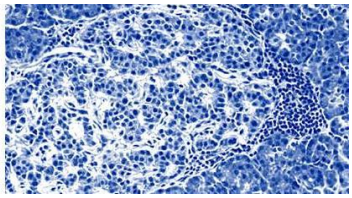


ISLET ISOLATION BY THE ISLET CORE LAB

PURPOSE: The purpose of this Standard Operating Procedure (SOP) is to outline procedures for processing pancreas and associated organs in conjunction with islet isolation for the nPOD Islet Isolation Pilot Program.

RESPONSIBILITY:
Managers and supervisors are responsible for making sure that technicians are properly trained and equipment and facility are maintained in good working order.

Laboratory personnel are responsible for reading and understanding this SOP and related documents and to perform these tasks in accordance with the SOPs. They are responsible for following clinical laboratory and tissue banking best practices.

SCOPE:
This SOP will be applied to all samples recovered through the nPOD Islet Isolation Pilot Program and designed to be used in conjunction with SOP 65.1 “nPOD Islet Isolation Case processing”.

I. INTRODUCTION

A. This procedure must be performed under the strictest sterile conditions possible.

B. Frequent changing of sterile gloves will be necessary to maintain sterility. Three people are needed throughout this procedure - one will remain sterile during the phase of organ manipulation and work within the hood (“sterile person”). The other “non-sterile persons” will pass and receive all necessary materials, reagents and supplies to the sterile person and document all necessary information throughout the procedure.

C. Utilizing sterile technique while opening sterile packaging involves removing the outer cover and placing the item inside the hood without touching the inside packaging. See SOP GP-005 (Sterile / Aseptic Technique).
Passage of sterile items from the non-sterile person to the sterile person implies a strict application of the following rules:
For all items with a double wrapping, the non-sterile person will open the outer layer and let the sterile person touch only the inner sterile pack.
For sterile items contained in one layer wrap, the non-sterile person will open the outer layer letting the sterile person directly hold the item(s).

This procedure will be followed at every stage when applicable in the following protocol.

D. The manufacturer, lot number and expiration dates of all media, reagents, enzymes or solutions used in this procedure must be documented. Throughout the procedure, this is best accomplished by placing all bottles or containers before use in one designated location, then recording the lot numbers and other related information at the beginning of the procedure.

II. MATERIALS & EQUIPMENT

A. For Islet Isolation:

Reagents & Media

For media or reagent preparation, see SOP IS-002 (Preparation of Reagents and Media for Human Islet Isolation). **Only use media with tamper-proof seal present unless freshly made on the day of use. If the media are customized, check the file for QC.**

a) RPMI, MediaTech or equivalent
b) Plain HBSS (undiluted), MediaTech or equivalent
c) Heat Inactivated Fetal Calf Serum, Sigma or equivalent)
d) Dithizone blend (DTZ) Solution (pre-made and stored at –20ºC)
e) Viaspan (UW solution) Dupont or equivalent
f) Heparin, Invitrogen or equivalent
g) Collagenase NB 1 GMP Grade, lyophilized, Serva
h) Neutral Protease NB GMP Grade, lyophilized, Serva
i) HEPES 1M
j) Anhydrous CaCl2 2.H2O
k) Sterile Water for Injection
l) Amphotericin B
m) Gentamicin
n) Cefazolin Sodium

Equipment, Instruments & Non Disposables

- Sterile Human Islet Isolation Pack, pre packaged with the following:

a) sub-set 1 for hood #1 (items are wrapped together) containing

1. Large stainless steel tray
2. Sterilized Rajotte's recirculation chamber
3. Medium stainless steel tray
4. Small stainless steel tray with surgical instruments:
   - ⇒ 1 Large sharp scissors & 1 small sharp scissors
   - ⇒ Hammer and chisel

⇒ 2 Metzenbaum dissecting scissors with blunt ends
⇒ 2 Forceps (small tip, blunt end)
⇒ 4 Mosquito or Klemmer clamps (2 straight, 2 curved)
⇒ 1 Large vascular clamp

b) sub-set 2 for hood #2 (items are wrapped together) containing

1. 500 ml stainless steel isolation chamber with lid and rubber ring
2. 1 screen (430 µM)
3. 7 glass beads
4. Stand with one clamp attached
5. Heating coil
6. Masterflex silicone tubing
   ⇒ 2 “size 16”
   ⇒ 1 “size 17”
7. Y-connector coupled to #16” tubing
8. Straight connector coupled to #16” tubing

c) basin pack

1. 2 sterile 1L bottles with lids
2. 1 sterile 500ml beaker covered with foil

- Sterile beakers covered with aluminum foil (3 x 1L, 6 x 500 ml)
- Portable pipet-aid (pipettor)
- Micropipettor for 100 µl and 1000 µl
- Heated water bath
- 2 refrigerated floor centrifuges
- CO_2 incubator (Standard 5% CO_2)
- Inverted light microscope
- 2 peristaltic pump drives with pump heads
- Temperature monitor with attached temperature sensor holders
- Trip balance
- Timer
- Biological Safety Cabinets / Hoods (#1, #2)
- Water recirculator -heating system
- Vacuum System
- Containers for red biohazard bags
- Plastic covered hammer

Disposables (or equivalent)

- Sterile 16,18, 20 or 22 gauge needles
- Sterile 250 ml conical centrifuge tubes
- Sterile wide mouth pipettes: 10 ml and 25 ml
- Sterile syringes with Luer-Lok: 10 ml, 30 ml and 60 ml
- Sterile wide mouth syringes

- Sterile bacteriological aspirating pipettes (without cotton)
- Sterile 14, 16,18, 20 or 22 gauge catheters
- Sterile 2.0 silk suture
- Sterile No. 10 scalpel
- Sterile 10 X 35 counting and petri dishes
- 3 sterile temperature probes
- Sterility tubes for Microbiology tests (see SOP IS-003, Contaminant and Sterility Testing for Islet Cells)
- Sterile 15 ml and 50ml conical tubes
- 3 Sterile Micro-volume extension sets
- Vacuum collection flask with 2 associated sterile tubing sets
- Nalgene sterile 500 ml and 1 L bottles filter system (0.2 µm size)
- Deionized sterile water, bottled (for waterbaths)
- Sterile disposable half sheets
- Sterile disposable gloves, gown, shoe covers, head cover, masks and face shield
- 1 liter of frozen sterile water for injection
- Biohazard bags
- Red sharps disposal containers
- Preprinted HP forms and sampling labels

Attachments

- Human Pancreatic Islet Isolation Data Sheet, SOP IS-001, Attachment I.

C. For Histology Tissue Processing.

Please refer to nPOD SOP 65.1 “nPOD Islet Isolation Pilot Case Processing”

III. PROCEDURE

A. Tissue Processing Laboratory Set Up

Please refer to nPOD SOP 65.1 “nPOD Islet Isolation Pilot Case Processing”

B. Islet Laboratory Set Up

1. Check the binder in the locked file containing the islet isolation records and assign the next consecutive number to the upcoming isolation.

2. Change into clean scrubs in the changing room and put on head cover, face mask, and shoe covers in that order. Street clothes cannot be worn under the scrubs.

3. Wash hands thoroughly with betadine.

4. Enter the clean room (Islet lab) and put on eye protection and non-sterile gloves.

5. Perform the following tasks:

    a) Switch the blower and light on in the hoods (ensure that ultraviolet UV light is off). Thoroughly wipe interior and exterior with 10% Clorox using wads of 4 X 4 gauze.

    b) After cleaning, allow blower to run for at least 10 minutes before using the hood.

    c) Turn on the power for the 2 refrigerated centrifuges, close the lids, and ensure that the temperature is set to 4°C.

    d) Ensure that fresh empty red bags and sharps containers are in place.

    e) Fill the water bath with deionized sterile water, turn on water bath and warm to reach and maintain 45°± 5°C.

    f) Turn on the water recirculation system, adjusting the heat to 38°C. Assure that the tubes are clamped.

    g) Record the manufacturer, lot number and expiration dates of all disposables (if not previously done) finalized to the isolation in section 1 of the Human Pancreatic Islet Isolation Data Sheet, SOP IS-001, Attachment I. Always check integrity of the packages.

    h) Thaw aliquots of dithizone (DTZ) working solution prepared as indicated in SOP IS-002 (Preparation of Reagents and Media for Human Islet Isolation).

    i) Place one peristaltic pump under hood #1 (right end), and a second one under hood #2 (left side).

C. Hood and Chamber Apparatus Set Up & Reagent Preparation

1. Exit the lab to the hand washing area, scrub and subsequently gown following SOP GP-006 (Scrubbing & Gowning Procedure).

2. All individuals performing the procedure must scrub and gown but one will be designated as “sterile” and the others as “non-sterile”.

3. Passage of items from the non-sterile person to sterile person will follow strict safety rules (SOP GP-005 Sterile/Aseptic procedure):

4. For sterilized packages with double layers, the non-sterile person will touch the external part of the outer layer only allowing the sterile person to touch the inner, sterile side.

5. For sterilized items contained in monolayer packages, the non-sterile person will carefully open the package, exposing directly the sterile item to the sterile person, within the hood flow area.

5. ***Sterile Person:***

a) Accept the inner sterile pack containing the sheets, open and cover at least 50% of the surface of hoods #1 and #2 while avoiding the non-sterile vented areas. The sheets will define the areas where only sterile items are placed and handled by the sterile person.

a) Accept sub-set #2 containing the digestion chamber and place it in hood #2.

b) Accept sub-set #1 in hood #1.

c) In hood #2: remove the wrap and place the chamber on its stand. Assemble the stand to its base and tighten the clamp approx. 15 inches from the surface. Insert the ends of the masterflex tubings 16” and 17” in the clamp.

d) Accept a sterile pack with a 500 ml beaker

6. ***Non-Sterile Person:***

a) Open the outer layer of the packs of sterile sheets and allow the sterile person to take the inner pack.

b) Open the outer wrap of the package “Human Islet Isolation Pack” and allow the sterile person to take sub-set #2 and place it in hood #2, and subsequently sub-set #1 in hood #1.

c) Open the outer wrap of a pack containing a sterile 500 ml beaker and pass it to the sterile person.

e) Open the inner wrap and place the beaker at the base of the stand. Insert the ends of the tubings in the clamp and pull them into the beaker. Use sterile gauze to secure tubings to the clamp.

f) Bring the coil to the border of the area covered with sterile sheet.

g) Identify the part of small (#16) tubing that connects the coil to the chamber. Bring this part to the border of the area covered with sterile sheets.

h) Place the temperature probe into the side port of the chamber. This is accomplished by turning the clear cap.

i) Bring the end of the probe to the border of the area covered with sheets.

j) Go to hood #1. Change gloves.

k) Open sterile set. Put the smallest tray (containing the instruments) on the right side of the largest tray. Place the medium-size tray nearby – it will later go inside the large one.

l) Accept the items from the non-sterile person and place them at the right side of the large tray.

d) Place the coil in the water bath.

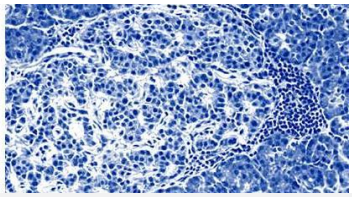
e) Take the tubing out of the sterile flow and connect it to the head of the peristaltic pump.

f) Open a wrap containing a sterile temperature probe and pass it to the sterile person.

g) Spray a temperature monitor with alcohol and place it in the hood, in the area not covered with sterile sheets. Insert the end of the probe in the temperature monitor.

h) Pass the following disposables to the sterile person:

- one 10 cc syringe with Luer-Lok
- one No.10 scalpel
- one 2.0 silk suture
- two sterile micro-volume extension sets
- sterility testing containers

D. Pancreas Cleaning & Cannulation

1. ***Sterile person:***

a) Receive the sterile saline pack, place it in the large tray and use the sterile scissors in the tray to cut off the plastic bag from around the frozen saline.

b) Use sterile hammer to further break the crushed block of ice into smaller pieces.

c) Place the medium tray inside the larger tray and instruct the non-sterile person to add approximately 300 ml of cold Hank's with 2% Fetal Calf Serum and antibiotic solution to the medium tray.

d) Using sterile technique, open the pancreas container and aspirate 15 ml of the procurement fluid (sample referred to as procurement solution) using a sterile syringe. Insert the syringe-needle in the microbiology bottles hold by the non-steril person.

e) Allow 1-2 minutes of Betadine contact and take the pancreas to the tray.

f) Clean the pancreas with Antibiotic/Antimycotic Solution and rinse with HBSS. Change gloves

g) Move the pancreas to a clean tray containing 2% HBSS. Remove the portion of intestines and the pancreas.

h) Clean off the fat and connective tissue using sterile scissors and forceps:

2. ***Non-Sterile person:***

a) Wrap a bag of frozen sterile saline (with outer bag intact) and break up with a hammer. Partially remove the outer bag and pass the inner bag to the sterile person under the hood.

b) Remove a bottle of cold Hank's plus antibiotic solution from the refrigerator when the sterile person requests it and follow their instructions on pouring.

c) Open the external plastic bag containing the pancreas and let the sterile person take the inner containers.

d) Hold the microbiology bottles (aerobic, anaerobic and fungi) to alow the steril person to fill with fluid and follow SOP IS-003 (Contaminant and Sterility Testing for Islet Cells).

e) Add Betadine to the pancreas solution (between 10 ml-20 ml).

f) Pour Antibotic solution and HBSS into sterile beakers when instructed.

g) Pour fresh 2% HBSS into tray when instructed.

h) While the organ is cleaned proceed with phases 1-10 of Section D (Enzyme preparation)

- Use the scissors in a “spreading, closing” motion to separate the connective tissue from the pancreas.
- Remove the spleen first, then the fat and connective tissue using a blunt dissection technique. When separating the duodenum from the head of the pancreas do not injure the intestine to avoid contaminations.
- Do not trim the pancreas too closely as leaks may develop.

i) Accept the 500 ml beaker. Open the wrap.

j) Place the cleaned pancreas in the beaker .

Take the organ and immediately put it in the tray with Hank’s with HA.

k) Note the amount of fat, presence of edema, whether pancreas is flushed, and the texture of the pancreas by gently examining it. Document information as soon as possible on Attachment I of SOP IS-001.

l) Use a No. 10 scalpel to cut one piece of pancreas at the junction Head and Body and one at the distal end of the tail. Each pieces should be about 1.5 cm wide at each division. Place both pieces of the pancreas in sterile Petri dish and transfer it together with tray containing spleen, fat, and duodenum

i) Open the outer wrap of a pack containing a sterile 500 ml beaker and pass it to the sterile person.

j) Weigh the beaker (containing the pancreas) and record the gross weight.

k) Return to hood #1 and remove the foil in the hood so that the sterile person can retrieve the organ.

l) Weight beaker plus foil and subtract it from gross weight. Do not re-use the beaker. Record this and all other required information on Attachment I, SOP IS-001.

m) Accept the tissue for histology and process it according to nPOD SOP 65.1 “ nPOD Islet Isolation Case Processing” using 6.2 paragraph as a starting point.

outside of the hood for histology processing.

m) Locate the pancreatic duct ends (head and body-tail). Estimate the size of the diameter and decide catheter size.

n) Remove the needle from the catheters.

o) Cannulate both the head and body portions with the catheters.

p) Secure the catheter in the duct of the pancreas body as follows:

- Insert the straight needle (2.0 black silk attached) not too close to the cannula around the point of catheter insertion.
- Reinsert the needle on the other side in the same manner and cut the needle off from the silk.
- Before tightening, reinsert the needles into the catheters to avoid crushing the plastic cannula and bend the needle (at the top) to prevent it from coming out of the tip of the catheter.
- Repeat the tying three times and place an additional tie after twice wrapping the silk around the catheter. Tie the loose ends of the silk into a loop.
- The catheter is secured when it does not slide out of the duct even after gently trying to pull it out.

q) Repeat this procedure (outlined in n) Open 2 catheters of the appropriate size and hand them to the sterile person.

section "n-q", above) for the head of the pancreas.	o) Transfer the bottle of enzyme solution in the water bath in hood #2 and insert the temperature probe end into its receptacle. Switch the monitor on and allow heating (time estimated for heating ~ 10')
r) Instruct the non-sterile person to warm up the enzyme approximately 5 minutes before completion of cleaning and cannulation.	
s) Once both catheters are in place and tied in, wait for the enzyme solution to be warmed up.	o) Open the outer wrap of the package containing the Rajotte's recirculation chamber and allow the sterile person to touch the sterile inner part.
t) Receive the sterile package containing the Rajotte's recirculation chamber, open it and place it on the right side of the hood. Allow the sterile sheet to cover the pump, placed behind the chamber.	p) Carefully touch the ends of the tubings and insert them in the recirculation-heating tubes (originating from the water recirculator-heater) clamped.
u) Turn the chamber so that the plastic tubings finalized for water recirculation face the external part of the hood.	q) Remove the clamp from the water recirculator-heating tubes to let water flow through the external part of the Rajotte's chamber. r) The flow should enter from the lower tube and flow out from the upper one.
v) Connect the micro-volume extension sets to the mobile stainless steel relay of the Rajotte's recirculation chamber.	
w) Pass the tubing to the non-sterile person and hold the relay while the non-sterile person connects the tubing to the pump head. Holding prevents	s) Connect the tubing to the pump.

abrupt movements of the system.

E. Enzyme Preparation

The final working solution will contain 1600 Units of Collagenase NB 1 and 200 Neutral Protease NB.

Procedure.

First preparation: Collagenase NB 1

Reconstitution of Collagenase NB 1 (allow approximately 45 minute to 1 hour)

1. Reconstitute the bottle of NB 1 with 10 ml of HBSS and heparin (10U/ml)(100µl). Keep the bottle in the refrigerator or on ice (2-8 ºC) for 30 minutes.

2. Prepare Calcium chloride at the final concentration of 11 mM:

3. Mix 0.39 g of CaCl2 anydrous or 0.52 g of CaCl2.2H20 with 35 ml of Hepes in a 50 ml conical. (Weigh conical and tare to maintain sterility.)

4. Filter the suspension thorugh a 0.22 micron filter in a sterile bottle and keep the bottle at 2-8 ºC.

5. Based on the specific activity reported for each lot take the equivalent of 1600 Units.

For example:

If the lot has 3200 Units/vial, resuspend in 10 ml and take
3200/1600= 2
Take 10 ml/2 = 5 ml of Collagenase solution

Units of activity per vial/1600 = X coefficient

10 ml solution/ X = Y ml to take for use

6. Transfer the calculated volume of Collagenase solution to the bottle containing Hepes.

Second preparation: Neutral protease

Reconstistute Neutral Protease right before use.

1. Add 10 ml of sterile water to the vial and mix.

2. As established before for the collagenase, take the equivalent of 200 units:

Units of activity per vial/200 = X coefficient

10 ml solution/ X = Y ml to take for use

3. Add the calculated amount of solution of Neutral Protease to the bottle containing Hepes/Collagenase and bring to 350 ml adding HBSS as needed.
Also add heparin to reach the final concentration of 10 U/ml.
Add a sterile temperature probe to the bottle.

4. Label the bottle with Enzyme solution, with date and time and store at 2-8 ºC.

5. When the cleaning of the organ is on the way to completion, transfer the bottle into waterbath. Allow ~10-15min to reach the temp. of 28-30ºC.

F. Pancreas Distention

1. Sterile Person:

Allow filling of the extension sets. Place the head and body of the pancreas in the

2. Non-Sterile Person:

a) When the temperature of the enzyme solution reaches 28-30^{O}C, remove the bottle of enzyme from the water bath.

b) Wipe and dry the bottle of enzyme. Move to hood #1.

Remove the lid and the temperature probe. Pour the contents on the upper part of the Rajotte's chamber. Keep the bottleneck on the side of the chamber (external).

Switch the pump on. Set the speed on setting 1 (20-30 ml/minute) and record the

open upper sterile tray of the Rajotte's recirculator.	time.
Connect the micro-volume extensions sets to the catheters. The gland will inflate and enlarge in size. a) Use sterile hemostats to clamp off the leaks b) Occasionally, a portion of the pancreas does not distend well. The poorly distended region may be injected intraparenchymally using an 18 or 20 Gauge needle attached to a 60 cc syringe. Allow the distension to be carried out for 10-15 minutes.	
Accept the 1 L beaker and open the wrap.	Open the outer wrap of a pack containing a sterile 1 L beaker and pass it to the sterile person.
Disconnect the extension sets from the catheters and transfer the sets into the beaker. Enzyme solution will be collected.	c) Write the time, augment the pump speed to setting 1.5 and collect all enzyme solution. d) Stop the pump.
c) Clean the pancreas from residual fat or connective tissue. <u>All tissue removed needs to be weighted.</u> d) Transfer the pancreas to the 1 L beaker (with enzyme). Cover the beaker with foil and transfer to hood #2.	

G. Pancreas Digestion

1. Sterile Person:	***2. Non-Sterile Person:***
a) Remove the aluminum foil from the beakers. Change gloves. Open the Ricordi's chamber b) Take the pancreas and place it in the chamber. Fill the chamber with collagenase. c) Set the screen into place and tighten the lid securely. d) Pour remainer of the enzyme solution into the 500 ml beaker placed in the ring stand and instruct the non-sterile person to begin pumping solution through the system.	a) Switch the pump on at a rate of 120-150 ml/min (setting 2.5) while the circuit is filling. b) Add more HBSS to the beaker in order to fill the system completely and to eliminate air from the circuit. c) As soon as the circuit is full and no air bubbles are observed in the system, adjust the pump speed to 80-110 ml/min (set 2).
e) Start manual shaking.	d) Write the time and temperature on Attachment I, SOP IS-001. e) When temperature reaches 34-36◦C,

f) Adjust the intensity of the shaking noting that it varies depending on the age of the donor. Harder on older donor tissue.

g) After 2-3 minutes from T=0, take a sample (1-2 ml) from the end of the large outlet tubing. Repeat sampling as many times as needed. Place the sample of digest in a small, 10 x 35 mm petri dish and pass to the non-sterile persons.

note the time and refer to as T=0.

f) Temperature in the chamber should not vary from 34-37°C during the digestion. Record requested information on Attachment I, SOP IS-001.

g) Add a few drops of dithizone (DTZ) solution to the digest and observe under the microscope. DTZ stains the islets red. Conversely, the acinar tissue appears unstained. Record all observations on Attachment I of SOP IS-001. Normally, fat cells are seen first, followed by acinar and an occasional islet fragment.

Clean hood #1. Remove all items used during the cleaning and distension of the pancreas.

Wipe the surface with alcohol and cover with sterile sheets.

Take 250 ml conicals with two racks.

Open the wraps of a sterile bucket and fill it with sterile ice.

Ice bags have to be prepared as described (section C). The non-sterile person will pour ice directly into the bucket.

Open the wraps of a sterile 1 L beaker, fill it with 500 ml RPMI 0.5 HA (Formulation,

	SOP-IS-002) and place it on ice. Put in a sterile 4L collection flask, open the wraps and labeled as “waste” h) Collection of cells should start when an increase in the amount of tissue liberated from the chamber is observed and most of the islets are free of the surrounding acinar tissue, but still intact.
h) When collection time is approaching, warn the non-sterile person to switch the pump off. i) Transfer the outlet tubing of the circuit from the beaker onto the 250 ml conical tube. j) When 75 ml of cell suspension have been collected (watch the mark), transfer the tubing to a subsequent tube.	i) Transfer one rack with 6 x 250 ml tubes under hood #1. Mark the level of 75 ml on each tube. Open the lids. Switch the pump off. Fill the beaker with 250-300 ml of HBSS kept at room temperature. Switch the pump on again.
Continue warm collection and manual shaking for approximately 10-15 minutes until the tissue released is reduced in volume.	Receive the 250 ml conicals containing 75 ml of digest. Spin for 30 seconds at 1000 RPM in a refrigerated centrifuge. After spinning, take the tubes to hood #2. Open the lid and pour the supernatant into the beaker, leave a small amount of liquid in the lip of the conical to resuspend the pellet. Close the lid, transfer the pellet under hood #1 and detach the pellet from the

Receive the bottles and open the inner wrap.	bottom of the conical tube using a quick circular motion and gently pour the pellet into the 1 L beaker. Return the tube under hood #2 for subsequent filling.
k) As the warm collection nears completion, instruct the non-sterile person to be ready to fill the collection bottles and the beaker with fresh RPMI 0.5 HA	Open the outer wrap of two sterile 1 L bottles. Pass to sterile person.
l) Stop the warm digestion process (switch to cold dilution).	j) When instructed by the sterile person, pour 500 ml of RPMI 0.5 HA in the beaker and in the collection bottles.

H. Dilution and Collection of the Digest

1. Sterile Person:	**2. *Non-Sterile Person:***
a) At this time, instruct the non-sterile person to switch the pump off and place the end of the larger tubing into the collection bottle.	a) Switch the pump off
b) Begin collecting digest solution into the collection bottles until 500 mL of fluid is obtained.	b) Turn the pump on and record the time. ***c)*** Remove heating coil from the water bath to allow the chamber to be cooled
c) Start to shake the chamber vigorously.	d) Fill the beaker with cold RPMI 0.5 HA as needed during the procedure.
d) Once the bottle has 500 mL, transfer the tube to a new bottle and continue the collection.	e) Tighten the lid on the bottle and transfer it to hood #1.
e) Sample the digest to determine if islets are still released. The dilution phase lasts 15-45 minutes or until islets are	f) Mix the cell suspension by shaking, then pour digest solution from the bottle into 250 ml conical tubes, cap and close tightly.

no longer detected in a sample.

When dilution is considered complete no more fresh RPMI 0.5% is added to the beaker and air is allowed to enter.

Shake the chamber upside down to favor tissue detachment from the mesh.

Open the chamber and check for residual tissue.

f) Weigh and record the weight of the undigested pancreas.

g) Balance the tubes and centrifuge them at 1000 rpm for 2 minutes at 4°C.

h) After centrifugation, bring the tubes to hood #1. Discard supernatant with a single smooth motion into a 4L collection flask labeled as “waste”. Leave a small amount of liquid in the lip of the conical to resuspend the pellet.

i) Detach the pellet from the bottom of the conical tube (as previously done). Pour it from the conical into the1L beaker containing 500 ml RPMI 10% FCS on ice.

j) Repeat steps d-g, above, using the entire digest until all the tissue is centrifuged and collected.

Once all of the tissue has been collected, resuspended well. Pour the cell suspension in new conicals. If clumps form, a filtration step might be useful. Using sterile gloves place a sterile filter system onto the edge of a 500 ml sterile beaker. Pour the resuspension through the filter and rinse the mesh with fresh fluid. When approximately 400 ml of fluid have been filtered, fill the conicals with the filtered suspension and continue filtration until completion using a new filter and a new sterile beaker.

k) Centrifuge at 1000 rpm for 2 minutes.

l) Set up the aspiration tubes and vacuum system under hood # 2. Connect the first aspiration tube to the vacuum receptacle under the hood and connect the end to the collection bottle.

Open a second sterile tube. Connect one end of it to the collection bottle while

maintaining the remaining end in the hood. Switch the vacuum on by turning the lift in the hood.
Open a sterile pipette (for aspiration) and connect it the end of the tube left in the hood. Put the pipette in a new sterile conical and use it over the following steps.

When the vacuum bottle is full replace the whole system (bottle, pipette and tube) before use.

m) Remove supernatant completely by aspiration.

n) Add 200ml UW to the cells and resuspend, incubate for 30 minutes.

o) Centrifuge at 1100 rpm for 2 minutes.

p) Remove supernatant completely by aspiration, measure the cell pellet volume and record it.

*If clumps form, filter as described on pg 19.

*A purification step may be added to reduce the final infused cell pellet. Purification will be carried out according to the procedure outlined in SOP-IS-011.

I. Counting of Islet Cells

1. The counting sample can be taken from the whole preparation or for each bag with a different purity after COBE. Mix the fresh final islet suspension well before taking a sample. Take out 500µl in a 5ml tube.

2. Add a small volume of PBS to cover the bottom of the counting dishes before taking duplicate 100 µl samples, using a Hamilton-Gilson P-100

pipette and place each sample in the 10x35 mm counting dish containing PBS.

3. Add a few drops of DTZ solution to the counting dish and allow a 2-minute incubation at room temperature to completely stain the islets.

4. Set the inverted microscope to 20X objective and visualize the calibrated grid for sizing the cell aggregates.

5. In addition to counting the stained aggregates the islets are divided into diameter (size) classes. 50 µm diameter range increments are used, without considering particles smaller then 50 µm since their contribution to the total volume of the preparation is insignificant.

6. The distance between two lines on the calibrated grid in the eyepiece will equal 50 µm. Using the eyepiece (20X total magnification), use the chart below as a guide:

Number of Lines	Size of Islets
2	50 µm
4	100 µm
6	150 µm
8	200 µm
10	250 µm

7. Count the number in each group using the manual cell counter and record numbers as well as the purity and conversion to islet equivalents on Attachment I, SOP IS-001 and in the computer islet isolation file.

8. Islet number (IN) and islet equivalent (IEQ) calculations can be done manually. The table below indicates the mean volume for each diameter class and the relative conversion factor into islets of 150 µm diameter. These factors make it possible to convert the total islet number from any preparation into islet equivalent (IEQ). In place of IEQ, the total islet volume of the final preparation can also be estimated.

islet diameter range (u)	**mean volume (u)**	**conversion into islets of 150 um diameter**

		(IEQ conversion factor)
50-100	294,525	n/6.00
100-150	1,145,373	n/1.50
150-200	2, 977,968	nx 1.7
200-250	6,185,010	nx 3.5
250-300	11,159,198	nx 6.3
300-350	18,293,231	nx 10.4
>350	27,979,808	nx 15.8

Note: If 500,000 islets at 150 u size and 100% purity are counted, there should be approximately 0.9 ml of packed pellet. If 500,000 islets at 150 u size and 50% purity are counted, there should be approximately 3.6 ml of packed pellet.

J. Purity assessment

Take a sample containing approximately 50 cell aggregates, add 1-2 ml of PBS and few drops of Dithizone solution.
Wait 1-3 minutes and count the red-stained aggregates as islets.
Express purity as the percentage of islet/non-islets.

K. Cleanup of the Laboratory

Clean up the laboratory as follows:

- Dispose of all used disposables.
- Wash all non-disposables thoroughly with soap and hot water, place in a large grey bin, place on a cart and deliver to the autoclave room.
- Decontaminate hoods and counters with 10% Bleach following SOP GP-001, Decontamination of Laboratory Work Areas and Equipment.
- Remove all visible spills from equipment with 10% Bleach following SOP GP-001, Decontamination of Laboratory Work Areas and Equipment.
- Disconnect and discard disposable sections of the vacuum collection system.
- Discard any opened, unused media.

- Close all sharps containers and red biohazard bags according to SOP GP-002, Disposal of Waste Materials, and remove from the laboratory.

IV. NOTES AND INTERPRETATION

- The manufacturers, lot numbers, and expiration dates of all reagents must be entered on Attachment I, SOP IS-001.

- All requested information on the Human Pancreatic Islet Isolation Data Sheet, Att. I, Att. II SOP IS-001, must be filled out entirely using current Good Manufacturing Practice (cGMP) for document record maintenance and correction.

V. RECORD REVIEW

Records will be reviewed by the Supervisor or designee as per SOP GP-008 (Supervisory Review of Records).

VI. RECORD RETENTION

Records will be maintained in the Islet Core Lab following the time period outlined in SOP GP-003 (Record Retention & Control of Documentation).

VII. REFERENCES

1. Ricordi C. at al. Islet Isolation Assessment in Pancreatic Islet Cell Transplantation by Ricordi C. Ed.R.G. Landes Co. 133-142, 1992.

Prepared by: _____ Date: _____

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