP kinase)
QRT-PCR: Gene expression (c-FOS, HIF1a, HMOX1, VEGF, GLUT2, INS, ISL1, GCK, IPF1)

Long Term Function
Kidney capsule transplant: Reversal of diabetes in NOD.scid mice
Clinical outcome in human transplant patients

Single Cell Flow Cytometry Analysis of Dispersed Pancreatic Islets

- Objectives of the UW-Madison Protocol
  - High sensitivity
  - Adequate specificity
  - Rapid (<3 hrs) – Completion on the day of transplant
  - Reliable and repeatable
  - Translatable (cell line → rodent → human islets)
  - Practical (Don't need a Ph.D. to do it!)
  - Cheap!

UW Islet Dispersion and Staining Protocol for Flow Cytometry Analysis

I. Islet Dispersion (2000 – 5000 IEQ)

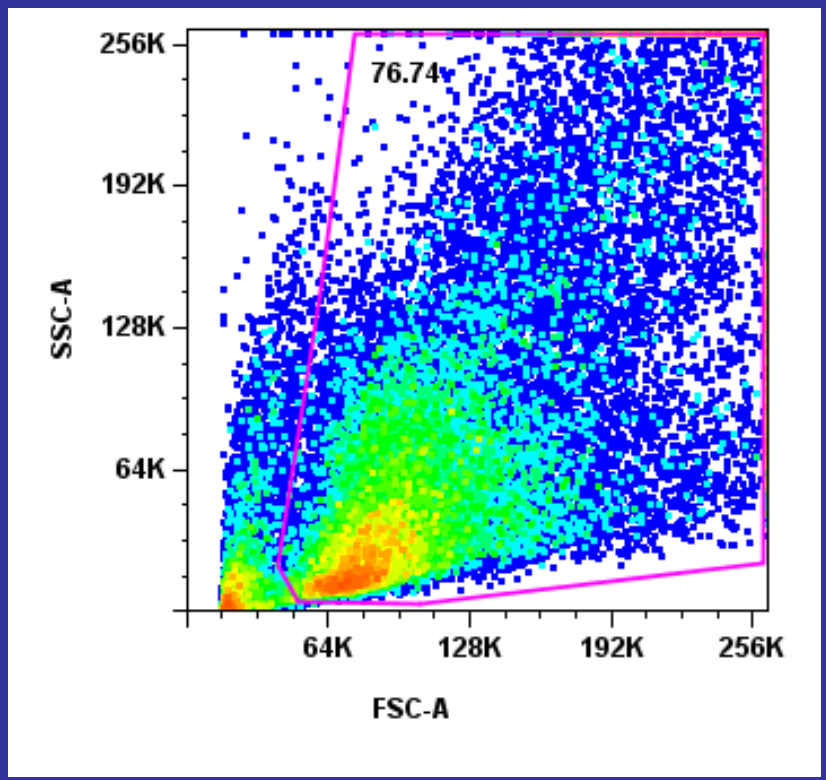
a. Pellet by centrifugation (1000 rpm, 1 min., no brake, room temp.)
b. Remove media, add 4.5 mL Trypsin [0.05%], EDTA [0.53 mM] in HBSS, incubate at 37°C for 4 min. with gentle agitation.
c. Pipet through a p1000 and then a p200 tip.
d. Add 5 ml of culture media containing 10% FBS.
e. Pipet suspension through a 40 μm filter.
f. Pellet by centrifugation (1000 rpm, 5 min., with brake, room temp.)
g. Wash 1x with islet staining buffer (MKRB, pH 7.4, containing 3.3 mM glucose).

II. Islet Staining (100,000 – 500,000 cells/tube)

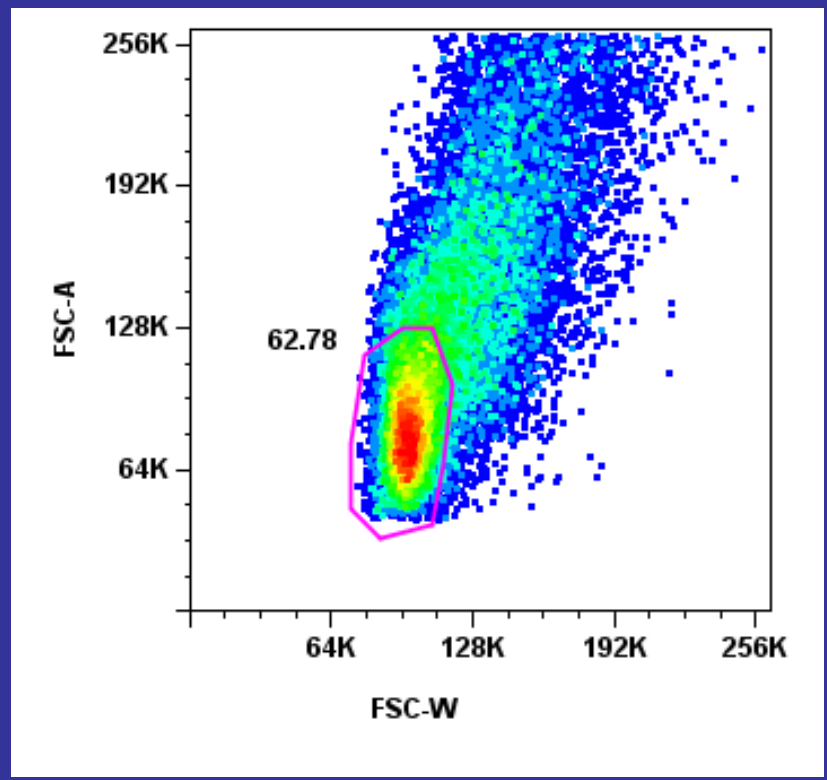
a. Suspend islet cells in pre-warmed islet staining buffer.
b. Add fluorescent probes.
c. Incubate at 37°C for 25 – 30 minutes.
d. Wash 2x with islet staining buffer.
e. Hold on ice prior to analysis.

Flow Cytometric Analysis of Dispersed Islets

Exclusion gating for debris



Inclusion Gating for Singlet Cells



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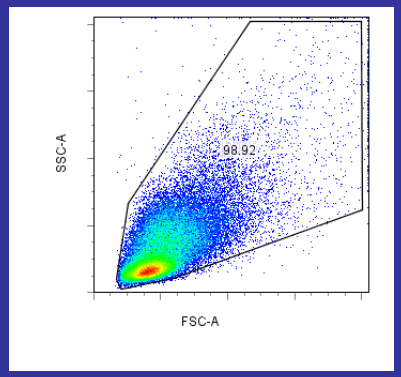
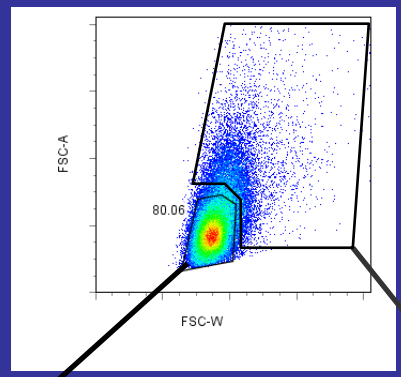
Phenotypic Analysis of Flow Sorted Human Islet Cells



Debris Gate 1



Singlet vs Aggregate Gating

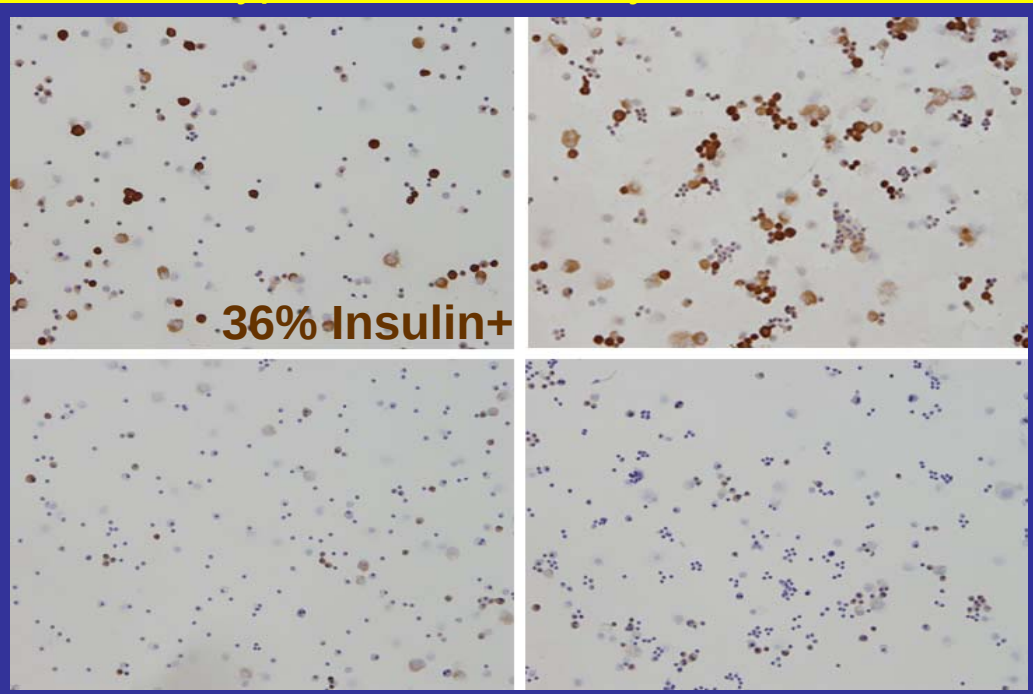
Singlets

Aggregates

Islet Cell Phenotype Assessment by Immunohistochemistry

Anti-Insulin + Hemotoxylin

2º Alone Control + Hemotoxylin



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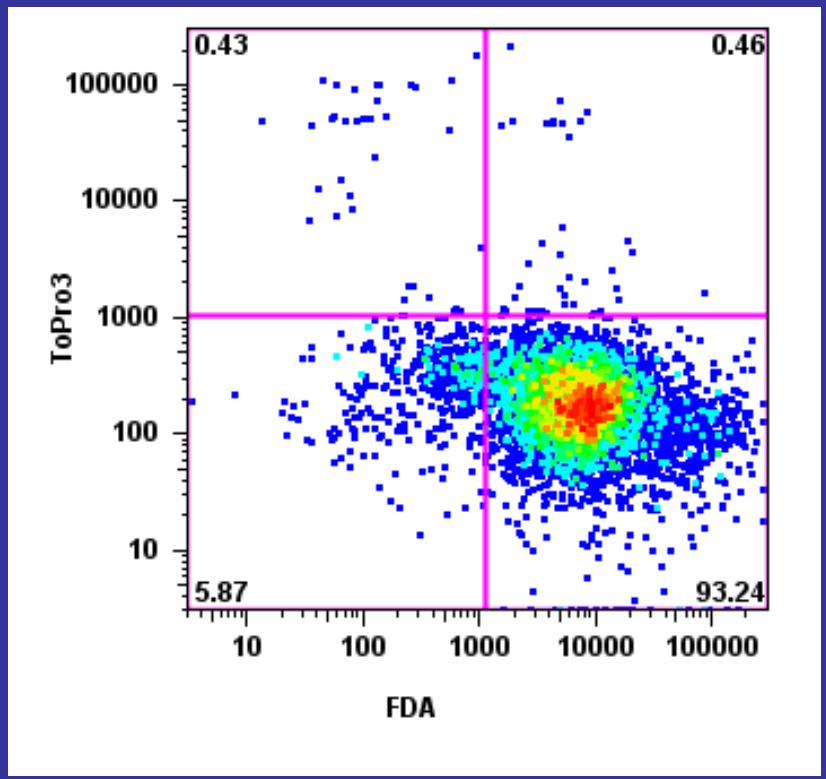
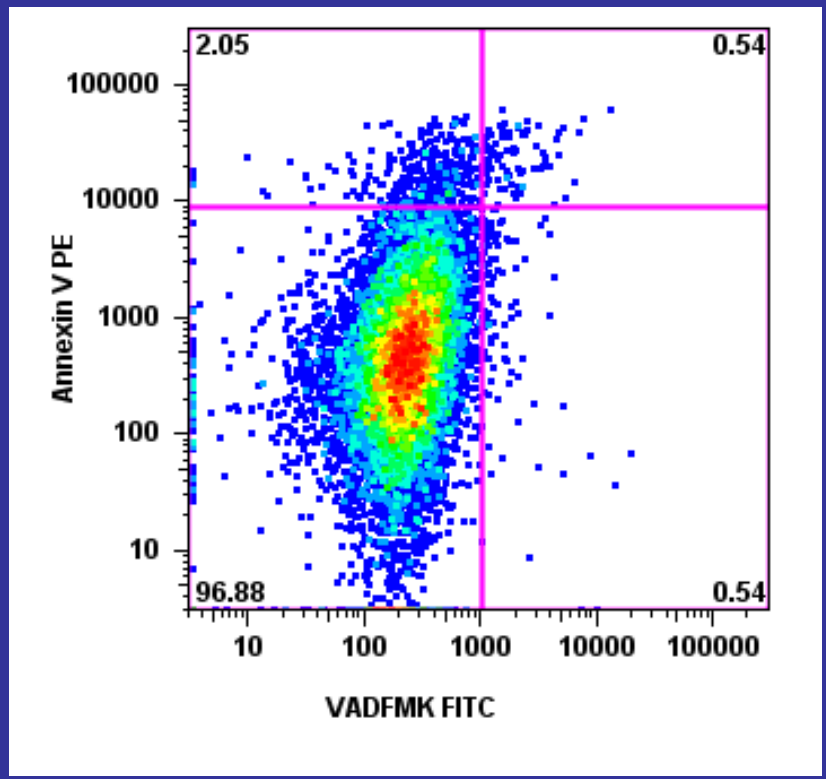
Viability



Apoptosis



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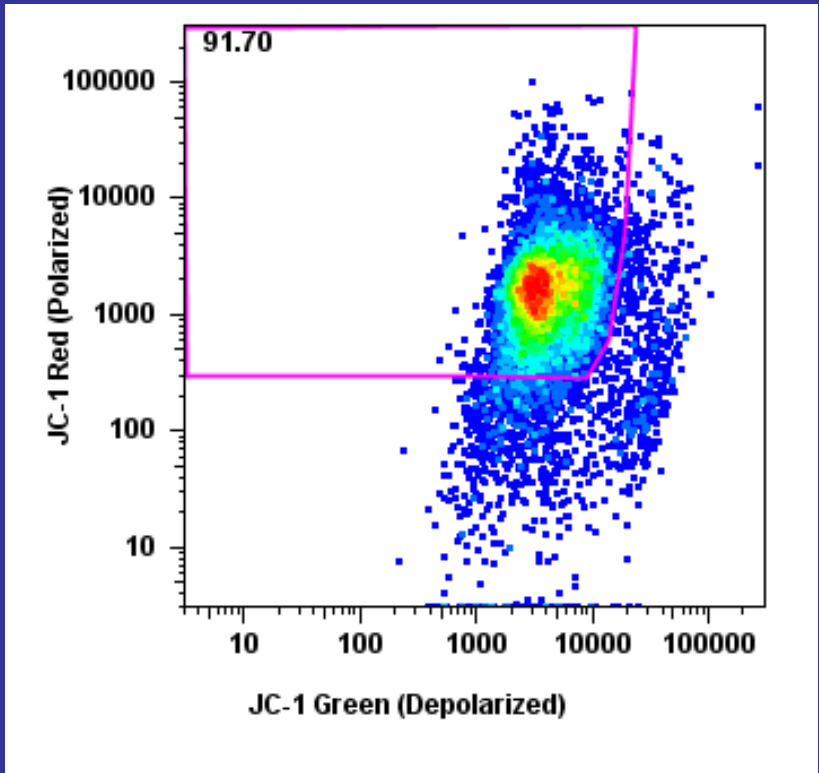
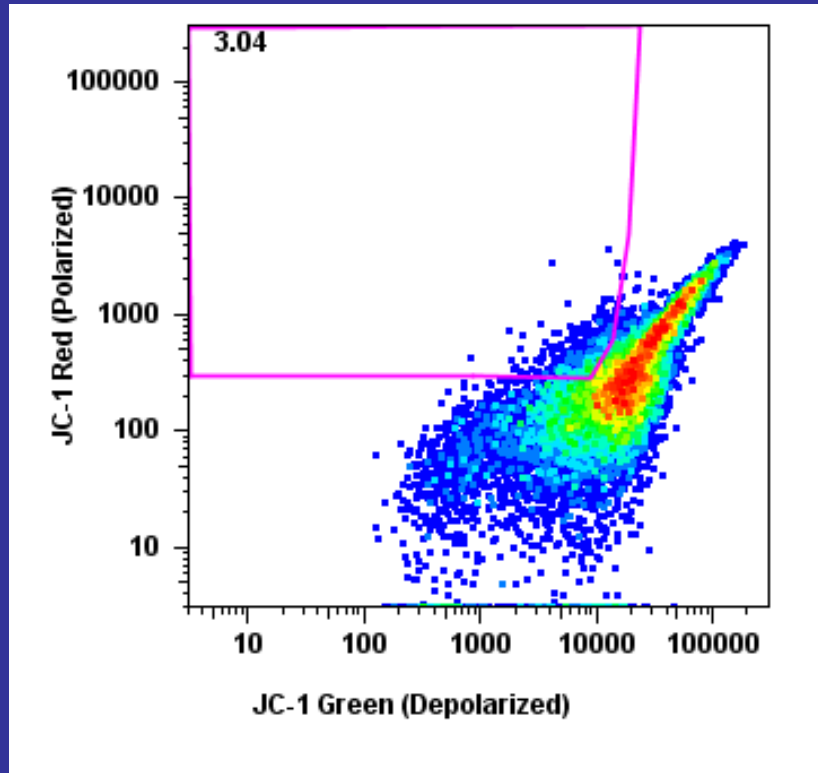
MMP^+ = JC-1 Red > Green



CCCP Treated Control



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UW Protocol for Islet Adenine and Pyridine Nucleotide Analysis by HPLC

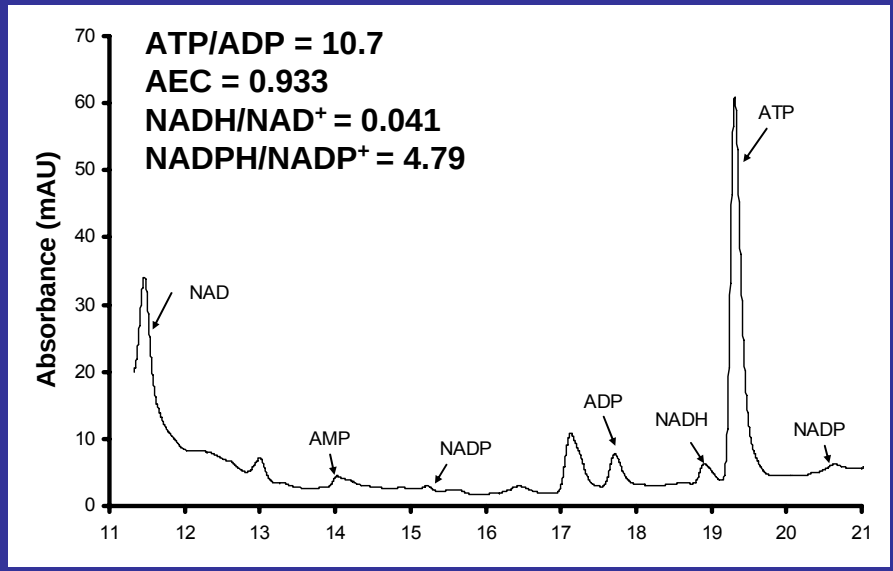
I. Islet Extract Preparation (1500 IEQ/assay) – Part I

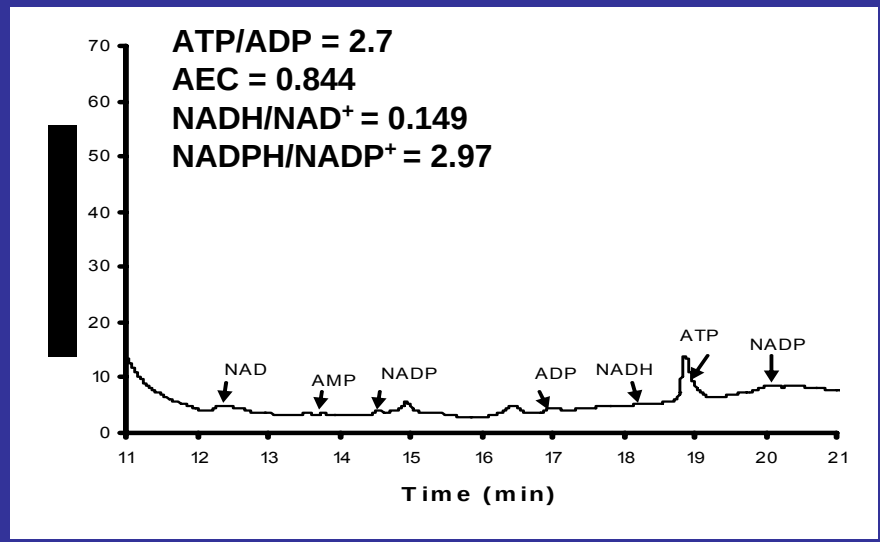
a. ATP samples (3x 500 IEQ each).
b. Centrifuge islets for 1 minute at 1000 rpm without braking in a swinging bucket centrifuge. Discard supernatant by pipetting (do not decant).
c. Resuspend islets in 6 mL of ice cold PBS + 66 mM EDTA.
d. Aliquot 1mL each into 3 microfuge tubes.
e. Pulse spin islets in a microcentrifuge to gently pellet the islets. Remove PBS + 66 mM EDTA solution by pipetting.
f. Add 300 µL ice cold PBS + 66 mM EDTA per tube.
g. Add 1 mL cold Phenol Chloroform Isoamyl Alcohol [34:24:1] to each tube.
h. Vortex tubes until no intact cells are visible (approx 30 seconds each).
i. Snap freeze in liquid nitrogen.
j. Store at -80°C or proceed immediately to Part II

• Islet Extract Preparation – Part II

a. Centrifuge for 5 min at 14,000g and 4ºC to separate phases.
b. Remove upper aqueous phase (~250 - 300uL) and place into a new tube.
c. *OPTIONAL: If a clear aqueous layer is not apparent, the sample can be divided into two equal volumes of 650 ul add 650 ul of PCI to each, vortex and centrifuge again to clear the aqueous of contaminating protein and lipid.*
d. Add 500uL ice cold water saturated diethyl ether.
e. Vortex on high for 30 sec.
f. Centrifuge for 5 min at 14,000g to separate phases.
g. Remove **LOWER** aqueous phase (~250 - 300uL) and repeat steps d – f.
h. Remove **LOWER** aqueous phase (~250 - 300uL) and place into a Millipore Microcon YM-100 filter tube.
i. Centrifuge at 1000xg for 30 min.
j. Discard the retentate and save the flow-through which should represent ~250 ul.
k. Run the flow-through immediately on HPLC.

Liquid Chromatography: A modified reverse phase HPLC method according to Noack et al. with an ion pairing reagent according to Childs et al. was used for nucleotide separations. A HP1100 series quad pump HPLC with a variable wavelength UV detector set to 254nm and an inline degasser were used. Discovery C-18 (250x4.6mm, 5µm, Sigma-Supelco, St. Louis, MO) and LC-18T (150x4.6mm, 3µm, Sigma-Supelco, St. Louis, MO) columns were used and kept at 25°C during the run. Analysis of the chromatograms was performed using HP Chemstation computer software. The flow rate was set to 1mL/min throughout the run.
Mobile phase A: 0.1M KH_2PO_4 with 4mM tetrabutylammonium hydrogen sulfate, pH 6.0 with a ramp gradient to B: A:methanol (70:30), pH 7.2 according to the following A/B timetable (T0 100/0, T2.5 100/0, T7 70/30, T12 40/60, T16 0/100, T24 0/100, and T25 100/0). Peaks were identified by co-elution with known chemical standards.





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UW Protocol for Islet Static Incubation Assessment of Glucose Stimulated Insulin Secretion

I. Islets were handpicked into oxygen (95%O2/5%CO_2) saturated basal Krebs-Ringer Bicarbonate Buffer.
II. Incubation at 37°C for 30 min. in a 5%CO_2/95% air incubator.
III. Groups of 8 – 10 islets from the equilibration cultures were then transferred to fresh oxygen saturated KRB containing either 3.3 mM or 16.7 mM glucose and incubated an additional 60 min. in a 37°C water bath with gentle shaking.
IV. Secreted insulin in the media was measured by ELISA and values normalized to extracted islet DNA.
V. Data are shown as a stimulation index (mean of secreted insulin from quintuplicate samples incubated with 16.7 mM/3.3 mM).

Summary Islet Quality Assessments Segregated by in vivo Potency

Donor Parameters	Potent (n=16)	Poor Functioning (n=18)	p value*
Age (yrs)	39.9 ± 11.2	47.4 ± 11.4	0.058
Gender (M/F)	9/7	12/6	.50#
BMI	32.8 ± 4.3	30.7 ± 5.0	0.20
Cold Ischemia Time (h:m)	6:49 ± 2:37	8:00 ± 3:10	0.24
Islet Quality Assessments			
Yield [IEQ/g pancreas]	7354 ± 4511	4486 ± 2304	0.026
% Purity (DTZ microscopy)	84.5 ± 8.5	86.2 ± 5.4	0.47
% Viability (Fluorescent Microscopy)	90.6 ± 4.0	91.6 ± 7.1	0.67
S.I. (GSIS)	5.0 ± 3.0	3.1 ± 1.7	0.024

Data shown are the Mean ± SD.
*P values were calculated by two-tailed Student's T test or #Fisher's exact test.

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UW Extended QC	Potent (n=16)	Poor Functioning (n=18)	p value*
% Viability (Flow cytometry)	89.5 ± 5.7	82.7 ± 10.1	**0.029**
% Apoptosis (Flow cytometry)	9.2 ± 6.5	11.1 ± 7.2	0.44
% Polarized Mitochondria (Flow cytometry)	85.2 ± 5.8	68.4 ± 11.4	**<0.0001**
ATP/ADP (HPLC)	9.1 ± 2.1	7.2 ± 2.3	**0.040**
AEC (HPLC)	0.927 ± 0.019	0.885 ± 0.082	0.29
Islet In Vivo Function			
Kidney capsule TX [1000 IEQ Dose] STZ-diabetic NOD.scid mouse	10/10 6/6 (human recipients)	0/18	<0.05#

Data shown are the Mean ± SD.
*P values were calculated by two-tailed Student's T test or #Fisher's exact test.

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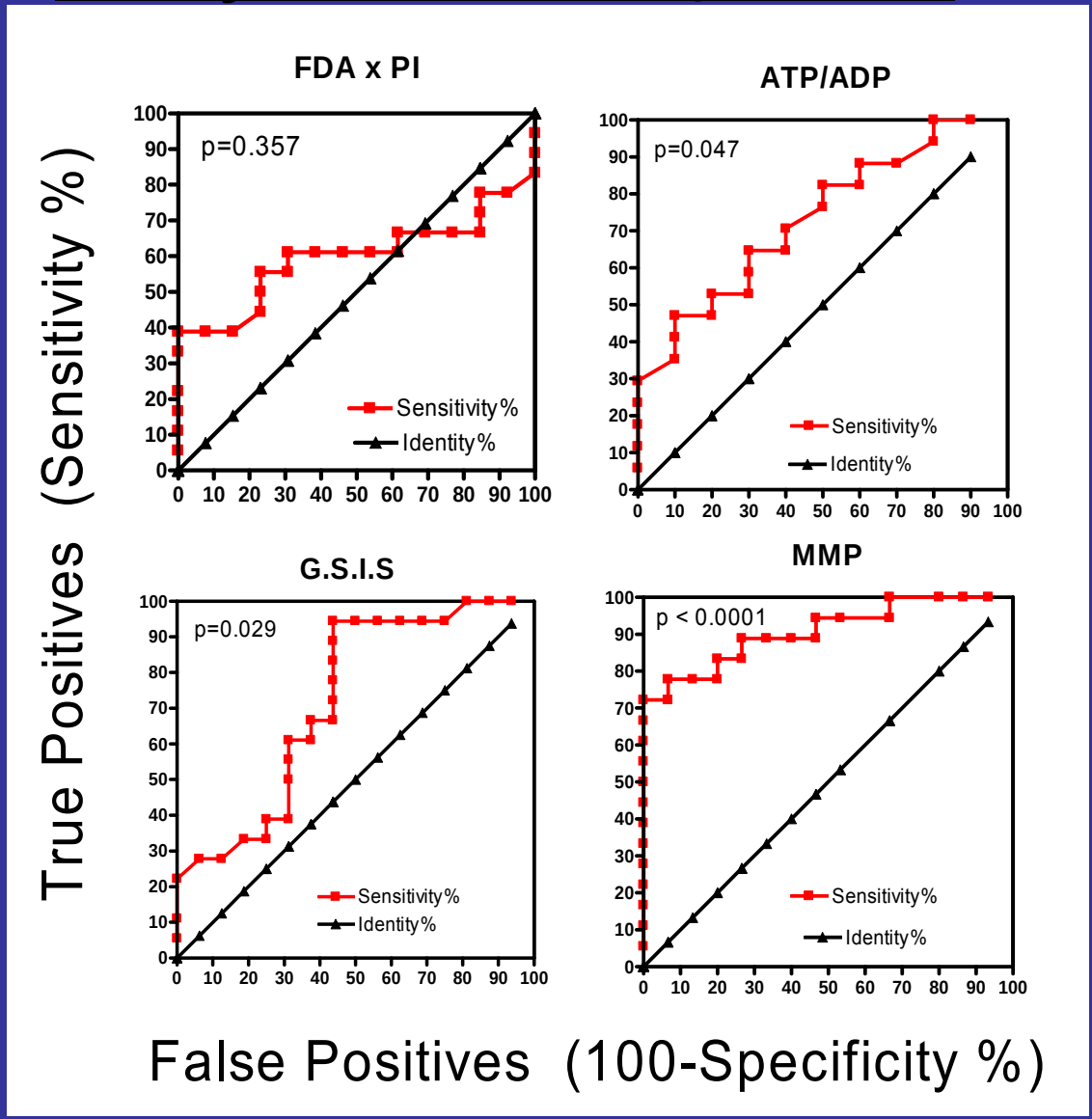
Receiver Operator Characteristic Curve Analysis of Islet QC Data



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Determination of a Single Islet Quality Score

Islet Quality Score[#] =

% MMP[+] (0.100) + GSIS S.I. (0.231) + ATP/ADP ratio (0.188) – 10.0

	Potent (n=16)	Poor Functioning (n=18)
Mean ± SD	1.27 ± 0.89	-1.10 ± 1.105

[#] Islet quality score derived using multivariate discriminant analysis (Statistica software).

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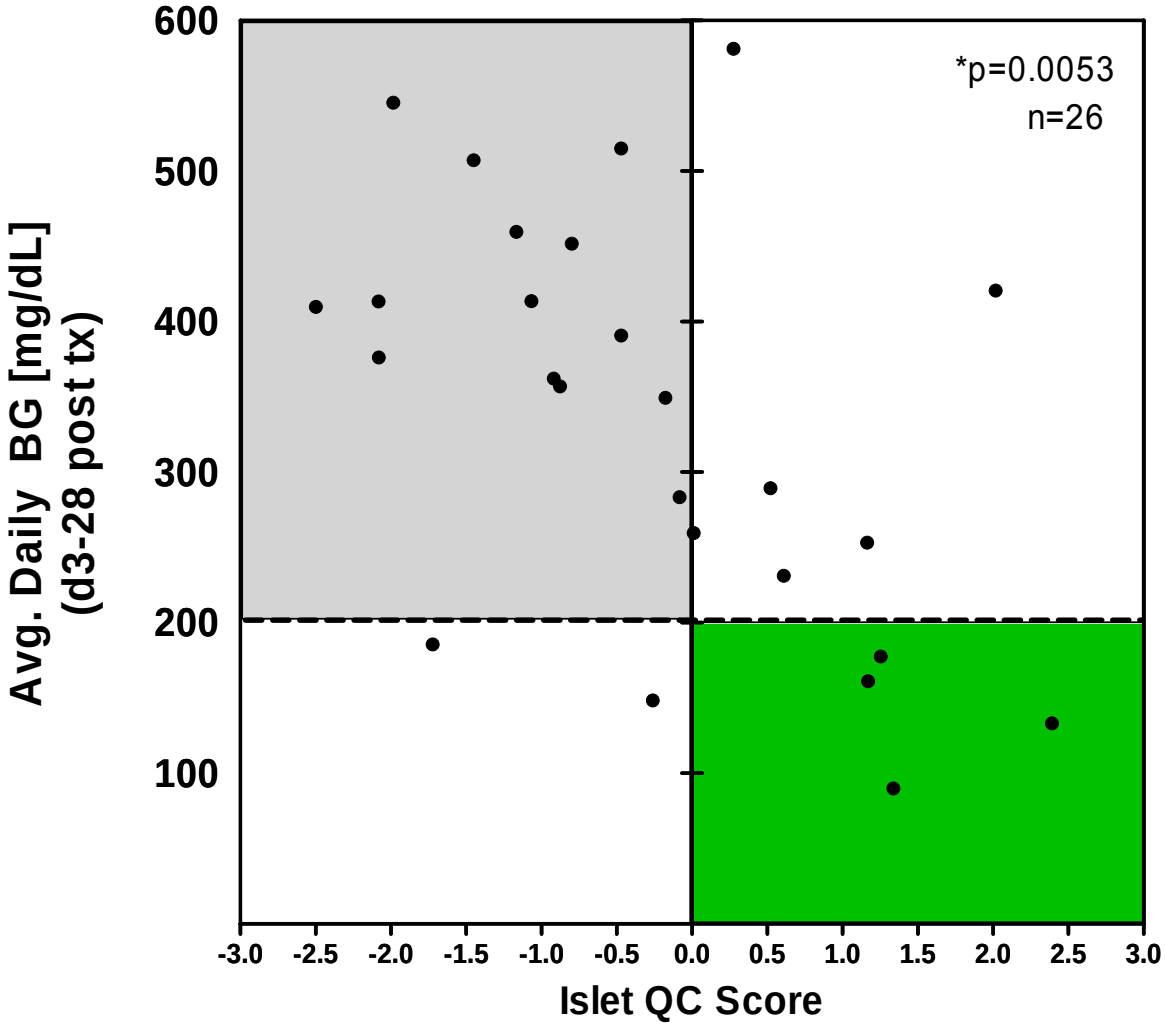
Islet QC Scores Correlate with In Vivo Function in STZ-Induced Diabetic NOD.scid Mice



* p value determined by Spearman's rank correlation

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Islet Preparation Classification Results Using the Canonical Q.C. Scores

	Potent (n=16)	Poor Functioning (n=18)	Overall Success Rate of Classification
3 Variable Model (MMP, GSIS S.I., ATP/ADP)	13/16 (81.3%)	16/18 (88.9%)	85.1%
2 Variable Model (MMP + GSIS S.I. or MMP + ATP/ADP)	14/16 (87.5%)	14/18 (77.8%)	82.4%
2 Variable Model (GSIS S.I. and ATP/ADP)	11/16 (68.8%)	15/18 (83.3%)	76.5%
1 Variable Model (MMP)	14/16 (87.5%)	14/18 (77.8%)	82.4%
1 Variable Model (GSIS S.I.)	9/16 (56.3%)	15/18 (83.3 %)	70.6%
1 Variable Model (ATP/ADP)	5/16 (31.3%)	14/18 (77.8%)	55.9%

Islet Infusion Quantity and Quality Assessment and Human Subject Outcomes

Human Subject	Islet Infusion No.	Islet Infusion Mass [IEQ/kg]	Islet QC Score	Post-Infusion Insulin Requirements [U/kg/day]		
				Pre-last Infusion	3 Months	Reduction (Δ)
1	1 2 (+ 7 mos)	12,786 15,297	ND 2.45	0.62 0.17	0.43 0	0.19 0.17
2	1 2 (+ 4 mos)	6849 16,337	ND 2.00	0.65 0.36	0.36 0	0.29 0.36
3	1 2 (+1 mos) 3 (+ 6 mos)	10,712 11,116 6467	1.37 0.083 ND	0.88 0.15	 0.15	 0
4	1 2 (+ 1 mos)	8654 17,013	-0.077 1.34	0.49 0.26	 0	 0.26
5	1 2 (+11 mos) 3 (+ 12 mos)	7318 12,547 10,791	ND ND 0.55	0.96 0.59 0.48	0.53 0.42 0.18	0.43 0.17 0.30
6	1 2 (+ 12 mos)	9898 7419	2.94 1.17	0.61 0.21	0.06 0	0.55 0.21
Mean ± SD		**11,214 ± 3491**	**1.33 ± 1.00**	**0.44 ± 0.26**	**0.19 ± 0.20**	**0.27 ± 0.15**

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UW-Madison Supplemental Islet Preparation Release Criteria

Standard Islet Release Criteria	
IEQ Dose/patient kg b.w.	≥ 4000
Viability (Fl. Micro.)	≥ 80 %
Purity (Micro.)	≥ 50 %
Endotoxin (LAL)	≤ 5 EU/kg b.w./hr
Gram stain	Negative

+

UW Extended Islet Quality Assessments	
Viability (Flow)	≥ 80%
Apoptosis (Flow)	≤ 10%
Mitochondrial Membrane (Flow)	≥ 80%
ATP/ADP (HPLC)	≥ 8
GSIS S.I. (16.7 mM/3.3 mM)	≥ 3.0
ISLET QC SCORE	> 0

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Conclusions

- **A critical balance must be found between assay sensitivity, specificity and practicality.**
- **Assessment of islet cell metabolic state (MMP, ATP, GSIS) provides data predictive of in vivo potency.**
- **A multi-parametric quality assessment model increases the likelihood of detecting poor quality islet preparations.**
- **Assay validation is a long-term ongoing process that depends on animal model and human clinical data.**

UW-Madison
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JDRF Juvenile Diabetes Research Foundation International
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