


DAIT, NIAID, NIH				
ASSAY INSTRUCTIONS				
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<p>Document Title:</p> <p style="text-align: center;">PURIFIED HUMAN PANCREATIC ISLETS, <i>IN VIVO</i> ISLETS FUNCTION</p>				

1.0 PURPOSE:

To describe the nude mouse bioassay procedure that will be use for in vivo potency assessment of lots manufactured for use in DAIT-sponsored CIT clinical studies. This assay measures the restoration of normoglycemia in chemically-induced diabetic mice transplanted with an aliquot of the Purified Human Pancreatic Islets product.

2.0 RESPONSIBILITY:

It is the responsibility of the Islet 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,
- assure that the site-specific procedure is executed, and
- maintain records of the execution of the site-specific procedure.

3.0 SCOPE:

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

4.0 INTRODUCTION

Diabetes is induced in euglycemic immunodeficient mice by administration of a beta cell toxic drug, for example streptozotocin (STZ). An aliquot from each purified human pancreatic islet product lot is transplanted under the left kidney capsule in two recipient mice. Subsequently, blood glucose levels are monitored for up to 28 days. Reversal of hyperglycemia and maintenance of euglycemia will provide evidence of functionality of the islet product. If diabetes has been reversed at 28 days post-transplant, a left nephrectomy is performed to remove the transplanted islets, with a rise in glucose levels indicating the reversal of diabetes was the result of the transplanted human islet product.

The assay procedure has four stages:

- Induction of diabetes in immunodeficient mice by administration of a beta cell toxic drug.
- Transplantation of an aliquot of the human islet product under the left kidney capsule of the mice rendered diabetic.
- Monitoring of blood glucose levels in mouse recipients to assess transplanted islet function.
- Nephrectomy of kidney containing transplanted islets to confirm biological activity/function of the transplanted islet product.

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5.0. Intra-facility Procedural Preference and Variation

There are a number of institutional, laboratory, and personal preferences in mouse strains, diabetes-inducing beta cell toxins, and surgical transplant procedures that should not have bearing on the goal of this assay, which is to assess the *in vivo* functionality of the prepared human islet product in diabetic mice.

The following procedure should be considered a general guideline for this assay. Certain aspects of this protocol should NOT be deviated from between testing centers, as delineated in Section 6.1. Other procedural variations from this protocol may occur without expected impact on the assay results. Please decide on and document your facility's practice in your institution's SOP.

Areas where strict compliance is requested are listed below. If there is personal, facility or institution IACUC objection to these, please notify the site principal investigator prior to conducting these assays.

6.0. PROCEDURE

6.1 Protocol components that are *not* to be deviated from:

6.1.1 Transplanted islets number and timing:

- 2000 IEQ of purified human islets product per mouse
- Islets will be transplanted into mice within 24 hours following sampling.
- Between isolation and transplant into mice, islets will be cultured in CIT Culture Media for 12 to 24 hours at 37°C and 5% CO₂, and for up to a total of 72 hours at 22°C and 5% CO₂.

6.1.2 Mouse recipient number:

- Two mice per human islet batch will be evaluated.

6.1.3 Definition of hyperglycemia:

- Blood glucose levels of > 350 mg/dL

6.1.4 Definition of diabetes:

- The first day of two consecutive readings of hyperglycemia (measured at least 3 days per week)

6.1.5 Definition of normoglycemia:

- Sustained blood glucose for at least three glucose determinations on different days of readings of ≤ 200 mg/dL

6.1.6 Mice housing and handling:

- Mice should be maintained in a VAF/SPF/sterile facility, in sterile housing, receiving sterile food and water, and handled in a sterile manner according to your institution's policies and procedures on immunodeficient mice. All reagents and instruments should be sterile prior to use.

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6.1.7 Assay length/timeline:

- Mice will be maintained for at least 4 weeks following transplantation. If mice have not had restoration of euglycemia at 28 days following transplant, mice may be sacrificed and discarded. If at 28 days mice are normoglycemic, the mice will receive left nephrectomy within the next 7 days. If such mice re-develop diabetes, they will be sacrificed and discarded. If nephrectomized mice remain normoglycemic, they can be sacrificed and discarded 14 days following nephrectomy. If mice have signs or symptoms of illness or infection, notify your veterinary staff, check blood glucose and proceed with your institution's routine policies (including sacrifice if need be) for supportive care vs. euthanasia.

6.1.8 Frequency, documentation and reporting of blood glucose determination:

Record blood glucoses at least 3 days per week. Record glucose concentrations in mg/dL. Viewing data presented graphically may be helpful.

6.2 Facility-specific variations:

Please record in institutional SOP the following information. If your group believes that their practice needs to deviate from an accepted alternative procedure or wishes to evaluate a novel alternative, please contact the protocol administrator.

6.2.1 Immunodeficient mice:

- Nude mouse of either sex.

Age/Weight:

- 4 – 12 weeks (correlates to approximately ~25 – 35g)

Please note in records: strain, immunodeficiency, genetic background, sex, age and weight.

6.2.2 Diabetogenic agent:

Diabetes can be induced in mice using a variety of beta cell toxins, two of the most common are streptozotocin and alloxan. There are various methods to prepare and administer these agents with the same end effect: beta cell death and diabetes.

For example, streptozotocin can be purchased as streptozotocin /citrate mixture (Zanosir) or mixed from individual constituents (various recipes).

- dose range 150 – 240 mg/kg
- usually given as a single dose or repeated once
- can be dose intravenously (IV) or intraperitoneally (IP)

Please record the specifics regarding your method to induce chemical diabetes.

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6.2.3 Glucometer:

- Numerous options and vendors for “fingerstick” products, please QC and check for accuracy per facility’s standards and operator’s manual.

6.2.4 Anesthesia:

- Options include inhaled (i.e. halothane) or parenteral (i.e. fentanyl/Versed or ketamine)
- Please defer to individual facility and IACUC for most appropriate drug and route.

6.2.5 Obtaining blood for glucose determination:

- The most standard method to obtain a drop of blood for glucometry is to securely scruff the mouse and clean the tail with 70% ethanol and dry. Nick the end of the tail with sterile scissors, razor blade or syringe needle. If necessary, milk the tail to obtain a drop of blood and draw up in glucometry strip or cuvette. Achieve hemostasis on the tail using pressure or brief cautery.
- Individual facilities and IACUCs may have additions and/or alternatives to this strategy to obtain blood for glucose determination. Please defer to local requests and policies.

6.3 Additional materials (all sterile unless notes):

- 15 mL conical tubes (i.e. Falcon)
- polyethylene tubing PE-50
- microfuge tubes
- polystyrene Petri dishes
- 1 mL syringe with 23 g needle or “Precision” syringe
- tabletop centrifuge
- parafilm
- sterile gloves
- appropriate personal protective devices and garb
- microsurgery hood
- microsurgery scissors
- microsurgery forceps
- cotton swabs
- gauze
- cautery
- 70% ethanol , dilute betadine solution (i.e. 1:10 v:v (betadine:sterile water)), or other skin disinfectant
- electric shaver (cleaned with alcohol or betadine)
- tape
- microsurgery table/plate
- dissecting microscope/magnifying glasses (loops)
- microsurgical staples/sutures (i.e. (i.e. 3-0 chromic gut or vicryl)
- HBSS (HEHPE-buffered saline solution)

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6.4 Preparation of chemically diabetic mice:

- 6.4.1 In a sterile manner, parenterally (IV or IP) administer facility-specific diabetogenic drug (i.e. streptozotocin or alloxan) at least 5-7 days prior to anticipated islet transplantation. Although only 2 mice will be transplanted, consider administering drug to 3 – 4 mice, as all treated mice may not be rendered diabetic and/or some mice expire from this treatment. Verify mice are normoglycemia prior to drug administration.
- 6.4.2 Maintain mice in sterile housing with access to adequate sterile food and water.
- 6.4.3 Check blood glucose levels daily to identify mice rendered diabetic and record.
- Mice not diabetic after 5 days may be retreated with diabetogenic drug.

Note: Mice rendered chemically diabetic will usually survive well for prolonged periods without the administration of exogenous insulin as not all beta-cell mass is destroyed, which prevents diabetic ketoacidosis. Be sure mice have adequate water supply and food. Some facilities prefer, and IACUCs require, treating such chemically rendered diabetic mice with exogenous insulin. Defer to local practice and regulations. If administering exogenous insulin, stop at least 2 days prior to transplant so hyperglycemia can be confirmed prior to transplant.

6.5 Islets Transplantation:

- 6.5.1 Islets Preparation (all to take place under sterile conditions)
- 6.5.1.1 On day of Purified Human Pancreatic Islets transplant, transfer 4000 IEQ from the containers for each product bag with a purity of 70% or greater. Place cells in a 37°C incubator with 5% CO₂ until staff is ready to perform murine islet transplant, which should occur within 24 hours following sampling.
- 6.5.1.2 To prepare islets, remove from incubator and transfer islets with a sterile pipet to a 15 mL conical tube and qs to 10-15 mL with HBSS. Allow islets to settle by gravity (10 min) or low speed centrifugation (i.e. 400g x 4 min). Gently mix by pipetting up and down. Wash twice using HBSS by allowing islets to settle for 10 minutes between each wash, and aspirating supernatant, or using low speed centrifugation (i.e. 400g x 4 min).
- 6.5.1.3 Aspirate supernatant, and then add 1 – 2 mL HBSS. Mix by gently pipetting up and down and swirling and then split the media and islets to two 1.5 mL microfuge tubes or two small sterile Petri dishes.
- 6.5.1.4 If using microfuge tubes, allow islets to settle and aspirate most of the supernatant. If using Petri dishes, add enough HBSS so that islets can be swirled to the center of the dish.
- 6.5.1.5 Draw up HBSS in precision syringe or 1 mL syringe with 23g needle. Attach syringe to a 10 cm length of gas sterilized polyethylene tubing PE-50, and flush, leaving ~250 µL of the HBSS in the syringe.

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6.5.1.6 Place distal end of tubing near islet pellet (if using microfuge tube) or in center of swirled islets (if using Petri dish). If using islets in microfuge tube, gently pipet up and down and stirring to loosen pellet. Gently draw up islets in tubing, being sure islets stay in tubing and do not enter syringe. Assure all islets are drawn up, which may be facilitated using magnification/microscopy.

6.5.1.7 Remove tubing from syringe, bend tubing in half, and push bent end in 15 mL conical tube so that both ends are at the opening of the tube. Screw cap on or seal conical tube with parafilm.

6.5.1.8 To concentrate islets at the bend of tubing, centrifuge tube at 250 – 400 rpm x 1 to 2 min in a tabletop centrifuge at room temperature.

6.5.1.9 Transplant the islets into the mice promptly (Section 6.5.3, below).

6.5.2 Mouse Preparation

6.5.2.1 Identify hyperglycemic mouse previously rendered diabetic. Re-check blood glucose and document.

6.5.2.2 Anesthetize mouse per facility regulations using parenteral or inhaled drugs.

6.5.2.3 When mouse is asleep, shave left flank with clippers. Clean area using 70% alcohol, dilute betadine solution, or other skin disinfectant.

6.5.2.4 Place mouse with left lateral side up on a sterile tray, placing a small amount of rolled gauze under mid section (to “prop out” left mid section), slightly outstretch mouse and tape down fore and hind feet. Keep close observation of respiratory effort.

6.5.2.5 At this time the mouse can be placed under a dissecting microscope, with appropriate lighting, with the rostral end pointing away from the operator.

6.5.2.6 Identify the intra-abdominal left kidney by sight or by a slight bulge. The kidney will be just caudal to the red/maroon spleen. Using sterile dissecting forceps and scissors make approximately 1 cm incision in skin and muscle fascia overlying kidney. Dab any blood with cotton swab or gauze.

6.5.2.7 Being careful to avoid bruising, tease the kidney with forceps and/or cotton swabs. Some prefer to surround the kidney with gauze to assist in keeping kidney outside the peritoneum. Be sure to prevent the kidney from drying out by occasionally applying drops of HBSS.

6.5.2.8 Make a small nick (3 – 5 mm) in the rostral OR caudal pole of the ventral side of the exposed kidney capsule with scissors or a syringe needle. (Rostal or caudal pole will depend on facility /operator preference, experience and comfort)

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6.5.3 Islets Transplantation

- 6.5.3.1 Re-attach precision syringe/1 mL syringe with 23 g needle and ~250 mL HBSS to PE tube containing settled islets. If there is air at the end of the tube, cut the tube below the air:water interface so that there is a continuous column of fluid between the syringe, tubing and islets.
- 6.5.3.2 Cut tubing just distal to the settled islets at a slight angle.
- 6.5.3.3 Moisten tubing and carefully insert into subcapsular space along the ventral side of the kidney to the opposite pole. This requires particular care and caution. Prior to this, some will use a moistened spatula or forceps to loosen the capsule from the parenchyma and form a track for the tubing.
- 6.5.3.4 Gently express the islets from the tubing by depressing the traditional syringe or closing the precision syringe. Using the microscope can facilitate assurance of proper placement and full ejection of islets from tubing.
- 6.5.3.5 Remove tubing. If islets are particularly clumped, they can be spread by gently pressing (with syringe tubing, cotton swab, etc).
- 6.5.3.6 Apply slight pressure to and/or cauterize the entrance of the capsule so islets will not flow out of subcapsular space.
- 6.5.3.7 Re-introduce the kidney into the peritoneum. Assure that both the muscle layer and the skin are sutured or stapled. (Failure to appropriately approximate and close the muscle layer will predispose to abdominal hernia.)
- 6.5.3.8 Clean any residual surface blood from coat. Consider applying a dab of triple antibiotic ointment and bandage.
- 6.5.3.9 Allow mice to recover from anesthesia in a new sterile cage, under a warming light or on a heating pad. Observe mice hourly until fully recovered and administer analgesic as determined by facility/IACUC.
- 6.5.3.10 Repeat with second mouse and second batch of islets.
- 6.5.3.11 Following recovery, check blood glucose of mice at least 3 times per week for 4 weeks. If staples or non-absorbable sutures are used, remove 5-7 days following transplant.

6.6 Nephrectomy. If at 28 days diabetes has been reversed following islet transplantation, nephrectomy will be performed to assure that the restoration of euglycemia is from transplanted islets. Perform nephrectomy within 7 days of the 28 day "end-of assay" timepoint.

- 6.6.1 Return mouse with islet transplant to procedure area and administer anesthesia. If fur has grown back, shave left flank again. Clean flank with skin disinfectant.
- 6.6.2 Place on surgical plate as before with the left side up, propping up mid-section, and tape down feet.

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- 6.6.3 Cut 1 cm vertical incision through skin and muscle at previous incision site.
- 6.6.4 Tease out kidney from peritoneum with forceps and cotton swab, using caution as adhesions around the kidney may have formed. If so, these may require cutting (using a blade, scissors, and/or cautery).
- 6.6.5 Using sutures or staples, ligate renal efferent and afferent renal vessels. Using scissors, blade, or cautery cut vessels and connective tissue between kidney and sutures/staples.
- 6.6.6 Remove kidney (may be processed for histochemistry as needed) and assure hemostasis. If bleeding, attempt to staple, tie, or cautery.
- 6.6.7 Close site and suture or staple, allow animals to recover as noted above, and return to sterile housing area.
- 6.6.8 Check blood glucose at least 3 times weekly. Mice can be sacrificed and discarded upon diagnosis of diabetes or 14 days post procedure, whichever comes first. Record findings.

7.0 RECORD REVIEW

Records will be reviewed as defined by the site-specific SOPs. At a minimum the operator supervisor and/or QA personnel should review the records.

8.0 RECORD RETENTION

Records will be maintained by the manufacturing facility following the time period specified in the site-specific SOP describing Record Retention and Record Archival System. Do not destroy any records without consulting previously with DAIT, NIAID, NIH.

9.0 REFERENCES

N/A