Apr 29, 2020 Version 1

Potency Test: Glucose Stimulated Insulin Release Assay V.1

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Integrated Islet Distribution Program[1]

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

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

Integrated Islet Distribution Program
Integrated Islet Distribution Program, City of Hope

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We use this protocol and it's working

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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.

GSIR Overview:
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.

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.

CITATION

NIH CIT Consortium Chemistry Manufacturing Controls Monitoring Committee ., NIH CIT Consortium . (2013). 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.

LINK

https://doi.org/pii:e900

Attachments

Prodo Media-Preparat...
441KB

Ciprofloxacin SDS-20...
64KB

PIMRTechNotes.pdf
863KB

Attachment 1-Solutio...
191KB

Attachment 2- Microt...
132KB

Attachment 3- GSIR W...
160KB

Attachment 4 Mercodi...
280KB

CIT GSIR by ELISA ni...
378KB

Guidelines

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):*** 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.

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

- ***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

MATERIALS

- HEPES **Fisher Scientific** **Catalog #**BP310-500
- Potassium Chloride **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**P9541
- DPBS **Invitrogen - Thermo Fisher** **Catalog #**14190
- Sodium hydroxide solution **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**S2770
- Hydrochloric acid solution 1.0 N **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**H9892
- Human AB Serum (ABS) HI **Gemini Bio-Products** **Catalog #**100-512; Heat Inactivated
- PIM(G)® (5 mL Glutamine/Glutathione) **Prodo Laboratories, Inc** **Catalog #**PIM(G)®
- Sodium Chloride NaCl **Fisher Scientific** **Catalog #**BP358-212 or equivalent
- Magnesium chloride hexahydrate **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**M2393
- Calcium chloride dihydrate **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**C7902
- Bovine Serum Albumin **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**A7906
- Sodium bicarbonate **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**S5761
- D-( )-Glucose **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**G7021
- Mercodia Human Insulin ELISA or equivalent **Mercodia** **Catalog #**10-1113-01
- PIM(R)® **Prodo Laboratories, Inc** **Catalog #**PIM(R)®

Additional Reagents required

- 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

Equipment

Falcon Tissue Culture Dishes with grid or equivalent

NAME

Tissue Culture Dishes

TYPE

Falcon

BRAND

25383-103

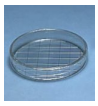
SKU

https://us.vwr.com/store/catalog/product.jsp?catalog_number=25383-103

LINK

60 × 15 mm petri dish with injection-molded 20 mm grid on outside

SPECIFICATIONS



Equipment

Eppendorf® Cell Culture Plates or equivalent

NAME

Cell Culture Plate

TYPE

Eppendorf®

BRAND

EP0030722019

SKU

https://www.sigmaaldrich.com/catalog/product/sigma/ep0030722019?lang=en&region=US

LINK

size 24 wells, surface treatment non-treated, flat bottom clear wells

SPECIFICATIONS

Equipment

Millicell® Cell Culture Insert

NAME

Cell Culture Inserts

TYPE

Millicell®

BRAND

PIXP01250

SKU

https://www.emdmillipore.com/US/en/product/Millicell-Cell-Culture-Insert-12mm-polycarbonate-12m,MM_NF-PIXP01250?bd=1

LINK

Millicell Cell Culture Insert, 12 mm, polycarbonate, 12 µm

SPECIFICATIONS

Equipment

Bottle Top Filters with PES Membrane or equivalent

NAME

0.2 µm Bottle Top Filter

TYPE

Thermo Scientific™ Nalgene™ Rapid-Flow™ Sterile Di

BRAND

09-741-09

SKU

https://www.fishersci.com/shop/products/nalgene-rapid-flow-sterile-disposable-bottle-top-filters-pes-membrane/0974109

LINK

0.2 µm membranes for sterile filtration; PES is the best membrane for cell culture fluids; lowest protein binding to maintain protein balance, lowest extractables to maintain media purity

SPECIFICATIONS

Equipment

Fisherbrand™ Pipette-Specific Tips

NAME

Pipette Tips, 100 to 1000uL or equivalent

TYPE

Pipet Tips

BRAND

02-681-182

SKU

https://www.fishersci.com/shop/products/fisherbrand-pipetter-specific-tips-natural-100-1000-l-101-6mm-long-bulk-pack/02681182#?keyword=true

LINK

100 to 1000uL

SPECIFICATIONS

Equipment

Gilson™ PIPETMAN Classic™ Pipets

NAME

Pipettor, 200 to 1000 uL

TYPE

Gilson or equivalent

BRAND

F123602G

SKU

https://www.fishersci.com/shop/products/gilson-pipetman-classic-pipets-8/f123602g

LINK

Pipettor, 200 to 1000 uL

SPECIFICATIONS

Equipment

Corning 4492 or equivalent

NAME

Sterile Polystyrene Serological Pipets

TYPE

Corning™ Stripette™ Wide-Tip Disposable Polystyren

BRAND

07-200-619

SKU

https://www.fishersci.com/shop/products/stripette-disposable-plastic-wrapped-polystyrene-serological-pipettes-2/07200619?searchHijack=true&searchTerm=07200619&searchType=RAPID&matchedCatNo=07200619

LINK

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

SPECIFICATIONS

Equipment

Fisherbrand™ Large-Orifice Pipet Tips, 1 to 200µL or equivalent	NAME
Genomic/Wide Orifice Pipet Tips	TYPE
Fisherbrand	BRAND
02-707-134	SKU
https://www.fishersci.com/shop/products/fisherbrand-large-orifice-pipet-tips-6/02707134	LINK
1 to 200µL	SPECIFICATIONS

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

1. For Glucose Stimulation:
   - Biological Safety Cabinet (BSC)
   - 37°C CO_2 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 × 75 mm
- 200 µl pipette tips
- Parafilm
- Microcentrifuge tubes

2. For Insulin Assay:
   - Aluminum foil to cover plate

3. For ELISA Insulin Assay

Note

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 CO_2 Incubator
- Microtiter Plate Rotator
- Vortex

Safety warnings

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

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 wa ter. 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

1 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.

2 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 thawed.

Note

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

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

- 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

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.

Note

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 % CO_2** 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:

- 5.96 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 KCl (final concentration 5 mM)
- 0.2033 g MgCl2•6 H2O (final concentration 1 mM)
- 1.0 g BSA (final concentration 0.1 %)

Note

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

9 QS to 1 L with distilled water and mix until dissolved.

10 Add 0.3675 g CaCl2•2 H2O (final concentration 2.5 mM) and stir the solution (the $CaCl_2$•2 H_2O may not completely dissolve until the solution pH is adjusted).

11 Check the pH of the solution and adjust to pH 7.3 to pH 7.5 using either [M] 1 N NaOH or [M] 1 N HCl as necessary.

12 Filter sterilize into a sterile 1 liter bottle using a 0.22 µm bottle top filter.

13 Divide into 18 X 50-ml aliquots in sterile 50 ml tubes.

Note

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 2 °C – 8 °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.

Note

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**

18 Prepare High Glucose ([M] 28 millimolar (mM)) Solution by making a 1:10 dilution of [M] 280 millimolar (mM) Glucose stock , using **Krebs Buffer Stock Solution**. Prepare as much solution as needed.

Note

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

19 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**

20 Prepare Low Glucose ([M] 2.8 millimolar (mM)) Solution by making a 1:10 dilution of [M] 28 millimolar (mM) High Glucose Solution , using **Krebs Buffer Stock Solution**. Prepare as much solution as needed.

Note

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

21 Label with preparation date, expiration date (*1 week after preparation*), and store at 2 °C – 8 °C.

Glucose Stimulation: **Plate preparation**

22 Label a 24-well plate with sample ID and date.

23 Add 1.0 mL [M] 2.8 millimolar (mM) Low Glucose Solution per well to wells *A1*, *A2*, *A3*.

24 Add 1.3 mL of [M] 2.8 millimolar (mM) Low Glucose Solution per well to wells *B1*, *B2*, *B3*.

25 Add 1.3 mL of [M] 28 millimolar (mM) High Glucose Solution per well to wells *C1*, *C2* and *C3*.

26 Using forceps, place one Millicell Cell Culture Plate Insert (insert) into well *A1*, *A2* and *A3*.

27 Cover plate with lid and incubate the plate to 37 °C in **5 % CO_2** incubator for 01:00:00 (to equilibrate pH and temperature of media).

28 Transfer 1.0 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**

29 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.

31 Transfer the islets to a 5 ml conical tube.

32 Add 1400 µL PIM(R) complete media to the tube.

Note

NOTE: *If 100 % of the 400 IEQ are cultured and there is overnight loss, there are 80 IEQ/300 µl 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 µl aliquot containing ~80 IEQ will be added to each insert in the next step). 400 IEQ ÷ 1500 µl (100 µl islets in PIM(R) Complete Media + 1400 µl PIM(R) Complete media) = 0.26 IEQ/ µl 0.26 IEQ/µl X 300 µl = 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*.

34 Slowly pipette 300 µL of ***well-mixed islet suspension*** into each insert in *row D*.

35 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 37 °C in **5 % CO_2**.

Glucose Stimulation: **Low Glucose Stimulation (Row B)**

37 Remove plate from incubator.

38 Transfer the inserts from *row A* to corresponding wells in *row B* (*B1*, *B2*, *B3*) as described below:

> Note
>
> *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
>
> 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*.

39 Take Zero Time media samples for Low Glucose media from wells B1, B2, B3:

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

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 % CO_2**.

41 After 1 hour, carefully remove the plate from incubator.

42 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 % CO_2**.

45 After 1 hour, carefully remove the plate from incubator.

46 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 – 8ºC** must be assayed **within 24 hours**, while samples stored at stable **-20ºC** can be assayed **within 3 months**. Place all samples in appropriate storage.

Note

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.

Note

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);

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

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

Preparation of Human Insulin ELISA Solutions

50

Note

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**

51 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.

Note

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 2 °C – 8 °C .

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

54

Note

Prepare immediately prior to use.

Note

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

For each microstrip used, mix 100 µL Conjugate Stock Solution with 1 mL Conjugate Buffer. Examples:

	# of Strips	Conjugate Stock Solution	Conjugate Buffer
	1	100 µl	1 ml
	6	600 µl	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**

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

Note

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

58 **Reconstitute Low and High Insulin controls as follows:**

- Add 500 µL distilled water of to each vial.
- Replace cap and let sit for 00:05:00.
- Mix vial to dissolve contents by swirling gently. Avoid producing bubbles.

59 Aliquot 95 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 (see *Attachment 3: Glucose Stimulated Insulin Release Worksheet*)

63 Bring all reagents and samples to Room temperature .

64 Prepare sufficient microplate wells to accommodate calibrators, controls and samples **in triplicate**.
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.

67 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** (350 µL) 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** (350 µL) 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** (350 µL) 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** (350 µL) 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 50 µL Stop Solution to each well. Place plate on a shaker for approximately 00:00:05 to ensure mixing.

72 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 For assay acceptance:

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

Note

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.

Citations

NIH CIT Consortium Chemistry Manufacturing Controls Monitoring Committee; NIH CIT Consortium. Functional assessment of purified human pancreatic islets: glucose stimulated insulin release by ELISA: A Standard Operating Procedure of the NIH Clinical Islet Transplantation Consortium
http://www.ncbi.nlm.nih.gov/pubmed/30615353