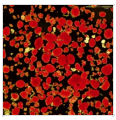

Isolation of Human Pancreatic Islets of Langerhans for Research

Version 2

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ABSTRACT

This protocol, and the five linked protocols, describe the equipment setup, solutions, and methodology for the isolation of pancreatic islets of Langerhans from human donor pancreas for research purposes. These protocols are employed by the Alberta Diabetes Institute IsletCore program in their work isolating, banking, and distributing human islets for research.

PROTOCOL STATUS

Working

We use this protocol in our group and it is working

BEFORE STARTING

Prepare all media and setup as described in Human Islet Isolation Media Preparation and IsletCore Equipment Setup for Human Islet Isolation protocols.

Media and Equipment preparation

1 Prepare all media as described in Human Islet Isolation Media Preparation protocol.
Set up equipment as described in IsletCore Equipment Setup for Human Islet Isolation protocol.
Set up COBE as described in Human Islet Purification - COBE Setup protocol.

Record Identifier Rxxx

2 An internal identifying case number will be assigned to each pancreas received by the ADI IsletCore, this identifier will be a sequential number from previous case and will be preceded by the letter R.

Technician personal protective equipment

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- The dissection, perfusion, digestion, and collection requires two trained individuals to complete. These will be refered to as lead and assistant technicians.
- The lead tech shall scrub in and don sterile personal protective equipment. (cap, mask, sterile gown, sterile gloves)
- The assistant tech is not required to wear sterile personal protective equipment but will be mindful of all sterile fields, handling and sterile equipment of the lead tech.
- The assistant tech shall wear personal protective equipment (lab coat and gloves).

Removal of the donor organ from the transport containers

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- The assistant tech shall then pour the 10% betadine into the first beaker, and ~200 ml of HBSS into the second and third beakers, and pour the remaining HBSS into the cutdown tray.
- The assistant tech shall obtain the packaged pancreas/spleen/duodenum from the transport cooler and cut the closure ties with scissors down to the final sterile barrier containing the pancreas.

- The lead tech will transfer the pancreas/spleen/duodenum within the final sterile barrier to the BSC and place it into the dissection tray.
- Remove pancreas/spleen/duodenum from package containing transport solution
- Place the procured organ(s) into the dissection tray and pour 200 - 500ml of the transport solution into the dissection tray.
- Record this time point as isolation start time.

Pancreas cut down and decontamination

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- Refer to video for further information: Removal of intrapancreatic bile duct
- Dissect away the spleen, major vessels (splenic artery/vein, mestenteric artery and vein) and any excess fat taking care to not damage the pancreas or the duodenum
- Locate the opening of the bile duct and dissect it away from the pancreas following the lenth of the duct to the main drainage point were the pancraetic duct enters the duodenum.
- Disect away the pancreas from the duodenum working towards the pancreatic duct.
- Expose the pancreatic duct in preparation of cannulation.
- Cut through the pancreatic duct close to the duodenum to allow easy location for cannulation.
- Place the trimmed pancreas into the first beaker containing the 10% betadine and allow to decontaminate for ~1 minute.
- Transfer the decontaminated pancreas to the second beaker and agitate/rinse. Transfer and repeat for the third beaker.
- Remove the dissection tray from the cutdown tray
- Remove and discard the sterile gloves. Don a new pair of sterile gloves
- Transfer the decontaminated and rinse pancreas to the cutdown tray

Pancreas cannulation

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- Refer to video for further information: Pancreas cannulation
- Identify the opening of the exposed main pancreatic duct
- Insert a 16gaude catheter into the pancreatic duct and fasten the opening edge of the duct with a bent internal needle to avoid cannula collapse.
- Using 3-0 silk suture tie around the opening of the caunulated duct and secure the canula by tieing of the wings of the canula.
- Inject HBSS into the pancreas via the canula to locate the panceatic duct passsge through the pancreas
- Locate the pancreatic duct at neck of the pancreas and using a scalpel cut the parenchyma to expose the main duct.
- Dissect the main duct bluntly from the surronding tissue and isolate the duct with two 3-0 silks.
- Using a scapal or microscissors cut a small hole in the exposed pancreatic duct to faciliate canulation.
- Insert a 16-gauge catheter towards the tail and tie off as discribed at the duct opening at the head.
- Insert a final 16-gauge catheter towards the head and tie off as discribed at the duct opening at the head.

Collagenase Perfusion of Pancreas

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- After cannulation, pat dry the pancreas with sterile 4x4 gauze and transfer the organ to a tared sterile 500ml nalgen container.
- Weight and record the organ in grams (the average weight of a human pancreas is ~90g but can vary a great deal depending on the donor age, height, weight, BMI, health, etc).
- Determine the dose of the non-specific protease by targeting the number of enzymatic units to 600 to 900 U/g of pancreas. This must be adjusted according to cold ischemia time, organ consistancy, and cardiac arrest/warm ischemia damage.
- Add the calculated non-specific protease volume to the prepared collagenase.
- Pour the collagenase/neutral protease solution in the perfusion tray.
- Prime the perfusion tubing set by pumping the collagenase solution at 100ml/min through both channels. Allow the solution to circulate through the tubing set and the tray creating an air bubble free complete circuit.
- Check for leaks in the tubing.
- Once the priming is complete shut off both channels.
- Transfer the cannulated pancreas to the perfusion tray.
- Clamp or plug the cannula at the opening of the main pancreatic duct.
- Attach perfusion channel "A" tubing to the mid-body cannula that is directed to the head.
- Attach perfusion channel "B" tubing to the mid-body cannula that is directed to the tail.
- Initiate the perfusion protcol that is pre-programed into the perfusion apparatus.
- Observe the perfusion of the pancreas making sure to determine thesuccessful perfusion of both the head and tail sections. Observe and clamp any leaks that may effect the distention quality.
- Following the perfusion protocol, collect and record the collageanse soltution volume.
- Remove the catheters from the pancreas.
- Take a biopsy sample from the pancreas following Human Pancreas Biopsy Collection, ex vivo protocol.

- Cut the pancreas into 10-15 equal pieces in preparation for digestion.
- Examine cut surfaces of pancreas for abnormalities and record any findings.
- Place the pancreas pieces into the kidney bowl containing the silicon nitride marbles.
- Record pancreas consistency (i.e. soft, normal, fibrotic)

Pancreas Digestion

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- Drain the primed auto-isolator tubing/ricordi chamber system by removing the inlet line from the priming solution bottle and inverting the ricordi chamber.
- Once the system is drained of priming solution, advance the protocol to step 2 (pause) and transfer the distended pancreas pieces and the marbles to the Ricordi chamber within the BSC.
- Install the sterile 500µm stainless steel mesh.
- Seal the Ricordi chamber and reattach to shaking arm.
- Begin filling digestion circuit with collagenase solution at 300mL/min. (Step 3 – fill as outlined in autoisolator section of IsletCore Equipment Setup protocol).
- Add DNase solution to a 250 mL conical containing collagenase solution and the Inlet 1 and Outlet 1 rods.
- Add additional perfusion and priming solution until all the air is removed and a compete circuit is achieved through inlet 1 and oulet 1 rods.
- Advance auto isolator to step 4 (digest 1) and begin timing the digest.
- Once the autoisolator reaches 37 ± 1 °C, advance to step 5 (digest 2). This may take from 3 to 6 minutes to achieve.
- Observe the amount of tissue flowing the through the system by looking through the inlet and outlet rods. Tissue is usually observed around the 8 minute mark.
- Once a sufficient about of tissue begins flowing through the system, the assistant tech shall take 1ml samples of the digest every 2 minutes. This is usually begins around the 10 minute mark. Samples are transfered to a 60mm petri dish, stained with dithizone, and observed under a stereo-microscope by the lead tech.
- Observe approximate islet yield, islet quality, and overall tissue volume within each sample.
- The lead tech shall determine the completion of the digest once the optimum amount of tissue within each sample has been reached based on pancreas size, quality, size, and donor age.
- Once the lead tech determines completion of the digest the auto-isolator must be advanced to step 6 (collection 1)

Tissue collection

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- Insert intake rod into the first bottle of dilution 2 solution and the collection outlet rod into the first of the twelve 250 mL conical tubes containing 100ml of dilution 1 solution. These 12 conical would have been stored and held on a circulating cold block - IsletCore Equipment Setup for Human Islet Isolation step 8.
- Document this time point as the the dilution time.
- Collect the tissue digest into twelve 250 mL conical tubes prefilled with 100 mL of dilution 1 solution, followed by empty 250 mL conical tubes.
- Once the first four conical tubes are full, centrifuge at 1100 rpm (282 x g) for 1 min @4˚C.
- Remove the supernatant, and transfer the resulting tissue pellets to a 1L bottle on ice containing ~200ml wash 1 media. Be sure to disrupt each pellet by gentle hand vortex prior to transfer to the wash 1 bottle.
- Following the first addition of 2L of Dilution 2 to circuit, advance auto isolator to step 7 (pause) and invert chamber.
- Advance the auto-isolator to step 8 (Collection 2) and allow air to enter the system for about 30 seconds.
- Continue collection/centrufigation/transfer throughout the collection step until all 6L of dilution solution 2 has been circulated through the system.
- After the last of the 6L of dilution solution 2/tissue has been centrifuged and transfered to the wash 1 media, transfer the resulting tissue suspension to four 250ml conical tubes.
- centrifuge at 1100 rpm (282 x g) for 1 min @4˚C
- Remove the supernatant and transfer and fully disrupt the resulting pellets in one 250ml conical tube with wash 1 media.
- Sample 100µl from the 250ml suspension and dithizone stain the sample for observation of the digested tissue. Take note of the islet numbers, size, and % trapped.
- Again, centrifuge at 1100 rpm (282 G) for 1 min @4˚C the 250ml suspension and remove the supernatant completely.
- Weight the resulting pellet by placing the tube containing the pellet on a tared balance. Record the tissue weight to the nearest 1/10 of a gram.
- Using UW divide the tissue into purification run aliquots of no more than 30g/aliquot, although the number of COBE runs is at the discrestion of the lead tech who will base the number of purifiction runs upon the tissue suspension quality (i.e. - exocrine density and trapped islets). Typically one purification run is sufficient but all of these factors might determine two runs.
- Add enough UW solution each aliquoted tissue volume for a total volume of 150ml. Place each tube containing the UW/tissue suspension on ice to await purification for at least 15 minutes.

Islet purification (COBE)

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- Refer to Human Islet Puritification - COBE Setup protocol.
- Set COBE 2991 speed at 1500 rpm, super out rate at 0 mL/min, and super out volume at 600 mL.
- Load Biocoll into cell processing bag installed in COBE. *NOTE: Ensure COBE bladder is not full before continuing process*
- Pour Biocoll (1.100 g/mL, 100 mL) into the front beaker of gradient maker, and turn on Masterflex pump.
- When Biocoll has reached bag, press START/SPIN.
- When all Biocoll has been loaded, press SUPER OUT, turn off pump, release tubing from pump head and increase super out rate to 100 mL/min.
- When Biocoll has filled the tubing and reached front beaker, clamp tubing in pump and press STOP/RESET.
- Set COBE speed at 3000 rpm and super out rate at 0 mL/min.
- Load Density gradients into bag. NOTE: Ensure COBE bladder is not full before continuing process. Pour the 'High' gradient into front beaker. Unclamp the haemostats that occludes the tubing between the front and rear beakers of the gradient maker. Allow a few ml of high density gradient to flow through to the rear beaker. Re-clamp again.
- Add the 'Low' gradient into rear beaker.
- Turn on the stir plate (set the stir plate to a speed that maximizes mixing but does not introduce air).
- Press "START SPIN".
- Once the COBE has reached full speed, turn on the pump set to 30ml/min.
- Allow the contents of the gradient maker to fully flow out until the last of the gradient reaches the point of the connection of the front beaker and the pump tubing by tilting the gradient maker and stir plate forward.
- Without the introduction of air, pour the UW/tissue suspension 50 ml at a time into the front beaker.
- Once the last 50 ml has been added to the gradient maker rinse the conical with 50ml of wash 1 (cap).
- Once the last of the tissue suspension is about to leave the gradient maker pour the rinse into the front beaker.
- When all of the rinse rinse solution is loaded into the COBE bag proceed as follows:
- Clamp main bag line with haemostats just above metal seal weight on the COBE
- Open the pump head.
- press SUPER OUT
- Release the haemostats to release pressure from COBE.
- Turn off the pump.
- Time the purification for 5 minutes.
- Open sterilized collection rod and place in the collection conical tube 1 containing 100 ml of Wash solution 2.
- Disconect the gradient maker tubing from the COBE inlet line and reconnect the COBE inlet line top the collection rod line.
- After 5 min increase super out rate to 100 mL/min.
- Collect tissue into each of the labelled 12 prefilled 250 mL conical tubes starting at tube 1.
- Centrifuge conical tubes at 280 xg for one minute in refrigerated centrifuge (4°C).
- Record the resulting pellet volume from each fraction and then transfer and disrupt each pellet a similarly marked (i.e. 1 to 12) 50ml conical tube using wash 2.

Purification Assesment

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- Suspend the pellets within each 50ml conical tube by gentle agitation and bring to 50ml volume using wash 2 solution.
- Sample 100µl each tube using a wide bore plug 200µl pipette tip.
- Transfer each sample to a sterile 24-well non-tissue culture treated multi-well plate.
- Stain each sample with dithizone to visualize islet content and purity and to make purity designations. The guidelines for purity designations are as follows:
- The "Top" layer (i.e. highest purity)
- The "Middle" layer may be created if a large percentage of trapped islets were observed during pre-purification islet quantification.
- The "Bottom" layer (i.e. lowest purity).
- Make a decision as to what fractions can be usable for research and what fractions can be discarded. Usually tube 1 contains no islets but only cellular debris. The highest purity fraction starts at tube 2 and decreases in purity, islet number and increased trapped islets, through to tube 12 which will typically contain only exocrine tissue.
- Once each fraction has been assigned a purity layer designation, centrifuge the conical tubes at 280 xg for one minute in refrigerated centrifuge (4°C).
- Aspirate supernatant without disturbing tissue pellet.
- Combine all pellets designated to the same layer to achieve one 100 mL conical tube per layer using supplemented CMRL. Record all pertinent data to the isolation batch record (See step 14)

Islet sampling

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- Refer to the Sampling of Human Islets for Quality Control protocol for specific sampling procedures.
- Once all islet samples have been taken, (Insulin, DNA, counts, immunohistochemistry), allow islets to gravity settle for at least 5 minutes.
- Remove 5ml of the supernatant and incubate for 1 week at 37°C (5% CO_2) as a post-isolation contamination control.

- Replace the 5 ml with culture media to bring the suspension volume back to 100ml.

Islet Culture

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- Transfer the islet suspension to the appropriate number of non-tissue culture treated 150mm x 15mm petri dishes. The number of isolated islets determines the number of culture dishes. Typically, 40,000 to 60,000 islet equivalents per dish.
- Add enough supplemented CMRL to bring each culture dish to a final volume of 35ml.
- Transfer all dishes to a 22˚C 5% CO_2 incubator until time of Human Islet Distribution to ADI IsletCore Recipient Labs.
- Record all applicable information on sampling and results in RedCap (IEQ, purity, %trapped)

Islet Batch record

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Isolation Batch Record				
Donor				
Human number			Isolation date	
Donor ID			Procurement start time	
Donating Centre			Cross clamp/withdrawal time	
Admin date			Arrival time	
age			Isolation start time	
Date of Birth			Cold ischemia time	
Sex			Perfusion start time	
weight (kg)			Digest start time	
Height (m)			Digest length (min)	
BMI			Dilution start time	
Race			Collection end time	
Cause of death			Purification start time	
NDD/DCD			In culture time	
warm ischemia time (min)				
Down time			**Notes**	
Diabetes?				
Serum glucose				
HbA1C				
Blood type				
Serology				
Media	**Lot**		Enzymes	
HBSS (priming)			Collagenase manufacturer	
HBSS (decon)			Collagenase	
Cut down Solution			Collagenase Catalgue #	
Perfusion			Collagenase Lot #	
Dilution 1			Collagenase reconsitution volume (ml)	
Dilution 2			Non-specific protease manufacturer	
Wash 1			Non-specific protease	
Wash 2			Non-specific protease Catalgue #	
BSA			Non-specific protease Lot #	
Nicotinamide			Non-specific protease reconsitution volume (ml)	
Ficoll			DNase manufacturer	
UW			DNase	
			DNase Catalgue #	
CMRL			DNase Lot #	
Date supplemented			DNase reconsitution volume (ml)	
ITS				

P/S				
BSA			**Digest**	
Perfusion			Perfusion start time	
Perfusion Start time			Collagenase volume post perfusion (ml)	
Transport sample taken?			Digest start time	
Spleen attached			Digest length	
Fat			Collection end time	
consistency			Collection volume	
flushed			Digest/Collection time	
pancreas weight (g)			undigested tissue weight (g)	
Leaks			Collect tissue weight (g)	
Pancreas divisum			Comments	
Sections pre-digest				
Biopsy sample taken?				

Purification						
Pellet weight (g)						
# of Cobe runs						
Cobe 1	**pellet PCV (ml)**		**purity**	**% trapped**		**fraction**
1						
2						
3						
4						
5						
6						
7						
8						
9						
10						
11						
12						
Cobe 2	**pellet PCV (ml)**		**purity**	**% trapped**		
1						
2						
3						
4						
5						
6						
7						
8						
9						
10						
11						
12						

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