



Version 3 ▼

May 13, 2021

Potency Test: Glucose Stimulated Insulin Release Assay V.3

Integrated Islet Distribution Program¹

¹Integrated Islet Distribution Program, City of Hope, Duarte, CA

1 Works for me

dx.doi.org/10.17504/protocols.io.buzhnx36

Integrated Islet Distribution Program Tech. support email: iidp-email@coh.org



ABSTRACT

The goal of the Integrated Islet Distribution Program (IIDP) is to develop a uniform standardized shipping method among all the subcontracted IIDP centers that will result in minimal loss of quality or quantity of shipped islets (as compared to control islets remaining at the IIDP center). The objective of this Standard Operating Procedure (SOP) is to be a model for site-specific SOPs that define the method for quantitative determination of insulin released after glucose stimulation for proving the potency of the human islet preparation shipped by the IIDP.

Integrated Islet Distribution Program (IIDP) (RRID:SCR_014387)

GSIR Overview:

 $After overnight \ culture \ at \ 37^{\circ}C \ (12-24 \ hrs), human \ is lets \ are \ incubated \ with \ media \ containing \ a \ relatively \ low \ and \ relatively \ low \ lo$ 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

Reference:

Purified Human Pancreatic Islets, Glucose Stimulated Insulin Release Determination by ELISA; DAIT, NIAID, NIH SOP Appendix, Document No. 3104, A03, Revision Number 03; Effective Date: 25 Oct 2010; Supersedes Date: 08 June 2010

NIH CIT Consortium Chemistry Manufacturing Controls Monitoring Committee .. NIH CIT Consortium . (2014). Functional assessment of purified human pancreatic islets: glucose stimulated insulin release by ELISA: A Standard Operating Procedure of the NIH Clinical Islet Transplantation Consortium.. CellR4- repair replacement, regeneration, & reprogramming https://doi.org/pii:e900

EXTERNAL LINK

https://iidp.coh.org/Investigators/Policies-Standard-Operating-Procedures

ATTACHMENTS

Prodo Media-Preparationand-Use-Instructions-1.pdf Attachment 4 Mercodia CIT GSIR by ELISA nihms-Insulin ELISA.pdf

Ciprofloxacin SDS-2014Jul22.pdf 995424.pdf

PIMRTechNotes.pdf Attachment 2- Microtiter Plate Layout.pdf

preparation Sheets.pdf

Attachment 3- GSIR Worksheet.pdf

DOI

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PROTOCOL CITATION

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Version created by Integrated Islet Distribution Program

KEYWORDS

Potency Test, Insulin Release Assay, Insulin, Release Assay, islets, human islets, Integrated Islet Distribution Program, IIDP, islet isolation, ELISA, Glucose Stimulated Release Assay, GSIR, Glucose stimulation, Stimulation Index, Enzyme-Linked Immuno-Sorbent Assay

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Responsibilities:

- It is the responsibility of the IIDP CC to both follow and ensure adherence to the procedures outlined in this SOP. In order to accomplish this, the IIDP CC will interact with the relevant personnel from each of the participating centers.
- It is the responsibility of each IIDP center to follow the procedures listed in this SOP and to work to the best of their ability to follow all requirements.

Definitions:

- Integrated Islet Distribution Program (IIDP) (RRID:SCR_014387): The IIDP is a contracted program commissioned and funded by the NIDDK to provide quality human islets to the diabetes research community to advance scientific discoveries and translational medicine. The IIDP consists of the NIDDK, the Project Officer (PO), the External Evaluation Committee (EEC) and the CC at City of Hope (COH). The IIDP CC integrates an interactive group of academic laboratories including the subcontracted IIDP centers.
- IIDP Coordinating Center (CC): Joyce Niland, Ph.D. is the Principal Investigator for the IIDP CC and leads staff from the Department of Research Information Sciences at COH to coordinate the activities of the IIDP and assist the participating centers and investigators in the distribution of human islets.
- Islet Equivalent (IEQ): An islet that is 150 µm diameter by mathematically compensating for their volumes.
- Glucose Stimulated Insulin Release (GSIR) Assay: A functionality assay that compares the amount of
 insulin secreted at a resting stage (low glucose concentration) of an islet preparation to the amount of insulin
 secreted during a stimulated stage (high glucose concentration) of an islet preparation. The resulting ratio is
 called the Stimulation Index.
- Stimulation Index (SI) Value: A measure of the ability of the human islet preparation to produce insulin when stimulated by an increase in the concentration of glucose.

- Enzyme-Linked Immuno-Sorbent Assay (ELISA): A sensitive immunoassay that uses an enzyme linked to an antibody or antigen as a marker for the detection of a specific protein, especially an antigen or antibody. This is the recommended assay for measuring Insulin content in the GSIR Assay.
- Radioimmunoassay (RIA): A highly sensitive and specific assay method that uses the competition between radiolabeled and unlabeled substances in an antigen-antibody reaction to determine the concentration of the unlabeled substance, which may be an antibody or a substance against which specific antibodies can be produced. This assay may be used in place of the ELISA for insulin measurement.

MATERIALS TEXT

MATERIALS

⊠HEPES Fisher

Scientific Catalog #BP310-500

Aldrich Catalog #P9541

⊠DPBS Invitrogen - Thermo

Fisher Catalog #14190

Sodium hydroxide solution Sigma

Aldrich Catalog #S2770

⋈ Hydrochloric acid solution 1.0 N Sigma

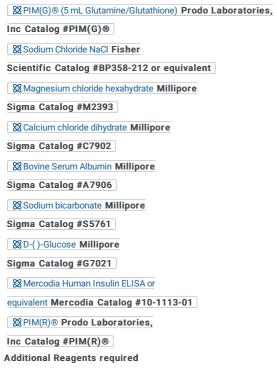
Aldrich Catalog #H9892

⊠ Human AB Serum (ABS) HI **Gemini**

Bioproducts Catalog #100-512; Heat Inactivated

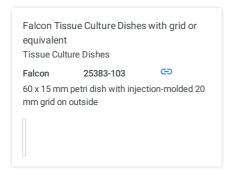


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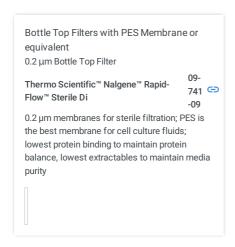
- Distilled Water
- Human Insulin ELISA kit (e.g. Mercodia)
- Calibrators
- Concentrate Wash Solution
- Conjugate Stock Solution
- Conjugate buffer
- Substrate TMB
- Stop Solution

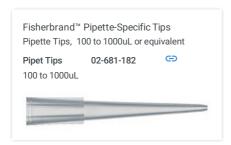
Equipment and Materials

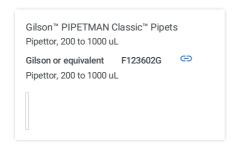












Corning 4492 or equivalent
Sterile Polystyrene Serological Pipets

07Corning™ Stripette™ Wide-Tip
Disposable Polystyren

519
Sterile, individually wrapped, and certified
nonpyrogenic and DNase-/RNase-free
Accuracy within ±2% at full volume

In addition, the following standard lab **equipment** is needed to perform the potency assay:

- 1. For Glucose Stimulation:
 - Biological Safety Cabinet (BSC)
 - 37°C CO₂ Incubator
 - Analytical Balance
 - pH Meter
 - Microscope
 - Conical tubes, 50 ml, sterile
 - Sterile tubes, 5 ml
 - 2 ml cryovials
 - Graduated cylinder
 - Petri dishes, 100 mm
 - 1 ml, 5 ml, 10 ml, and 25 ml pipettes
 - Stir bar
 - Weigh paper
 - 6 ml polypropylene tubes, 12 x 75 mm
 - $200\,\mu l$ pipette tips
 - Parafilm
 - Microcentrifuge tubes
- 2. For Insulin Assay:
 - Aluminum foil to cover plate
- 3. For ELISA Insulin Assay

Note: If using an RIA for insulin determination all of the following equipment may not be necessary.

- Microplate absorbance reader with attached computer and printer
- 37°C CO₂ Incubator
- Microtiter Plate Rotator
- Vortex

SAFETY WARNINGS

https://dx.doi.org/10.17504/protocols.io.buzhnx36

Please see attached SDS (Safety Data Sheet) for hazards and safety warnings.

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Ciprofloxacin Hydrochloride

Precautionary statements:

- P280 Wear protective gloves and eye/face protection
- P305 + P351 + P338 IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
- P337 + P313 If eye irritation persists: Get medical advice/attention.
- P273 Avoid release to the environment.

GemCell™ U.S. Origin Human Serum AB

GemCell™ human serum AB is collected from healthy male donors of the AB serotype at FDA-licensed facilities in the United States

Hazardous Components:

- Biohazard contains human source material. Handle as though capable of transmitting infectious agents.
- · Toxicity: Not Established.

Target Organs/Systems: Product could possibly irritate the skin, eyes and respiratory system. Do not ingest this product.

HEPES: Emergency Overview

Causes eye, skin, and respiratory tract irritation. Use personal protective equipment. Ensure adequate ventilation. Wash off immediately with plenty of water for at least 15 minutes. Obtain medical attention. Rinse immediately with plenty of water, also under the eyelids, for at least 15 minutes. Obtain medical attention. Move to fresh air. If breathing is difficult, give oxygen. Obtain medical attention. Do not induce vomiting. Obtain medical attention.

ELISA Kit-Stop solution

The Stop Solution contains <5% Sulphuric acid.

The Stop Solution is labeled:

Danger:

H318 - Causes serious eye damage.

H315 - Causes skin irritation.

P280 - Wear protective gloves. Wear eye or face protection.

P264 - Wash hands thoroughly after handling.

P302 + P352 + P362 + P364 - IF ON SKIN: Wash with plenty of soap and water. Take off contaminated clothing and wash it before reuse

P332 + P313 - If skin irritation occurs: Get medical attention.

P305 + P351 + P338 + P310 - IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. Immediately call a POISON CENTER or physician.

Culture Media Preparation

- Culture media that is to be used for overnight culturing is prepared as described below.
 - The Prodo Labs PIM(R) should be stored between 2° and 8°C upon receipt.
 - The (heat inactivated) Gemini AB serum and the PIM(G) vials should be stored at -5° to -20°C.
- The Ciprofloxacin powder can be stored on the shelf but filter sterilized suspension aliquots should be stored at -5° to -20°C.

Pre-Preparation of Ciprofloxacin Powder for Addition to Media

- Remove 0.5 gm (500mg) of ciprofloxacin hydrochloride from the bottle and QS to 50mL with distilled water. This will give a stock concentration of 10mg/ml.
- Mix with a stir bar and stirring plate until totally dissolved.
- Filter sterilize the solution using a 0.2µM filter. Aliquot into sterile tubes, 5mL samples, label, and freeze for later use.
- The expiration date of the solution is indicated on the Certificate of Analysis and/or the bottle. Document expiration date as date of CoA.
- Diluted solution is good for 1 year frozen (if less than CoA expiration date) and 1 month

Record preparation on Attachment 1: Solutions Preparation Sheet, of this SOP.

Prepare 1 bottle of PIM(R) media prior to the collection by adding the following:

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- Thaw and add 5 mL of PIM(G).
- Add 25 mL of AB serum (5%v/v).
- Add 0.5 mL of prepared sterile ciprofloxacin aliquot.
- Once all additives have been added to the bottle of PIM(R), it is now referred to as PIM(R) complete.

Note: If a prepared media bottle is to be used from a previous isolation, it must have been filter sterilized at the end of the previous use. The media will expire within 30 days, once it has been fully supplemented.

Record preparation on Attachment 1: Solutions Preparation Sheet, of this SOP.

Islet Sample Preparation

4 On the day of islet shipment, remove at least 400 IEQ (calculate using islet count per volume) from the purest layer using sterile technique.

The formula will be:

$$TotalIEQ/(TotalmL*1000\mu L)=xIEQ/\mu L$$
 then $400IEQ/xIEQ/\mu L=y\mu L$

- 5 Place islets in a 100 mm Petri dish, add 15 mL PIM(R) Complete, label, and cover.
- 6

Place the dish in a 37°C, 5 % CO2 incubator.

7 Culture islets for **© 12:00:00** - **© 24:00:00**.

Glucose Stimulation Reagent Preparation: Krebs Buffer Stock Solution

- 8 Combine in a 1 liter volumetric flask:
 - **3.96 g HEPES powder** (final concentration 25 mM)
 - **6.72 g NaCl** (final concentration 115 mM)
 - **2.02 g NaHCO3** (final concentration 24 mM)
 - **0.3728 g KCI** (final concentration 5 mM)
 - **0.2033 g MgCl2•6 H20** (final concentration 1 mM)
 - 1.0 g BSA (final concentration 0.1 %)

Record preparation on Attachment 1: Solutions Preparation Sheet, of this SOP.

- 9 QS to 11 L with distilled water and mix until dissolved.
- Add **_0.3675 g CaCl2•2 H20** (final concentration 2.5 mM) and stir the solution (the CaCl_{2•2} H₂O may not completely dissolve until the solution pH is adjusted).

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Check the pH of the solution and adjust to pH7.3 to pH7.5 using either [M]1 N NaOH or [M]1 N HCI as necessary.
       Filter sterilize into a sterile 1 liter bottle using a 0.22 µm bottle top filter.
  12
  13 Divide into 18 X 50-ml aliquots in sterile 50 ml tubes.
          NOTE: Reserve remaining Krebs Buffer for 280 mM stock glucose below.
  14 Label as Krebs Buffer Stock Solution with preparation date, expiration date (4 weeks after preparation), and store at
         82°C - 88°C.
Glucose Stimulation Reagent Preparation Stock Solutions: 280 mM Glucose Solution
  15 Add □3.0 g D-(+)-Glucose to □60 mL Krebs Buffer Stock Solution.
          Record preparation on Attachment 1: Solutions Preparation Sheet, of this SOP.
  16 Filter sterilize into a sterile 100 ml bottle, using a 0.22 μm bottle top filter.
  17 Divide into 10 X 6-ml aliquots, using sterile 15 ml tubes. Label with preparation date, expiration date (4 weeks after
        preparation), and store at § 2 °C - § 8 °C.
Glucose Stimulation Reagent Preparation Stock Solutions: High Glucose (28 mM) Solution
  Prepare High Glucose ( [M] 28 Milimolar (mM) ) Solution by making a 1:10 dilution of
        [M] 280 Milimolar (mM) Glucose stock, using Krebs Buffer Stock Solution. Prepare as much solution as
        needed.
          Record preparation on Attachment 1: Solutions Preparation Sheet, of this SOP.
       Label with preparation date, expiration date (1 week after preparation), and store at § 2 °C - § 8 °C.
Glucose Stimulation Reagent Preparation Stock Solutions: Low Glucose (2.8 mM) Solution
       Prepare Low Glucose ([M]2.8 Milimolar (mM)) Solution by making a 1:10 dilution of
        [M] 28 Milimolar (mM) High Glucose Solution , using Krebs Buffer Stock Solution. Prepare as much solution
        as needed.
          Record preparation on Attachment 1: Solutions Preparation Sheet, of this SOP.
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Label with preparation date, expiration date (1 week after preparation), and store at 8 °C - 88 °C. Glucose Stimulation: Plate preparation Label a 24-well plate with sample ID and date. 23 Add 1.0 mL [M]2.8 Milimolar (mM) Low Glucose Solution per well to wells A1, A2, A3. Add 1.3 mL of [M]2.8 Milimolar (mM) Low Glucose Solution per well to wells B1, B2, B3. 25 Add 1.3 mL of [M]28 Milimolar (mM) High Glucose Solution per well to wells C1, C2 and C3. Using forceps, place one Millicell Cell Culture Plate Insert (insert) into well A1, A2 and A3. 26 27 Cover plate with lid and incubate the plate to \$37 °C in 5 % CO2 incubator for @01:00:00 (to equilibrate pH and temperature of media). 28 Transfer 10 mL of each glucose solution into a marked tube (High Glucose Control and Low Glucose Control) and store at § 4 °C - § 8 °C until assay completion. Glucose Stimulation: Preparation of islets Remove the Petri dish containing islets from the incubator. 30 Collect the islets from the Petri dish by centering the tissue in the dish and removing the islets in 100 μl PIM(R) complete media, using a wide bore micropipette. Transfer the islets to a 5 ml conical tube. Add 1400 µl PIM(R) complete media to the tube. **NOTE:** If 100 % of the 400 IEQ are cultured and there is overnight loss, there are 80 IEQ/300 μ l in this suspension

NOTE: If 100 % of the 400 IEQ are cultured and there is overnight loss, there are 80 IEQ/300 μ l in this suspension of islets. There will be a loss of islets during the culture, so the recommended target of 50 – 75 IEQ per well is likely to be achieved. (A 300 μ l aliquot containing ~80 IEQ will be added to each insert in the next step). 400 IEQ ÷ 1500 μ l (100 μ l islets in PIM(R) Complete Media + 1400 μ l PIM(R) Complete media) = 0.26 IEQ/ μ l 0.26 IEQ/ μ l 300 μ l = 79.9 IEQ per well (with no islet loss).

Glucose Stimulation: Basal Equilibration (Row A) 33 Place an insert in each of the empty wells D1, D2, D3. Slowly pipette $\square 300 \ \mu I$ of *well-mixed islet suspension* into each insert in *row D*. Place insert on sterile gauze to drain liquid out completely. Transfer insert with islets to the wells A1, A2, A3. 36 Replace the lid on the plate and incubate for (01:00:00 at 8 37 °C in 5 % CO2. Glucose Stimulation: Low Glucose Stimulation (Row B) 37 Remove plate from incubator. Transfer the inserts from row A to corresponding wells in row B (B1, B2, B3) as described below: 38 Row A allows islets to equilibrate from the culture media to Krebs basal media. Samples from these wells in Row A are discarded at the end of the procedure. 38.1

Slowly and gently, lift the insert up from its corresponding well, using forceps.

NOTE: Bumping insert will cause the islets to release insulin resulting in false stimulation results.

- 38.2 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.
- 38.3 When the insert looks well-drained, gently blot the bottom of the insert on sterile gauze before gently setting the insert in the corresponding well in row B.
- 38.4 Repeat the above steps from 38.1-38.3 for all inserts from row A to row B.
- Take Zero Time media samples for Low Glucose media from wells B1, B2, B3:

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39.1 Allow the level of liquid to equilibrate between inside and outside of each insert (now in its corresponding well).

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- 39.2 Lift each insert and drain the liquid back into its corresponding well. Do not blot the insert on sterile gauze.
- 39.3 Remove 300 μl of media from each well (from the well, not the insert). Transfer media to corresponding microcentrifuge tubes labeled "Zero time Low Glucose", *B1*, *B2*, or *B3*, Lot#, Date, Tech.
- 39.4 As soon as the sample is collected, immediately replace the insert in its corresponding well. Repeat this for each of the inserts in *row B*.
- 40

Replace the lid on the plate and incubate for **© 01:00:00** at **§ 37 °C** in **5 % CO₂**.

- 41 After 1 hour, carefully remove the plate from incubator.
- Remove inserts from *row B* wells and transfer each into the corresponding well in row *C* as described above.

 Collect all media from each well in row B to corresponding microcentrifuge tubes labeled "Low Glucose", *B1*, *B2*, or *B3* and store at § 4 °C § 8 °C until assay completion.
- 43 Take Zero Time media samples for High Glucose media as described in steps 39.1 through 39.4 above but transfer media to tubes labeled "Zero time - High Glucose".

Glucose Stimulation: High Glucose Stimulation (Row C)

44

Replace lid on plate and incubate for **© 01:00:00** at § 37 °C in 5 % CO₂.

- 45 After 1 hour, carefully remove the plate from incubator.
- Remove inserts from row C wells and transfer each into the corresponding well in row D as described above. Collect all media from each well in row C to corresponding microcentrifuge tubes labeled "High Glucose", C1, C2, or C3.

Sample Storage

47 According to Mercodia (kit manufacturer), 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. Place all samples in appropriate storage.

Note, if RIA is the assay to be used for insulin determination, store as the RIA protocol recommends.

Assay Insulin Samples

48 Assay insulin samples using an ELISA assay kit (such as Mercodia) within 10 days of broadcast.

If RIA is the assay to be used for insulin determination, results should be available and entered into the database within 10 days of broadcast.

Assay Results

49 Results should be reported on the website under Potency Assay as Stimulation Index (SI);

 $Stimulation\ Index(SI) = \left(egin{array}{ll} rac{Insulin\ concentration\ after\ high\ glucose\ concentration\ stimulation\ insulin\ concentration\ after\ low\ glucose\ low\ glucose\ low\ glucose\ low\ glucose\ low\ glucose\ low\ glucose\ glucose\ glucose\ glucose\ gluc$

Results from each of three samples are averaged and reported within 10 days of broadcast.

Preparation of Human Insulin ELISA Solutions

50

Note: It is not required to use the ELISA for measuring insulin content of media. If using an RIA however, standards should be established and dilutions should be made in accordance with the assay's recommendations.

Human Insulin ELISA Kit

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

Preparation of Human Insulin ELISA Solutions: Wash Solution

Prepare Wash Solution by adding **800 mL distilled water** and **40 mL Concentrate Wash Solution** to a clean 1 l bottle. Mix the solution well.

Record preparation on Attachment 1: Solutions Preparation Sheet, of this SOP.

- 52 Label the bottle: Wash Solution with Preparation Date, Expiration Date (4 weeks after preparation), Lot #, and preparer's initials.
- 53 Store at A 2 °C A 8 °C.

Preparation of Human Insulin ELISA Solutions: Anti-insulin Conjugate Solution

54

Prepare immediately prior to use.

Record preparation on Attachment 1: Solutions Preparation Sheet, of this SOP.

For each microstrip used, mix $\square 100 \ \mu l$ Conjugate Stock Solution with $\square 1 \ mL$ Conjugate Buffer . Examples:

Α	В	С
# of Strips	Conjugate Stock Solution	Conjugate
		Buffer
1	100 μΙ	1 ml
6	600 μΙ	6 ml
12	1.2 ml	12 ml

- 55 Label the bottle: Conjugate Solution with Preparation Date, Expiration Date (1 day after preparation), Lot #, and preparer's initials.
- 56 Store at & 2 °C & 8 °C.

Preparation of Human Insulin ELISA Solutions: Preparation of Controls

Remove the Diabetes Ag Control vials from the refrigerator and allow them to equilibrate to A Room temperature for © 00:05:00.

Record preparation on Attachment 1: Solutions Preparation Sheet, of this SOP.

- 58 Reconstitute Low and High Insulin controls as follows:

 - Replace cap and let sit for **© 00:05:00**.
 - Mix vial to dissolve contents by swirling gently. Avoid producing bubbles.
- 59 Aliquot **395 μl** of **"Low Control"** into 5 1-ml microcentrifuge tubes.
 - Label as: "Insulin Low Control, 95 μl" with Preparation Date, Expiration Date (3 months after preparation), Lot #, and preparer's initials.
 - Store at < § -20 °C .
- 60 Aliquot **95 μl** of "**High Control**" into 5 1-ml microcentrifuge tubes.
 - Label as: "Insulin High Control, 95 μl" with Preparation Date, Expiration Date (3 months after preparation), Lot #, and preparer's initials.
 - Store at < § -20 °C .
- 61 Establishment of acceptable ranges for a new lot of controls using manufacturer's procedures.

Sample Dilution

- 62 Supernatants collected from the glucose challenge wells are diluted using DPBS 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.
 - 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.

Human Insulin ELISA Procedure

63

See Attachment 3: Glucose Stimulated Insulin Release Worksheet

Bring all reagents and samples to § Room temperature

- Prepare sufficient microplate wells to accommodate calibrators, controls and samples in triplicate. 64 Record the plate location of samples on Attachment 2: Microtiter Plate Layout.
- 65 Pipette 25 μl each, calibrators, controls and samples into appropriate wells. Pipette directly onto the bottom of the well. Change tips between each standard, control and sample.
- 66 Add 100 µl Enzyme Conjugate to each well.



Cover the plate and incubate on a plate shaker at @800 rpm, 01:00:00 at & 18 °C - & 25 °C.

68

Wash manually: fill each well completely with Wash Buffer (□350 μl) 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.

- 68.1 Fill each well completely with Wash Buffer (\blacksquare 350 μ I) using a squeeze bottle. Turn the plate upside down over a sink and shake to discard liquid completely. (1/6)
- 68.2 Fill each well completely with Wash Buffer (\bigcirc 350 μ l) using a squeeze bottle. Turn the plate upside down over a sink and shake to discard liquid completely. (2/6)
- 68.3 Fill each well completely with **Wash Buffer** (350 µl) using a squeeze bottle. Turn the plate upside down over a sink and shake to discard liquid completely. (3/6)
- 68.4 Fill each well completely with Wash Buffer (\blacksquare 350 μ I) using a squeeze bottle. Turn the plate upside down over a sink and shake to discard liquid completely. (4/6)
- 68.5~ Fill each well completely with Wash Buffer ($_{\blacksquare}350~\mu l$) using a squeeze bottle. Turn the plate upside down over a sink and shake to discard liquid completely. (5/6)
- 68.6 Fill each well completely with Wash Buffer (350 µl) using a squeeze bottle. Turn the plate upside down over a sink and shake to discard liquid completely. (6/6)
- 68.7 Invert and tap the plate firmly against absorbent paper.
- 69 Add 200 µl TMB Substrate Solution into each well.



70

Cover plate with aluminum foil to protect it from direct light and incubate for © 00:15:00 at

§ Room temperature (§ 18 °C - § 25 °C).

71 Add 30 pl Stop Solution to each well. Place plate on a shaker for approximately 00:00:05 to ensure mixing.

72 (8

Read optical density at 450 nm using bi-chromatic measurement with reference at 600 - 690 nm within © 00:30:00.

73

Record the data on Attachment 3: Glucose Stimulated Insulin Release Worksheet, of this SOP, and perform calculations required.

Quality Control, Interpretation and Release Criteria

74 L

For assay acceptance:

- All control values must be within their 2 SD established ranges
- $\,\bullet\,$ Standard values must be within ± 15 % of their nominal values
- Triplicates of standards, controls and samples must have Coefficients of Variation (CV) ≤ 20 %

For an unacceptable assay: If the assay is unacceptable for any of these reasons, sample values may not be reported. The assay should be investigated and repeated.