Islet Assessment for Transplantation

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Abstract

Purpose of review—There is a critical need for meaningful viability and potency assays that characterize islet preparations for release prior to clinical islet cell transplantation (ICT). Development, testing, and validation of such assays have been the subject of intense investigation for the past decade. These efforts are reviewed, highlighting the most recent results while focusing on the most promising assays.

Recent Findings—Assays based on membrane integrity do not reflect true viability when applied to either intact islets or dispersed islet cells. Assays requiring disaggregation of intact islets into individual cells for assessment introduce additional problems of cell damage and loss. Assays evaluating mitochondrial function, specifically mitochondrial membrane potential, bioenergetic status, and cellular oxygen consumption rate (OCR), especially when conducted with intact islets, appear most promising in evaluating their quality prior to ICT. Prospective, quantitative assays based on measurements of OCR with intact islets have been developed, validated and their results correlated with transplant outcomes in the diabetic nude mouse bioassay.

Conclusion—More sensitive and reliable islet viability and potency tests have been recently developed and tested. Those evaluating mitochondrial function are most promising, correlate with transplant outcomes in mice, and are currently being evaluated in the clinical setting.

Keywords
Oxygen consumption rate; viability; potency; release criteria; marginal mass; purity

Introduction

Islet cell transplantation (ICT) is emerging as a promising approach for the treatment of selected patients with type 1 diabetes mellitus [1–8]. ICT is currently in a phase III multicenter clinical trial [9] to determine if it will become the standard of care. There is an urgent need for reliable assays that characterize the islet product for release prior to transplantation. Development of such assays is mandated by the Federal Drug
Administration (FDA) and has been the subject of investigation for the past decade. To provide a framework for understanding the current state of the art, this article first reviews the numerous approaches that have been proposed and tested, than focuses on the most recent results and promising assays for clinical ICT.

**Current specifications for lot release prior to clinical islet transplantation**

The FDA mandates that for any cellular and tissue-based product, the manufacturer must be able to demonstrate that it can be safely and reproducibly manufactured [10]. This is generally done by characterizing the product and establishing specifications for product release. Lot release specifications for islet products include demonstration of safety (i.e., sterility, mycoplasma, pyrogenicity/endotoxin, and freedom from adventitious agents) and assessments of several key product characteristics that include, but are not limited to, identity, purity, viability, and potency. The current specifications for release of islet products within the United States are summarized in Table 1. These specifications function to exclude preparations that are contaminated, highly impure, grossly damaged, or do not contain significant numbers of islets. It is currently accepted that these specifications provide reasonable estimates of islet safety, identity, and purity, but do not provide meaningful measures of viability or potency of the preparation [12–15]. Therefore the establishment and validation of useful islet viability and potency tests is urgently needed. The sections that follow focus on existing and emerging islet viability and potency tests, including those that are based on measurements of oxygen consumption rate (OCR), which appear to be the most promising.

**Limitations of the tests currently used for islet lot release prior to clinical transplantation**

Many of the methods currently used to assess islet preparations were developed nearly 20 years ago [11]. The advantages and limitations of tests currently used for islet product release, which were recently discussed in detail [16], are summarized in Table 2A.

**Sampling from an islet suspension**

An important issue in characterizing islet preparations, relevant to all assays, is sampling. Obtaining a sample from a suspension that is representative of the whole preparation is critical [11,16]. Nevertheless, maintaining a homogeneous islet suspension while sampling is challenging, as islets settle rapidly. Differences in size and density of aggregates can lead to significant differences in settling velocity and exacerbate this problem. The extent of the systematic error introduced during this type of sampling is unknown. To minimize random error during sampling, multiple replicates should be collected. However, the additional time and analysis required for collection of multiple samples, the concern about introducing contamination and removing islets that otherwise could be transplanted to benefit the recipient all pose limitations. Currently, only duplicate samples of 100 µL (derived from a 100-mL islet suspension) are collected and counted, an amount that may not represent the entirety of the preparation.

**Measurements of the amount of islet tissue**

Quantification of the total amount of islets in an islet preparation is critical because it ultimately determines the islet dose that is transplanted. The method most widely used currently is manual, visual counting of islet equivalents (IE) under a light microscope following dithizone (DTZ) staining to determine the total volume of islet tissue and its purity. This method has advantages and limitations (See Table 2A) that are described in detail elsewhere [11,16]. Methods for estimating the total number of cells or volume of
tissue in a preparation include measurements of intracellular deoxyribonucleic acid (DNA), cellular nuclei counts, large particle flow cytometry [17], and packed tissue volume. These methods do not provide islet- or β-cell specific information, so they require an independent estimate of the purity (fractional volume of islet tissue or β-cells). Such estimates can be obtained using a variety of methods [16], including morphological analysis with electron and/or light microscopy [18*], immunohistochemistry with laser scanning confocal microscopy, or laser scanning cytometry [19]. Recent studies indicate that conventional DTZ staining overestimates purity by 20–30% as compared to measurements with electron and light microscopy [20] and total number of IE by as much as 90% [18*] as compared to recently-developed, more accurate methods that combine nuclei counting with light microscopy.

**Measurements of viability**

The current viability assay used for clinical islet product release is based on assessing membrane integrity with fluorescein diacetate/propidium iodide (FDA/PI) (See Table 1). Characteristics and limitations of this assay are outlined in Table 2A and detailed elsewhere [11,16]. A major limitation of this assay is that it does not reflect true viability because it may not account for cells undergoing early apoptosis or dying by other modes of cell death, during which cells have not yet developed damage to their cell membrane. Furthermore, it does not correlate with the diabetic nude mouse bioassay (NMB) or clinical ICT outcomes.

**Measurement of islet function (potency)**

The β-cells within the islets have a specific, dedicated function, the dynamic release of insulin in response to a glucose stimulus. Therefore, one would expect that assessment of islet function should be straightforward, particularly if the insulin secretion rate of a preparation can be easily measured. Measurements of basal and glucose-stimulated insulin secretion (GSIS) could theoretically be used to provide a meaningful measure of the amount of viable and functional IE (or β-cells) in a preparation if one assumes that insulin secretion from an islet population is relatively constant when normalized on a per viable IE or per viable β-cell basis. Unfortunately, GSIS does not correlate with clinical transplant outcomes [14–15,21]. There are several likely reasons for this persistent finding. Stresses associated with pancreas preservation, islet isolation, and islet purification may lead to extensive degranulation and/or insulin leakage (from dead or dying islet cells). Conceivably, islets that do not secrete insulin at expected rates, but are nonetheless viable, may recover when transplanted into the recipient. In other words, low GSIS may not necessarily imply irreversibly impaired secretory function and, thus, GSIS does not correlate with clinical outcomes. Furthermore, insulin leakage from dead or damaged cells may be difficult to account for (because this contribution to the total insulin cannot be reliably estimated), may interfere with proper calculation of insulin secretion rate and stimulation index, and may complicate the interpretation of the results of the GSIS assay. Insulin secretion is also particularly sensitive to the local partial pressure of oxygen (pO₂) and assay procedures usually do not account for that [22].

**The mouse bioassay as an in vivo islet potency test and a surrogate islet potency validation tool**

According to the FDA [10], a suitable potency assay is one that demonstrates that the clinical product possesses the specific ability to provide the desired clinical effect. The diabetes reversal (DR) resulting from islets engrafted under the kidney capsule of immunodeficient nude mice correlates with clinical transplant outcomes and is currently accepted as the gold standard for testing islet potency [14–15,23–24]. However, the time (days to weeks) required for this assay to produce interpretable results renders it
retrospective. Nonetheless, correlation of real-time, in vitro tests with transplant outcomes in the NMB can establish other such tests as acceptable surrogate potency tests. Several recently-proposed islet potency tests are therefore being judged based on their ability to predict DR in the NMB [19,25–36*,**]. Even though the NMB is the premiere method available to researchers for the assessment of islet potency, it suffers from numerous limitations (See Table 2B). These limitations include the length of time required to obtain a meaningful outcome, the complexity of the surgical procedure, the difficulties in maintaining diabetic mice and timing diabetes induction with the unpredictable availability of human islets, the negative impact of impurities on outcome [15,37–39], the transplant site (kidney capsule), which may be more prone to the presence of impurities and/or dead tissue than the clinical transplant site (the liver), and the inability to account for immune rejection or the effects of immunosuppressive drugs that are present in the clinical setting. There have been recent attempts [40–41] to provide other in vivo islet potency tests that are alternatives to the diabetic NMB. These alternatives can potentially overcome some, but not all of the limitations of the NMB.

Desired characteristics of islet potency tests

The assays under consideration for use as potency tests for islet characterization prior to clinical transplantation should be reliable, cost-effective, operator-independent, reproducible, and transferable to other labs, work with relatively small (yet representative) islet numbers (100–500 IE), not require islet handpicking (which may bias the results), and should be able to provide real-time results (i.e., completed within hours). Given the heterogeneity of islet preparations and the intrinsic difficulties in characterizing them, assays that possess all of the desired characteristics may be very difficult to develop. This difficulty is reflected in the fact that, despite the intense effort dedicated to develop, implement, and validate a number of assays over the past decade, consensus behind any single assay has not yet been reached. Key viability and potency assays under consideration for the assessment of clinical islet preparations are described next.

Islet viability and potency tests under consideration

Table 2C summarizes some of the more recently explored assays used in islet quality assessment, highlighting their key strengths and identified weaknesses. Despite the landscape of flavors available to researchers, many of these assays are most valuable when used in the study of individual cells rather than cell aggregates.

Islets are three-dimensional, multi-cellular aggregates composed of several different cell types, including the β-, α-, δ- and PP-cells. Most assays used to assess cellular viability, apoptosis, or mitochondrial health, have been designed for suspensions or cultures of individual cells, not aggregates. Consequently, the development of techniques to study the quality of an islet preparation provides unique challenges. Because the diameter of an islet equivalent is 150 µm, it is necessary to consider mass transport limitations, particularly if an assay relies on the availability of molecular oxygen. The relatively large size of the islet makes fluorescence microscopy difficult, subjecting any such analysis to background signal and operator bias that is simply unique to the study of intact multi-cellular clusters. To circumvent some of the islet shape and size limitations, techniques have been developed to break apart the islets. Digestion with serine proteases and mechanical agitation may be used to dissociate islets into suspensions of their constituent cells, but these techniques result in significant damage to the cells and possibly death by anoikis [43–44], leading to the loss of as much as 50% of the original cell populations [45–47]. To minimize the problems associated with islet disaggregation, gentler formulations have been created and used [19]. Yet, it is unclear whether the negative effects of dissociating individual islet cells from one
another can be fully minimized. Furthermore, islet preparations have varying amounts of impurities, which complicates the use of any technique designed with the expectation that the studied tissue is comprised entirely of islets. Differentiating the non-endocrine tissue from the islets poses additional difficulties.

**Cell Membrane integrity tests**

These assays interrogate the integrity of the cellular plasma membrane and rely on differential staining using newer combinations of both cell membrane permeable and impermeable dyes [12,16], but have been unable to fully obviate the problems encountered with the current viability stains used prior to product release (i.e., FDA/PI). In fact, some of these proposed stains introduce new issues, such as islet toxicity [16]. 7-aminoactinomycin D (7-AAD, a membrane impermeable dye) has been used on disaggregated cells in combination with flow cytometry (FACS) to enable quantification of the fraction of cells that are viable by membrane integrity, but the method nonetheless requires the undesirable dissociation of the intact islets [16]. An alternative approach relies on sequential staining of membrane compromised cells within intact islets using 7-AAD. After initially staining with 7-AAD, the nuclei of the entire preparation are released from intact islets using a detergent and subsequently counted by hemacytometer or FACS [48–49]. The initial count (of non-viable cells) is divided by the second count (of total nuclei) to present a ratio equivalent to fractional viability (FV). This technique bypasses the limitations associated with islet disaggregation of multi-cellular spheroids, such as islets; however, as a membrane integrity test, it only accounts for dead cells with compromised cell membranes [16,49].

**Other cell death and mitochondrial assays**

Several assays attempt to characterize the degree of apoptosis within islet preparations [29*, 31**]. These assays may depend on the timing of the measurement as it relates to the onset of apoptosis. The magnitude and timing of the responses may also vary between cell types and the unique nature, intensity, and duration of encountered stresses [16]. Importantly, these cell death markers may not be reliable indicators of irreversible damage. Even though mechanistic information regarding the cell death process can be obtained, individual assays may not capture all dying cells and still suffer from limitations that are related to islet size and its three-dimensional structure. A recent report describes a method to study several apoptosis and cell death-related markers (including VADFMK, Annexin V, and Fura Red) simultaneously using FACS and shows that this sort of multi-parametric analysis may more reliably characterize the quality of an islet sample [29*]. Another paper [31**] describes an elegant approach to combine fluorescence imaging of mitochondrial membrane potential (MMP) and Ca$^{2+}$ leakage with measurements of insulin secretion, determined by enzyme-linked immunoabsorbent assay (ELISA). The system involved perfusing a microfluidic chip containing intact islets. The future of islet quality assessment may continue to leverage these types of multimodal techniques in the attempt to map a quality “fingerprint” of islet preparations prior to their consideration for transplantation.

Assays have also been developed to probe the state of mitochondrial health, which span a range of relevant indicators, through assessing the ability of a cell to reduce tetrazolium salts [16,33], to replenish ATP [27*,28,30*,42**], or to maintain MMP [16,19,31**]. Tetrazolium assays like MTT have fallen slightly out of favor because many variables or conditions, not limited to mitochondrial activity, can affect the ability of a preparation to reduce tetrazolium salts [16]. In contrast, tests that measure the relative abundance of high energy phosphates (or the ADP/ATP ratio) have reportedly shown promise in predicting ICT outcome in mice [27*,28]. However, the ADP/ATP ratio must be interpreted with caution, because the concentrations of these metabolites fluctuate rapidly with changing conditions. Furthermore, as recently pointed out [42**], the ADP/ATP ratio does not reflect the true
viability of an islet preparation and unlike the ATP/DNA ratio fails to account for non-viable cells containing no ADP or ATP. Additionally, even though ATP and ADP measurements are simple, inexpensive, and quick to obtain, islet ADP measurements based on luminescence may be unreliable as they have frequently provided negative concentration estimates [42**]. MMP dyes are used as surrogate measures of mitochondrial health, in that they preferentially accumulate in healthy and polarized mitochondria. Both laser scanning cytometry [19] and the microfluidic system described earlier [31**] have been used to correlate MMP with the quality of preparations composed of dissociated and intact islets, respectively.

**Oxygen Consumption Rate (OCR)**

Measurements based on OCR, which is related to mitochondrial function, have been extensively used to assess the viability and health of cells in a variety of fields [50–55], including islets [26,32–34**,56,57**,58] and β-cell lines in tissue engineered constructs [59–61]. Several groups have recently focused their efforts on characterizing islet viability and potency using OCR measurements and in some cases correlating these measurements with outcomes in the NMB [26,32,34**,57**]. Reports on islet respiratory activity include measurements based on scanning electrochemical microscopy [27*] and oxygen sensitive phosphorescence lifetime or fluorescence intensity in a variety of configurations [26,32–34**,56,57**,58]. The instrumentation and methodologies employed along with the strengths and limitations of each approach are outlined in Table 3. The approach for indirectly measuring OCR using fluorescence intensity in a multi-well plate oxygen biosensor system (OBS) has the distinct advantage of being high-throughput and convenient but in its current form suffers from several major limitations that prohibit its reliable use [16,26]. Recent efforts to bypass some of the inherent limitations of the OBS [62] may enable more reliable and effective use of this method in islet potency assessment.

Recently published data obtained with the most basic approach, using optical pO\(_2\) sensors in stirred microchambers [33], demonstrate that transplanted OCR (OCR\(_{TX}\), a measure of the amount of viable tissue) and OCR/DNA (a measure of viability) are sufficient when used in combination to predict outcomes in diabetic mice transplanted with rat [56–57**], porcine [33], and human [26,32,34**,58] islets. These studies suggest that information on the functional capacity of the islets or β-cells is not necessary for predicting transplantation outcomes in mice. In fact, the most recently reported study with rat islets transplanted in immunosuppressed diabetic mice [57**] clearly demonstrated this relationship between OCR\(_{TX}\) and OCR/DNA of the transplanted islets and diabetes reversal in mice. When the results of these transplantations were plotted such that the ordinate was OCR\(_{TX}\) and the abscissa was OCR/DNA of the transplanted islet sample, the data segregated into three regions: (1) an upper and right-most portion, where diabetes was reversed in all animals, (2) a lower left, where diabetes was not reversed in any animals, and (3) a narrow band in the middle in which both outcomes were represented. In this study, sensitivity and specificity analyses on OCR\(_{TX}\) and OCR/DNA exhibited values of 93% and 94%, respectively, in predicting diabetes reversal. Importantly, the marginal mass for DR was not fixed [57**] but rather depended on OCR/DNA, and increased from 2,800 to over 100,000 IE per kilogram recipient body weight (KgBW) as OCR/DNA decreased. These findings are consistent with reports that neither OCR\(_{TX}\) nor OCR/DNA, when used individually, correlated with transplant outcomes in mice [15,63].

Correlation of transplantation outcomes with rat islets was substantially better than that obtained with human islet preparations [32]. There are several likely explanations for this finding, which include: (a) the absence of non-islet tissue in rat preparations, (b) the large fraction of nonviable tissue at low OCR/DNA, and (c) the large number of human islets, in
contrast with the small number of rat islets, required to reverse diabetes in mice. The predicted probabilities of DR with rat islet transplants were sharply defined with a large domain at 100% cure, whereas the analogous plot for human islet transplants [32] had angled contours of roughly constant slope with virtually no domain of 100% cure, although such a domain might have been attainable if there had been preparations of higher OCR/DNA. The absence of data in the high OCR/DNA range was a limitation of the study with human islets [32].

Data obtained with a porcine-to-non-human primate (xenogeneic) model suggest that sustained insulin independence is dependent on both OCR\textsubscript{TX} per KgBW and OCR/DNA (unpublished observations). Interestingly, initial data obtained with pure and impure clinical autologous and single-donor, allogeneic islet transplants suggest that in these cases (especially islet auto-transplants), the OCR dose normalized per KgBW alone may sufficiently correlate with clinical outcomes (unpublished observations).

Of particular interest are attempts to extract information on islet potency based on glucose-stimulated OCR, which may be more representative of β-cells and their functional capacity [26–27*,34**,56,58]. This index has been represented either as a ratio of the measured OCR in the presence of high glucose divided by the OCR in low glucose (OCR\textsubscript{hglc}/OCR\textsubscript{lglc}) or simply the difference in measured OCR in the presence of high and low glucose (ΔOCR\textsubscript{glc}) [34**,56,58]. Publications detailing these procedures report reasonable correlations with the NMB and suggest that there may be an advantage in using these indices for clinical islet potency assessment. It remains to be seen if the challenges associated with widespread implementation and inherent limitations of these complicated methodologies [16] can be overcome and whether the promising results attained with research models will translate into the clinical setting. Work currently under way with clinical auto- and allo- and pre-clinical xeno- transplant models is expected to provide further insight into these issues and help identify and establish islet potency tests that are truly predictive of transplant outcomes.

**Conclusion**

The islet product release criteria that screen preparations before clinical allogeneic ICT are currently unable to predict post-transplant success from failure. More sensitive and reliable islet viability and potency tests have been recently developed and tested. Those assessing mitochondrial function, particularly those that measure the OCR of an islet preparation, appear to be the most promising and correlate with transplant outcomes in the NMB. These tests are currently being evaluated in the clinical setting and preliminary results are encouraging. Assays that characterize cell composition and molecular profiles may be useful in further defining the islet product and may provide useful information on islet immunogenicity and pro-inflammatory potential. The recent clinical success in reversing diabetes with single-donor, allogeneic transplants, will further enhance our ability to define potency tests and islet characteristics that are predictive of transplant outcome.

**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ICT</td>
<td>Islet cell transplantation</td>
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<tr>
<td>OCR</td>
<td>Oxygen consumption rate</td>
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<tr>
<td>FDA</td>
<td>Federal Drug Administration</td>
</tr>
<tr>
<td>EU</td>
<td>Endotoxin unit</td>
</tr>
<tr>
<td>IE</td>
<td>Islet equivalent(s)</td>
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</table>

*Curr Opin Organ Transplant. Author manuscript; available in PMC 2010 December 1.*
DTZ  
Dithizone

FDA/PI  
Fluorescein diacetate/propidium iodide

NMB  
Nude mouse bioassay

DNA  
Deoxyribonucleic acid

GSIS  
Glucose-stimulated insulin secretion

pO₂  
Partial oxygen partial pressure

DR  
Diabetes reversal

SLM  
Standard light microscopy

FM  
Fluorescence microscopy

C  
Calcein AM

EH  
Ethidium homodimer

EB  
Ethidium bromide

SYTO®  
Green membrane permeable fluorescent dye

AO  
Acridine orange

FACS  
Fluorescent-activated cell sorting (or flow cytometry)

7-AAD  
7-aminoactinomycin D

VADFMK  
Membrane permeable caspase ligand (inhibitor)

PS  
Phosphatidylserine

MTT  
Tetrazolium salt, 3-(4;5-dimethylthiazol-2-yl)-2;5-diphenyl tetrazolium bromide

ATP  
Adenosine triphosphate

ADP  
Adenosine diphosphate

MMP  
Mitochondrial membrane potential

FV  
Fractional viability

ELISA  
Enzyme-linked immunosorbent assay

ΔOCR_{glc}  
Defined as the measured increment in OCR when stimulated by glucose

OCR_{TX}  
Transplanted OCR, which represents viable islet dose

OCR/DNA  
Measure of OCR normalized to DNA represents the FV of cellular/islet preparation

OCR_{GS}  
Glucose-stimulated OCR

OCR_{hglc}/OCR_{lglc}  
Defined as the Stimulation Index a ratio of OCR measured at high glucose concentrations (16.7 or 33.3 mM) to OCR measured at high glucose concentrations (2.8 or 5.6 mM)

ΔOCR_{glc}/DNA  
Defined as the OCR Index a ratio of the estimated ΔOCR_{glc} normalized to DNA

OBS  
BD Biosciences Oxygen Biosensor System®

KgBW  
Kilogram body weight (of transplant recipient)
Acknowledgments

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References


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describes a new more quantitative technique for measuring the amount of islet tissue in a preparation


30. Kim JH, Park SG, Lee HN, Lee YY, Park HS, Kim HI, et al. ATP measurement predicts porcine islet transplantation outcome in nude mice. Transplantation 2009;87(2):166–169. [PubMed: 19155969] This paper presents correlations between ATP measurements and transplant outcomes in mice. However, the technique presented in this paper appears to depend heavily on the amount of islets in the sample (significant differences were observed between pooled ATP normalized to 10 hand-picked islets versus 1000 IE).

31. Mohammed JS, Wang Y, Harvat TA, Oberholzer J, Eddington DT. Microfluidic device for multimodal characterization of pancreatic islets. Lab on a chip 2009 Jan 7;9(1):97–106. [PubMed: 19209341] This paper combines the measurement of several viability parameters in the assessment of the quality of a preparation. This microfluidic approach, involving intact and not dissociated islets, may prove particularly useful in representing the quality of a small number of islets.


34. Sweet IR, Gilbert M, Scott S, Todorov I, Jensen R, Nair I, et al. Glucose-stimulated increment in oxygen consumption rate as a standardized test of human islet quality. Am J Transplant 2008;8(1):183–192. [PubMed: 18021279] This paper reports on differences between measured basal and glucose-stimulated oxygen consumption rates ($\Delta$OCR$_{glc}$) and their ability to predict transplant outcome in the NMB. Eventhough it is reported that an advantage of this technique is the ability to distinguish islet potency in impure preparations the correlations with the NMB were established using pure (hand-picked islets) preparations.


42. Suszynski TM, Wildey GM, Falde EJ, Cline GW, Maynard KS, Ko N, et al. The ATP/DNA ratio is a better indicator of islet cell viability than the ADP/ATP ratio. Transplantation proceedings 2008;40(2):346–350. [PubMed: 18374063] This paper demonstrates that the ATP/DNA ratio is a better measure of viability than the ADP/ATP ratio in preparations containing varying proportions of viable and non-viable cells and islets. Despite these findings, ATP/DNA suffers from similar limitations as the ADP/ATP ratio, in that, ATP levels fluctuate rapidly and often reversibly.


### TABLE 1

Product release criteria for clinical islet preparation

<table>
<thead>
<tr>
<th>TYPE OF TEST</th>
<th>PRODUCT TEST</th>
<th>SPECIFICATION</th>
<th>TYPE OF SAMPLE</th>
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<tbody>
<tr>
<td>Safety</td>
<td>Endotoxin</td>
<td>&lt; 5 EU/kg</td>
<td>Supematant of islet suspension in transplant media</td>
</tr>
<tr>
<td></td>
<td>Gram stain</td>
<td>No organisms detected within limits of assay</td>
<td></td>
</tr>
<tr>
<td>Identity</td>
<td>Islet count (IE/kg)</td>
<td>5,000–20,000 (1st transplant)</td>
<td>Islets in transplant media</td>
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<tr>
<td></td>
<td></td>
<td>3,000–20,000 (Re-transplants)</td>
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<tr>
<td></td>
<td>Purity</td>
<td>≥ 30%</td>
<td></td>
</tr>
<tr>
<td>Viability</td>
<td>Dye exclusion (FDA/PI)</td>
<td>≥ 70%</td>
<td>Islets after overnight culture and in transplant media</td>
</tr>
<tr>
<td>Potency</td>
<td>Glucose stimulated insulin release (ELISA)</td>
<td>Stimulation Index &gt;1</td>
<td>Islets after overnight culture</td>
</tr>
</tbody>
</table>

EU = Endotoxin unit 
IE = Islet equivalent, defined as a volume of islet tissue equal to that of a sphere having a 150 µm diameter [11] 
DTZ = Dithizone 
FDA/PI = Fluorescein diacetate/propidium iodide
### TABLE 2

#### TABLE 2A

**Strengths and limitations of assays currently used prior to islet product release for clinical transplantation**

<table>
<thead>
<tr>
<th>ASSAY</th>
<th>STRENGTHS</th>
<th>LIMITATIONS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Islet count (IE)</td>
<td>Relatively easy to perform Counts Experienced islet isolation centers have standardized procedures</td>
<td>Visual assessment of 3D islet in 2D planes contributes to error Sample may not be representative of whole preparation Presence of contaminant tissue (e.g., exocrine cells, ganglia, etc) may complicate counts</td>
</tr>
<tr>
<td>Purity (DTZ)</td>
<td>Stain differentiates between exocrine and islet tissue Relative ease of use Rapid assessment</td>
<td>Visual assessment of 3D islet in 2D planes contributes to error Provides no information regarding viability of preparation</td>
</tr>
<tr>
<td>Cell membrane integrity (FDA/PI)</td>
<td>Relative ease of use Can be performed prospectively Fractional viability can be estimated by dye exclusion</td>
<td>Visual assessment of 3D islet in 2D planes contributes to error Impossible to identify irreversibly damaged cells whose plasma membranes have not yet been permeabilized FDA may be additionally cleaved by lipases or esterases from non-endocrine tissue, over-estimating the true islet viability Visual counting is operator dependent Background fluorescence (with certain combinations or high concentrations of dyes) can obscure approximations Counterstain may not provide enough contrast Dyes rely on diffusion to penetrate into islet core Lack of correlation with mitochondrial function assays, NMB and clinical outcomes Does not discriminate endocrine (islet) from exocrine (contaminant) tissue</td>
</tr>
<tr>
<td>Glucose-stimulated insulin secretion (GSIS)</td>
<td>May provide information regarding potency of islet preparation</td>
<td>Unable to predict true islet potency or transplant outcome Islets may not be as responsive to glucose stimulus <em>in vitro</em> but may still reverse diabetes <em>in vivo</em> Difficult to account for degranulation of β cells following glucose stimulus or “leaky” cells with damaged plasma membranes</td>
</tr>
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### TABLE 2B

**Strengths and limitations of the diabetic nude mouse bioassay**

<table>
<thead>
<tr>
<th>ASSAY</th>
<th>STRENGTHS</th>
<th>LIMITATIONS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nude mouse bioassay (NMB)</td>
<td>Most reliable <em>in vivo</em> assessment of islet potency Results correlate with clinical outcome</td>
<td>Assay can only be used retrospectively (days to weeks for outcomes) Impure preparations may yield false negative transplant outcomes The severity and duration of the diabetic state of the mouse affects the predictive outcome of the assay Islets are transplanted into the kidney capsule, not into the hepatic portal system (thereby not fully representing the current clinical protocol) Mice are susceptible to developing other conditions (e.g., infection) that can also affect outcome Does not account for immunologic rejection or the effect of immunosuppressive agents on islets The assay carries several practical challenges (e.g., induction of diabetes needs to be timed with islet isolation)</td>
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## TABLE 2C
Advantages and disadvantages of assays being under consideration for clinical islet quality assessment

<table>
<thead>
<tr>
<th>ASSAY</th>
<th>REFERENCES</th>
<th>ADVANTAGES</th>
<th>DISADVANTAGES</th>
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<td><strong>Membrane integrity tests</strong></td>
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</tr>
<tr>
<td>SLM/FM C/EH SYTO®/EB AO/PI</td>
<td>[12,15,16]</td>
<td>Similar advantages as FDA/PI (See Table 2A)</td>
<td>Similar disadvantages as FDA/PI (See Table 2A)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(See Table 2A)</td>
<td>Certain dyes are chemically unstable or form crystals which can manifest as visual artifacts</td>
</tr>
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<td></td>
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<td></td>
<td>Some stains may exhibit greater sensitivity in detecting islet cell membrane damage</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(fragmentation, e.g., C).</td>
</tr>
<tr>
<td>FACS 7-AAD† Topro3†</td>
<td>[16,19,29]</td>
<td>Minimizes diffusion limitations</td>
<td>Requires dissociation (except sequential staining with 7-AAD) of islet aggregates, resulting in irreversible cell damage and loss (i.e., anoikis)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>More quantitative</td>
<td>Relative subjectivity in gating cellular subpopulations in FACS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Allows possibility of β-cell specificity</td>
<td>Requires expensive equipment and training</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Membrane integrity can be approximated using the 7-AAD sequential staining procedure</td>
<td></td>
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</tr>
<tr>
<td><strong>Other cell death and mitochondrial assays</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caspase activation (VADFMK†)</td>
<td>[25,29]</td>
<td>Detects early apoptotic events</td>
<td>Provides “snapshot” of early apoptotic events, but may not detect late apoptotic or necrotic cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rapid measurement</td>
<td>May not account for caspase independent mechanisms of cell death</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>May require dissociation of islets</td>
</tr>
<tr>
<td>PS Externalization (Annexin V†) DNA Fragmentation (TUNEL†) Ca²⁺ Leakage (Fura Red†)</td>
<td>[25,29,31]</td>
<td>May detect both apoptosis and necrosis</td>
<td>Difficult to use prospectively, because the assay may require histological staining and subsequent analysis (i.e., Annexin V)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>May require dissociation of islets</td>
</tr>
<tr>
<td>Reduction potential Tetrazolium salts</td>
<td>[16,33]</td>
<td>Detects reducing capacity of islets</td>
<td>Reduction of salts involves complex reactions and may reflect local pO₂ changes or differences in the compositions of cell culture media</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Relative ease of use</td>
<td>Accumulation of insoluble byproduct of reduction reaction (in MTT assay) is toxic to assayed cell preparation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Inexpensive</td>
<td></td>
</tr>
<tr>
<td>Bioenergetic status ADP/ATP ATP/DNA ATP/protein ATP/IE</td>
<td>[27–28,30,42]</td>
<td>Relative ease of use</td>
<td>ATP concentrations fluctuate rapidly (short half-life) and are sensitive to transient changes in local conditions (i.e., glucose levels, pO₂, pH)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Inexpensive</td>
<td>Islets are difficult to assay because of differences between environmental conditions experienced by cells located in the core versus the periphery</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Low islet requirement (~100 IE)</td>
<td>ADP/ATP measurements do not account for non-viable cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ATP and ADP play a particularly critical role in insulin secretion (i.e., islet function)</td>
<td>ADP measurements by luminescence assay may be unreliable</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Can be performed on intact islets</td>
<td></td>
</tr>
<tr>
<td>Mitochondrial membrane potential IC-1 TMRE† Rh123</td>
<td>[16,19,31]</td>
<td>Detects loss of mitochondrial polarization, which occurs during early apoptosis and during necrosis</td>
<td>Difficult to quantify absolute changes in MMP</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>May require dissociation of islets</td>
</tr>
</tbody>
</table>

IE = Islet equivalent, defined as a volume of islet tissue equal to that of a sphere having a 150 µm diameter [11]

DTZ = Dithizone

FDA/PI = Fluorescein diacetate/propidium iodide

NMB = Nude mouse bioassay
Abbreviations and Legend

SLM = Standard light microscopy
FM = Fluorescence microscopy
C = Calcein AM, green membrane permeable fluorescent dye
EH/EB = Ethidium homodimer or ethidium bromide, red-orange membrane impermeable fluorescent dye
FDA = Fluorescein diacetate, membrane permeable dye that fluoresces green after cleavage by non-specific esterases
PI = Propidium iodide, red membrane impermeable fluorescent dye
SYTO® = Green membrane permeable fluorescent dye
AO = Acridine orange, green membrane-permeable fluorescent dye
IE = Islet equivalent, defined as a spherical aggregate of pancreatic endocrine cells of 150 µm diameter
FACS = Fluorescent-activated cell sorting (or flow cytometry)
7-AAD = 7-aminoactinomycin D, membrane-impermeable fluorescent dye
VADFMK = Membrane permeable caspase ligand (inhibitor)
PS = Phosphatidylserine
MTT = Tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
DNA = Deoxyribonucleic acid, measured using commercial fluorimetric assay
ATP = Adenosine triphosphate
ADP = Adenosine diphosphate
MMP = Mitochondrial membrane potential

† Assay has been used on islets in conjunction with FACS, which requires the dispersal of islet clusters. Dissociating islets typically involves harsh enzymatic digestion with serine proteases that results in the disruption of cell-matrix interactions, cellular damage and death (e.g., anoikis). It is important to note that FACS analysis in itself is associated with inherent limitations, including the relative subjectivity of gating cell sub-populations, the large sample required for analysis (~1000s IE), high cost of equipment, extensive training and complex methodology that is susceptible to error.
# TABLE 3

Current methodologies used in the measurement of oxygen consumption rate

<table>
<thead>
<tr>
<th>SYSTEM</th>
<th>METHOD</th>
<th>REFERENCE</th>
<th>MEASURED QUANTITIES</th>
<th>ASSAYED TISSUE</th>
<th>ADVANTAGES</th>
<th>DISADVANTAGES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Perifusion bioreactor</td>
<td>Phosphorescence</td>
<td>[56]</td>
<td>ΔOCR&lt;sub&gt;glc&lt;/sub&gt;</td>
<td>Rat islets</td>
<td>Real-time assessment of transient dynamics (e.g., glucose responsiveness, Ca&lt;sup&gt;2+&lt;/sup&gt; blockade, protein synthesis inhibition)</td>
<td>Complex system with limited to use in research</td>
</tr>
<tr>
<td></td>
<td>lifetime</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>[34]</td>
<td>ΔOCR&lt;sub&gt;glc&lt;/sub&gt;</td>
<td>Human islets</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>OCR&lt;sub&gt;TX&lt;/sub&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stirred microchamber</td>
<td>Fluorescence quenching</td>
<td>[33]</td>
<td>OCR/DNA</td>
<td>βTC3 cells</td>
<td>Quantitative, rapid and prospective assessment of an islet preparation</td>
<td>May not differentiate between OCR attributed to islets or other cells in a preparation</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>OCR/cell</td>
<td>Rat islets</td>
<td>Operator independent</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>OCR/cell</td>
<td>Porcine islets</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>[32]</td>
<td>OCR&lt;sub&gt;TX&lt;/sub&gt;</td>
<td>Human islets</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>OCR&lt;sub&gt;DNA&lt;/sub&gt;</td>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td>[57]</td>
<td>OCR&lt;sub&gt;TX&lt;/sub&gt;</td>
<td>Rat islets</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>OCR&lt;sub&gt;DNA&lt;/sub&gt;</td>
<td></td>
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</tr>
<tr>
<td>Static culture</td>
<td>Fluorescence</td>
<td>[58]</td>
<td>OCR&lt;sub&gt;GS&lt;/sub&gt;</td>
<td>NHP islets</td>
<td>Simple, inexpensive and rapid assessment</td>
<td>May not provide accurate estimates of OCR</td>
</tr>
<tr>
<td></td>
<td>microplate reader</td>
<td></td>
<td>OCR&lt;sub&gt;hglc/OCR&lt;sub&gt;lglc&lt;/sub&gt;&lt;/OCR&gt;</td>
<td>Human islets</td>
<td></td>
<td>Limited experience with its use in this application</td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>Complex theoretical estimation of pO&lt;sub&gt;2&lt;/sub&gt;</td>
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<td></td>
<td>OCR&lt;sub&gt;GS&lt;/sub&gt;/OCR&lt;sub&gt;DNA&lt;/sub&gt;</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>[26]</td>
<td>OCR ΔOCR&lt;sub&gt;glc&lt;/sub&gt;/DNA</td>
<td>Human islets</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

OCR = Oxygen consumption rate
ΔOCR<sub>glc</sub> = Defined as the measured increment in OCR when stimulated by glucose (3 – 20 mM)
OCR<sub>TX</sub> = Transplanted OCR, which represents viable islet dose
DNA = Deoxyribonucleic acid, measured using commercial fluorimetric assay
OCR/DNA = Measure of OCR normalized to DNA, represents the fractional viability of a cellular/islet preparation
OCR<sub>GS</sub> = Glucose-stimulated OCR
OCR<sub>hglc/OCR<sub>lglc</sub></OCR> = Defined by the authors of the paper<sup>37</sup> as the Stimulation Index, a ratio of OCR measured at high glucose concentrations (16.7 or 33.3 mM) to OCR measured at basal concentrations (2.8 or 5.6 mM)
ΔOCR<sub>glc</sub>/OCR<sub>DNA</sub> = Defined as the OCR Index, a ratio of the estimated ΔOCR<sub>glc</sub> normalized to DNA.