


<b>DAIT, NIAID, NIH</b>				
		<b>SOP APPENDIX</b>		
<b>Document No.</b> 3104, A03	<b>Revision Number</b> 03	<b>Effective Date</b> 25 October 2010	<b>Supersedes Date</b> 08 June 2010	<b>Page 1 of 11</b>
<b>Document Title:</b>				
<b>PURIFIED HUMAN PANCREATIC ISLETS, GLUCOSE STIMULATED INSULIN RELEASE DETERMINATION BY ELISA</b>				

**PURPOSE:** To be a model for site-specific SOPs that define the method for quantitative determination of insulin released after glucose stimulation by the Purified Human Pancreatic Islets product manufactured for use in the CIT trials.

**RESPONSIBILITY:** It is the responsibility of the Islet Cell Processing Principal Investigator or designee to:

- establish a site-specific SOP based on this document,
- train the site personnel in the execution of the site-specific procedure,
- validate the site-specific procedure,
- assure that the site-specific procedure is executed, and
- maintain records of the execution of the site-specific procedure.

**SCOPE:** This SOP applies to all trained personnel participating in the CIT consortium manufacturing the Purified Human Pancreatic Islets product for use in DAIT-sponsored clinical studies.

## I. INTRODUCTION

After overnight culture at 37°C (12 – 24 hrs), human islets are incubated with media containing a relatively low concentration of glucose (2.8 mM) and a sample of the supernatant is taken. Then the same islets are incubated with media containing a higher glucose concentration (28 mM) and a sample of the supernatant is taken. The amount of insulin present in both the supernatant samples is measured using a commercially available Human Insulin ELISA kit. The stimulation index is calculated by dividing insulin concentration of the supernatant from the 28 mM glucose incubation by the insulin concentration of the supernatant from the 2.8 mM glucose incubation.

## II. DEFINITION

**Stimulation Index:** A measure of the ability of the Purified Human Pancreatic Islets product to produce insulin when stimulated by an increase in the concentration of glucose.

$$\text{Stimulation Index (SI)} = \frac{\text{Insulin concentration after high glucose concentration stimulation}}{\text{Insulin concentration after low glucose concentration stimulation}}$$

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### III. EQUIPMENT AND MATERIALS

#### A. Equipment

##### Glucose Stimulation

- Biological Safety Cabinet
- 37°C 5% CO<sub>2</sub> Incubator
- Analytical Balance
- Sterile micropipettes (10-100 µL, 20-200 µL, and 100-1000 µL ranges)
- Wide mouth pipette tip and extender (Fisher, Cat # 02-707-134 and 02-681-183 autoclave before using).
- Pipette Aid automatic pipettor or equivalent
- Sterile individual wrapped transfer pipettes (Samco Scientific Cat # 204-1S)
- pH Meter
- Microscope
- Sterile gauze sponges (Tyco Healthcare Group Cat # 2187)
- Sterile forceps

##### Insulin Assay

- Microplate strip washer, automated or manual system
- Microplate absorbance reader with attached computer and printer
- Microtiter Plate Rotator
- Vortex
- Micropipettors in 20-200 µL, and 200-1000 µL ranges (repeating or multichannel optional)

#### B. Supplies and Materials

##### Glucose Stimulation

- 0.2 µm bottle top filter (250 mL, 500 mL or 1 liter)
- Syringe filter unit (Millipore Cat # SLGP033RB)
- Conical tubes, 50 mL, sterile
- Conical tubes, 15 mL, sterile
- 2 ml cryovials
- Graduated cylinder
- Petri dishes, 100 mm
- 1 mL, 5 mL, 10 mL, & 25 mL pipettes
- 24 well non-tissue culture treated multi-well plate
- Millicell Cell Culture Plate Inserts, 12 mm (Millipore, PIXP01250)
- Stir bar
- Weighing paper
- 6 mL polypropylene tubes, 12 x 75 mm
- 1-200 µL pipette tips
- Parafilm
- Microcentrifuge tubes

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C. Reagents and Media

Glucose Stimulation

- HEPES powder (EMD Chemicals Cat # 5320, FW 238.3 or equivalent)
- NaCl (J T Baker Cat # 405801, FW 58.44 or equivalent)
- NaHCO<sub>3</sub> (Fisher Biotech Cat # BP328-1, FW 84.01 or equivalent)
- KCl (Mallinckrodt Cat # 6845, FW 74.55 or equivalent)
- MgCl<sub>2</sub> · 6 H<sub>2</sub>O (Sigma Cat # M2670, FW 203.3 or equivalent)
- CaCl<sub>2</sub> · 2 H<sub>2</sub>O (Sigma Cat # C3881, FW 147 or equivalent)
- Bovine Serum albumin (BSA, Sigma Cat # A2153 or equivalent)
- Deionized water
- D-(+)-Glucose, (dextrose) (Sigma Cat. #7021 or equivalent)
- Hydrochloric acid, HCl, 1N solution, Fisher Scientific or equivalent
- Sodium hydroxide, NaOH, 1N solution, Fisher Scientific or equivalent

Insulin Assay

- Mercodia, Insulin ELISA, CAT. #10-1113-01 and #10-1113-10
- Mercodia Diabetes sample buffer for sample dilution , Cat. # 10-1195-10
- Mercodia, Diabetes-antigen Control (Low, High)/ Human, Unassayed, CAT #10-1134-01

D. Attachments

1. Attachment I, Microtiter Plate Layout
2. Attachment II, Static Incubation & Insulin Release Worksheet
3. Attachment III, Solution Preparations

**IV. ISLET SAMPLE PREPARATION**

This test is performed on a sample of islets taken before culture (purest fraction), and from the final Purified Human Pancreatic Islets product (Bag #1).

A. Pre-culture islet sample

1. On the day of islet isolation, remove 400 IEQ (calculate using islet count per volume) from the purest layer using sterile technique.
2. Prepare CIT Culture Media as described in the Production Batch Record and DAIT SOP 3106.
3. Place islets in a 100 mm Petri dish, add 15 mL of CIT Culture Media, label and cover.
4. Place the dish in a 37°C, 5% CO<sub>2</sub> incubator.
5. Culture islets for 12-24 hours.
6. Test islets according to Section VI of this SOP, below.

B. The samples collected from the Post-culture High Purity Purified Human Pancreatic Islets product require no further culture. Test according to Section VI, below.

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## V. GLUCOSE STIMULATION REAGENT PREPARATION

### A. Stock Solutions

#### 1. Krebs Buffer Stock Solution

- Record preparation on Attachment III of this SOP
- Combine in a 1 liter volumetric flask:
  - 5.96 g HEPES powder (final concentration 25 mM)
  - 6.72 g NaCl (final concentration 115 mM)
  - 2.02 g NaHCO<sub>3</sub> (final concentration 24 mM)
  - 0.3728 g KCl (final concentration 5 mM)
  - 0.2033 g MgCl<sub>2</sub> · 6 H<sub>2</sub>O (final concentration 1 mM)
  - 1.0 g BSA (final concentration 0.1%)
  - q.s. to 1 L with deionized water and mix until dissolved.
- Add 0.3675 g CaCl<sub>2</sub> · 2 H<sub>2</sub>O (final concentration 2.5 mM) and stir the solution (the CaCl<sub>2</sub> · 2 H<sub>2</sub>O may not completely dissolve until the solution pH is adjusted).
- Check the pH of the solution and adjust to 7.3 to 7.5 using either 1 N NaOH or 1 N HCl, if necessary.
- Filter sterilize into a sterile 1 liter bottle using a 0.22 µm bottle top filter.
- Divide into 19 X 50-mL aliquots in sterile 50 mL tubes.  
*NOTE: Reserve remaining Krebs Buffer for 280 mM stock glucose below.*
- Label as:
  - Krebs Buffer Stock Solution
  - P: *Preparation Date*
  - E: *Expiration Date* (12 weeks after preparation)
  - Tech: *Preparer's Initials*
  - Store at 2 – 8°C

#### 2. 280 mM Glucose Solution

- Record preparation on Attachment III of this SOP.
- Add 2.5 g of D-(+)-Glucose to 50 mL of Krebs Buffer Stock Solution.
- In a biological safety cabinet, filter sterilize into a sterile 50 mL sterile tube using a 0.22 µm syringe filter.
- Divide into 10 X 5-mL aliquots, using sterile 15 mL tubes.
- Label as:
  - 280 mM Glucose Stock Solution, 5 mL
  - P: *Preparation Date*
  - E: *Expiration Date* (4 weeks after preparation)
  - Tech: *Preparer's Initials*
  - Store at 2 – 8°C

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3. High Glucose (28 mM) Solution:
  - a) In a biological safety cabinet, prepare High Glucose (28 mM) solution by adding 5 mL of 280 mM Glucose stock into 45 mL Krebs Buffer Stock Solution (1:10 dilution) in a 50 mL sterile tube. Prepare as much solution as needed.
  - b) Label as:
    - High Glucose Solution, 28 mM
    - P: *Preparation Date*
    - E: *Expiration Date* (4 week after preparation)
    - Tech: *Preparer's Initials*
    - Store at 2 – 8°C
  
4. Low Glucose (2.8 mM) Solution:
  - a) In a biological safety cabinet, prepare Low Glucose (2.8 mM) Solution by adding 5 mL of 28 mM High Glucose Solution into 45 mL Krebs Buffer Stock Solution (1:10 dilution) in a 50 mL sterile tube. Prepare as much solution as needed.
  - b) Label as:
    - Low Glucose Solution, 2.8 mM
    - P: *Preparation Date*
    - E: *Expiration Date* (4 week after preparation)
    - Tech: *Preparer's Initials*
    - Store at 2 – 8°C

## VI. GLUCOSE STIMULATION

**NOTE:** Make sure to perform all procedures below in a biological safety cabinet and use sterile techniques.

### A. Plate Preparation

1. Label a 24-well plate with sample ID and date.
2. Add 1.0 mL 2.8 mM Low Glucose Solution per well to wells A1, A2, A3.
3. Add 1.0 mL of 2.8 mM Low Glucose Solution per well to wells B1, B2, B3.
4. Add 1.3 mL of 2.8 mM Low Glucose Solution per well to wells C1, C2 and C3.
5. Add 1.3 mL 28 mM High Glucose Solution per well to wells D1, D2 and D3.
6. Using forceps, place one Millicell Cell Culture Plate Insert (insert) into well A4, A5 and A6.
7. Cover plate with lid and incubate the plate to 37°C in 5% CO<sub>2</sub> incubator for 1 hour (to equilibrate pH and temperature of media).

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B. Preparation of islets

1. Remove the Petri dish containing islets from the incubator.
2. Label two 15 mL sterile tubes with 1 and 2, respectively.
3. Transfer islets and culture media from the Petri dish to tube 1 using a 10 mL pipette.
4. Wait until islets are settled. Use a sterile 200  $\mu$ L wide mouth pipette to transfer islet suspension from tube 1 to tube 2. Depending on the number of IEQ in Petri dish, the calculation of islet suspension will be as follows:  
 If there are 400 IEQ in tube 1, make two 200  $\mu$ L transfer from tube 1 to tube 2.  
 If there are 300 IEQ in tube 1, make one 200  $\mu$ L and one 100  $\mu$ L transfers from tube 1 to tube 2.  
 Make sure to pick islet pellet up first and then culture media. The final IEQ concentration in tube 2 should be always 100 IEQ/100  $\mu$ L.

C. Basal Equilibration (Row B)

1. Gently mix islet suspension well in tube 2.
2. Slowly pipette 100  $\mu$ L of well-mixed islet suspension into each insert in well A4, A5 and A6.
3. Close the lid on the plate. Check islets under microscope to make sure there is reasonable number of islets per insert. Otherwise repeat islet transfer.
3. Place insert on sterile gauze to drain culture media out completely. Transfer inserts with islets to the wells A1, A2, and A3 to rinse islets in order to remove remaining culture media as much as possible.
4. Immediately lift each insert and drain liquid back into corresponding well. Blot the insert on sterile gauze to drain out liquid completely. Transfer inserts with islets to the well B1, B2, B3.
4. Replace the lid on the plate and incubate for 1 hour at 37°C in 5% CO<sub>2</sub>.
5. Remove the plate from incubator.

D. Low Glucose Stimulation (Row C)

1. Transfer the inserts from row B to corresponding wells in row C (C1, C2, C3) as described below:
  - a) Slowly, lift the insert up from its corresponding well, using forceps.
  - b) Allow liquid to drain from each insert back into the well by “wicking” the liquid from the mesh bottom of the insert against the side of the well.
  - c) When the insert looks well-drained, blot the bottom of the insert on sterile gauze before gently setting the insert in the corresponding well in row C.
2. ZERO TIME SAMPLE
  - a) Allow the level of liquid to equilibrate between inside and outside of each insert (now in its corresponding well).
  - b) Lift each insert and drain the liquid back into its corresponding well. Do not blot the insert on sterile gauze.

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- d) Remove 300 µL of solution from each well (from the well, not the insert). Transfer solution to corresponding microcentrifuge tubes labeled “Zero time – Low Glucose”, C1, C2, or C3, Lot#, Date, Tech.
  - c) As soon as the sample is collected, immediately replace the insert in its corresponding well. Repeat this for each of the inserts in row C.
3. Replace the lid on the plate and incubate for 1 hour at 37°C in 5% CO<sub>2</sub>.
  4. Remove the plate from incubator.
  5. Remove inserts from row C wells. Drain the liquid back to well completely. You may use a sterile transfer pipette (one per insert) to collect remaining liquid at the bottom of the insert. Blot the bottom of the insert on sterile gauze. Transfer each into the corresponding well in row D., Collect all solution from each well in row C to corresponding microcentrifuge tubes labeled “Low Glucose”, C1, C2, or C3, Lot#, Date, Tech.
- E. High Glucose Stimulation (Row D)
1. ZERO TIME SAMPLES. Collect as described in Section D.2, above. Transfer samples to microcentrifuge tubes labeled as “Zero time – High Glucose”, D1, D2, or D3, Lot#, Date, Tech.
  2. Replace the lid on the plate and incubate for 1 hour at 37°C in 5% CO<sub>2</sub>.
  3. Remove the plate from the incubator. Remove inserts from their corresponding wells, thoroughly draining all liquid back into wells as described in step 5 of section D..
  4. Transfer inserts to empty wells to save for other assays, if desired.
  5. Immediately transfer all solution from each well to corresponding microcentrifuge tubes labeled “High Glucose”, D1, D2, or D3, Lot#, Date, Tech.
- F. Sample Storage
1. Samples must be transferred to an appropriate storage temperature as soon as the samples are collected.

**NOTE:** *For the purposes of this assay, samples should be stored at 4-8°C if assayed within 24 hours, or at -20°C if assayed within two weeks.*

2. According to Merckodia (kit manufacturer), serum samples stored at 4-8°C must be assayed within 24 hours, while samples stored at stable -20°C can be assayed within 3 months.  
**NOTE:** *Stability of insulin in glucose solutions used in this SOP has not been established.*

## VII. PREPARATION OF HUMAN INSULIN ELISA SOLUTIONS

- A. The following reagents come in the Human Insulin ELISA kit, ready for use:
- Insulin Standards (S0-S5)
  - Microplate strips coated with murine anti-insulin
  - Substrate TMB Colorless Solution (light sensitive)
  - Stop Solution

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B. Prepare Wash Solution by adding 800 mL of deionized water and 40 mL of Wash Buffer 21x to a clean 1 L bottle. Mix the solution well and label the bottle:

- Record preparation on Attachment III of this SOP.
- Label the bottle: Wash Solution  
P: *Preparation Date*  
E: *Expiration Date* (4 weeks after preparation)  
Lot #: *(manufacturer's lot #)*  
Tech: *Preparer's Initials*  
Store at 2 – 8°C

C. Prepare Anti-insulin Conjugate Solution immediately prior to use.

- Record preparation on Attachment III of this SOP.
- For each microstrip used, mix 100 µL of Enzyme Conjugate 11 X with 1 mL of Enzyme Conjugate Buffer.  
Examples:

# of Strips	Enzyme Conjugate 11 X	Enzyme Conjugate Buffer
1	100 µL	1 mL
6	600 µL	6 mL
12	1.2 mL	12 mL

Label the bottle: Anti-insulin Conjugate Solution

P: *Preparation Date*  
E: *Expiration Date* (1 day after preparation)  
Lot #: *(manufacturer's kit lot#)*  
Tech: *Preparer's Initials*  
Store at 2 – 8°C

D. Preparation of Controls

1. Remove the Diabetes Ag Control vials from the refrigerator and allow them to equilibrate to room temperature for 5 minutes.
  - Record preparation on Attachment III of this SOP.
  - Reconstitute controls as follows:
    - Add 500 µL of deionized water to each vial.
    - Replace cap and let sit for 5 minutes.
    - Mix vial to dissolve contents by swirling gently. Avoid producing bubbles.
2. Aliquot 95 µL of “Low Control” into 5 1-mL microcentrifuge tubes.



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3. Label as: Insulin Low Control, 95  $\mu$ L”  
Lot #: (manufacturer’s lot #)  
P: Date of preparation  
E: Date of expiration (3 months after preparation)  
Tech: Preparer’s Initials  
Store at < -20°C

4. Aliquot 95  $\mu$ L of “High Control” into 5 1-mL microcentrifuge tubes.

5. Label as: Insulin High Control, 95  $\mu$ L  
Lot #: (manufacturer’s lot #)  
P: Date of preparation  
E: Date of expiration (3 months after preparation)  
Tech: Preparer’s Initials  
Store at < -20°C

6. Establishment of acceptable ranges for a new lot of controls

The manufacturing site’s established procedure, or the following process, may be used.

a) For each new lot of High and Low controls, acceptance ranges must be established before the current lots of controls expire or are consumed.

**NOTE:** *Acceptable assays of the new control lots must have control values established for the previous control lots within the means  $\pm$  2 SD ranges.*

b) Reconstitute three vials of High control and three vials of Low control as described in Section D, 1, above.

c) Aliquot 80  $\mu$ L from each reconstituted control vial into six 1 mL v-vials, making a total of 18 X 80  $\mu$ L aliquots of high control and 18 X 80  $\mu$ L aliquots of low control from each reconstituted vial.

d) Sixteen X 80  $\mu$  L aliquots from each vial will be used to establish the ranges for the new control lots. The remaining two X 80  $\mu$  L aliquots from each vial may be used as controls in future assays.

e) On each of two days perform two independent insulin assays. In each assay, test triplicate samples from four 80  $\mu$ L aliquots of the new lots of High and Low controls. Forty-eight insulin results will be obtained for the new lots of High and Low insulin controls.

f) Calculate the mean and standard deviation of the 48 insulin results for each control level.

g) The acceptable control ranges are the means  $\pm$  2 standard deviations for High and Low controls. When the ranges for new lots of controls have been established, the same lot can be reconstituted aliquoted, and the same ranges used for the acceptance of assay results.

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E. Sample Dilution

Supernatants collected from the glucose challenge wells are diluted using Diabetes Sample Buffer for the ELISA. The commonly used dilution factors are 1:50 and 1:100. To select an optimal dilution factor, the insulin concentration detected by ELISA should be in the range of 31.8 to 191 mU/L. Experience from CIT laboratories shows that the dilution factor 1:20 is often the optimal one. It is recommended to select a dilution factor as low as possible and use the same dilution factor for the corresponding samples of both low and high glucose concentration.

**VIII. HUMAN INSULIN ELISA ASSAY PROCEDURE**

- A. Bring all reagents and samples to room temperature.
- C. Prepare sufficient microplate wells to accommodate calibrators, controls and samples in triplicate. Record the plate location of samples on Attachment I, Microtiterplate Layout.
- D. Pipette 25  $\mu$ L each, calibrators, controls and samples into appropriate wells. Pipette directly onto the bottom of the well. Change tips between each standard, control and sample.
- E. Add 100  $\mu$ L of Enzyme Conjugate to each well.
- F. Cover the plate and incubate on a plate shaker at 800 rpm for 1 hour at 18 – 25°C.
- G. Wash manually: fill each well completely with Wash Buffer (350  $\mu$ L) using a squeeze bottle. Turn the plate upside down over a sink and shake to discard liquid completely. Repeat 5 times. After the final wash, invert and tap the plate firmly against absorbent paper. Or, wash the plate six times with an automatic plate washer.
- H. Add 200  $\mu$ L of TMB Substrate Solution into each well.
- I. Cover plate with aluminum foil to protect it from direct light and incubate for 15 minutes at room temperature (18–25°C).
- J. Add 50  $\mu$ L Stop Solution to each well. Place plate on a shaker for approximately 5 seconds to ensure mixing.
- K. Read optical density at 450 nm using bi-chromatic measurement with reference at 600 – 690 nm within 30 minutes.
- L. Record the data on “Static Incubation & Insulin Release Worksheet”, Attachment II of this SOP, and perform calculations required. File the three completed and reviewed Attachments and any printouts with the corresponding Master Batch Production Record (MBPR) for the lot of Purified Human Pancreatic Islets product tested. Record the test results in the MBPR; and on the Certificate of Analysis for the final Purified Human Pancreatic Islets product.

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## **IX. QUALITY CONTROL, INTERPRETATION & RELEASE CRITERIA**

- A. For assay acceptance:
- All control values must be within their 2 SD established ranges.
  - Standard values must be within  $\pm 15\%$  of their nominal values.
  - Triplicates of standards, controls and samples must have Coefficients of Variation (CV)  $\leq 20\%$ .
- B. If the assay is unacceptable for any of these reasons, sample values may not be reported. The assay must be investigated and repeated. Notify the Lab Manager if the assay is unacceptable, or if the stimulation index from an acceptable assay is less than one.
- C. Calculation of Stimulation Index (SI)  
Insulin value (IV); Well C (C); Well D (D).

$$SI = \text{mean of individual SI} = [(IVD1/IVC1) + (IVD2/IVC2) + (IVD3/IVC3)]/3$$

## **X. RECORD REVIEW**

The supervisor or designee must review each and all records.

## **XI. RECORD RETENTION**

Records will be stored, maintained, reviewed and archived following the site specific SOP. Do not discard any record without prior notification to DAIT, NIAID (the study sponsor).

## **XII. REFERENCES**

- A. Cell Transplant Center, DRI, University of Miami, School of Medicine, Miami, FL.
- B. SOP IL4004.00 – Islet Isolation Laboratory, Department of Surgery, Emory University, Atlanta, GA.
- C. Mercodia, Insulin ELISA, Directions for Use, Catalog #10-1113-01 and #10-111010, Document #31-3107, Revised 2007-08-07.
- D. Glucose Stimulated Insulin Release/DNA Quantitation Assay SOP. Minnesota Molecular & Cellular Therapeutics Program, Minnesota, Minnesota.
- E. Mercodia, Diabetes-antigen Control (Low, High)/Human, Directions for Use, Catalog #10-1134-01, Document #31-3118, Revised 05-09-27.