Chapter 13

Isolation of Porcine Pancreatic Islets for Xenotransplantation

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Abstract

This chapter deals with a technique for isolating intact islets of Langerhans from the pig pancreas based on our experience performing approximately 750 isolations. The procedure we describe involves identification of an optimal donor pancreas, purification and in vitro culture of islets, diabetes induction in recipients, and transplantation of islets and their immunomodulation. Besides the sophistication of the technical equipment employed, the major factors influencing the isolation outcome are the pig breed, the number and morphology of the islets in the donor pancreas, the quality of the collagenase/neutral protease, and the skill of the team members.

Key words: Pig breed, Pancreatic islet isolation, Collagenase, Neutral protease, Porcine islet of Langerhans, In vitro culture, Diabetes induction, Islet transplantation, Microencapsulation, Immunomodulation

1. Introduction

The species *Sus scrofa* (pig) is favored for isolating intact islets of Langerhans from the pancreas. Among the reasons for this are the following: pig insulin is well tolerated by diabetic patients and differs from human insulin in only one amino acid; pigs breed well and are easy to keep and their gastrointestinal tract resembles that of humans. Compared to isolating human islets, however, it can present greater difficulties. One difficulty is posed by the variation in the number, size, and morphology of islets within the pancreas among the different pig breeds, a phenomenon that has been reported by us and many other groups. The reasons for these

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variations remain unknown and are currently being investigated with the support of federal and local breeding institutions. If pigs with good-quality islets are available, isolation of their islets is easy as we describe in detail below. This is particularly the case since high-quality enzymes can be obtained that disintegrate the pancreatic tissue but preserve the islets. This chapter focuses on the process for selecting the best possible pancreas for islet isolation, and describes the purification and in vitro culture of the islets. Only briefly discussed here are diabetes induction, in vitro testing of islet function, transplantation into diabetic animals, and microencapsulation to immunomodulate the xenogeneic immune response in the recipient, since these subjects are extensively covered elsewhere in the literature.

2. Materials

2.1. Media, Solutions, and Supplements (with Modifications for Use in Experiments) in Chronological Order of Their Application

1. Hanks' balanced salt solution (HBSS, 500 mL) to be used as manufactured to transport organs.
2. Fresh dithizone (DTZ) solution: Add 9 mL of HBSS to 10 mg DTZ (Sigma-Aldrich, Taufkirchen, Germany) dissolved in 1 mL dimethylsulfoxide (DMSO, 1-mL aliquot stored at −20°C until use). The solution is then passed through a 0.2-µm filter. This solution must always be prepared freshly. Prepare as described to discriminate between islets and acinar tissue (1).
3. Double-concentrated University-of-Wisconsin (UW-2×) stock solution: Dissolve 250.78 g lactobionic acid (Fluka, Steinheim, Germany) in 2,187.5 mL of distilled water and adjust pH to 7.0 with 5 M KOH. Add 47.6 g KH_2PO_4, 8.6 g $MgSO_4$, 124.78 g raffinose (Sigma-Aldrich), 472.5 mg allopurinol (Sigma-Aldrich), 6.4 g glutathione reduced (Sigma-Aldrich), 9.3 g adenosine (Sigma-Aldrich), and 350 g HES 200.000/0.5 (Fresenius Kabi, Bad Homburg, Germany), vortex until the solution is clear, adjust to pH 7.4 with 5 M NaOH, fill up to 3.5 L with distilled water, pass solution through a 0.2-µm filter, and store in 1-L glass flasks at 4°C until needed.
4. Single concentrated UW solution (UW-1×): Dissolve 135 mg allopurinol in 500 mL of distilled water (5 min sonic bath), fill up to 1 L with distilled water, transfer solution to a 2-L sterile glass flask, add 1 L UW-2×, and adjust pH (7.4–7.5) as above and density ($\delta = 1.048$) with density meter DMA 35N (Anton Paar, Graz, Austria). Store at 4°C until needed.
5. Enzymatic digestion solution: Prepare 0.5 mM trypsin inhibitor pefabloc SC (Roche Diagnostics, Mannheim, Germany), 2.5 mM $CaCl_2$ (to stabilize collagenase activity) in 200 mL

UW-1× solution; subsequently add 4 PZ units of NB8 collagenase (Nordmark, Uetersen, Germany) and 0.65–0.7 DMC units of neutral protease (Nordmark) per gram of pancreas. Vortex gently (no foam!) until all ingredients are dissolved. Prepare solution immediately before use, keeping it at room temperature.

6. KH_2PO_4 buffer stock solution (1 M): Dissolve 136.09 mg KH_2PO_4 in 1 L of distilled water and pass through a 0.2-μm filter. Store at room temperature.
7. HBSS solution with 13% fetal calf serum (FCS) (HBSS-13%, to stop enzymatic digestion): Add 65 mL of pretested heat-inactivated FCS (Cell Concepts, Umkirch, Germany) and 12.5 mL of KH_2PO_4 buffer stock solution (25 mM) to 500 mL of HBSS and adjust pH to 7.4 with 5 M NaOH. Store on ice until needed.
8. HBSS solution with 10% FCS (HBSS-10%, to wash, digest, and purify islets): Prepare solution as just described, but use 50 mL of heat-inactivated FCS instead of 65 mL. Store on ice until needed.
9. Nicotinamide solution (to restore islet viability and function after isolation): Dissolve 3.1 g nicotinamide (Sigma-Aldrich) in 15 mL of UW-1× solution, pass through a 0.2-μm filter, and store at 4°C until needed.
10. HAM's F12 islet culture medium (for in vitro culture of isolated islets): Add 50 mL of heat-inactivated pretested FCS (10%), 5 mL of penicillin/streptomycin (1%), 5 mL of amphotericine B (1%), 5 mL of glutamine (1%), and 3.1 g nicotinamide (50 mM) to 500 mL of HAM's F12 medium (Cell Concepts).
11. Fluorescein diacetate (FDA) solution (to test viability of islets): Dissolve 1 mg FDA (Sigma-Aldrich) in 1 mL of acetone. Prepare freshly and store in the dark.
12. Propidium iodide (PI) solution (to mark dead cells): Dissolve 5 mg PI in 10 mL of phosphate-buffered saline (PBS). Store in aliquots of 500 μL at 4°C.
13. Wash solution (WS, for islet purification gradient) (460 mL): Mix 230 mL of UW-2× and 230 mL of OptiPrep solution (Progren, Heidelberg, Germany), transfer to sterile flasks, and adjust optical density to $\delta = 1.206$.
14. Low-density solution (LDS) for islet purification gradient (309 mL): Mix 85 mL of WS and 224 mL of UW-1×, transfer to sterile flasks, and adjust optical density to $\delta = 1.090$.
15. The beta cell toxin streptozotocin (STZ, Sigma-Aldrich) for inducing diabetes in rats and mice.

16. Citrate buffer to dissolve STZ: A 1 M stem solution C is prepared from solution A and solution B as follows: To prepare 10 mL of solution A, add 2.1 g citric acid monohydrate to 0.9% NaCl solution (pH 1.7). To prepare 10 mL of solution B, add 2.94 g trisodium citrate dihydrate to 0.9% NaCl solution (pH 7.8). To prepare a 1 M stem solution C, mix 6.3 mL of solution A with 8.2 mL of solution B (pH 4.5). Dilute stem solution C with 0.9% NaCl solution 1:10 to produce a 0.1 M solution C.

2.2. Harvesting Donor Organs at Slaughterhouse or Farm

1. Two 10-L polystyrene transportation boxes filled with chipped ice.
2. Four 50-mL Falcon tubes filled with 20 mL HBSS transportation medium and 100 mL HBSS as reserve.
3. Sterile examination gloves, face masks, protective clothing, and rubber boots.
4. Sterile surgical tools for organ preparation, including small and large scissors and forceps (Aesculap, Tuttlingen, Germany), disposable scalpels, and cotton wool swabs.
5. Four sterile stainless steel kidney trays (AKULA Medizintechnik, Lauf, Germany) for on-site organ preparation.
6. Eight sterile plastic bags (2 L, four bag-in-bag), each double bag filled with 300 mL sterile HBSS, and metal clips to close bags.
7. Waterproof permanent markers and documentation sheets.
8. Four 45-cm-long 18 G Cavafix® Certo® 255 catheters (Braun, Melsungen, Germany).
9. Surgical suture material.
10. Clean plastic transportation basins (50 L) to collect the visceral organ package in toto.
11. Large sterile cotton sheets to cover preparation area or desk.
12. 70% Ethanol for on-site disinfection.

2.3. Microscopic Prescreening for a Suitable Organ

1. Dewar vessel with 2 L liquid nitrogen (–196°C) and safety attire and equipment to protect from splashing nitrogen.
2. Standard laboratory light microscope with ×10 and 20 objectives.
3. Cryostat Leica CM 3050S (Leica, Wetzlar, Germany) with microtome stainless steel blades S35 (Feather Safety Razor Co., Ltd., Medical Division, Hartenstein, Wuerzburg, Germany).
4. Standard glass slides and glass cover slides for histology (Histobond™, Marienfeld, Lauda-Koenigshofen, Germany).

5. Cotton wool swabs, disposable scalpels, small scissors and forceps to prepare tissue blocks, large forceps to transfer cryotubes to nitrogen in Dewar vessel.
6. Tissue Tek deep freeze medium (Sakura Finetek Europe B.V., Zoeterwoude, the Netherlands).
7. Cryotubes (2 mL, Brand, Wertheim, Germany).
8. Ten milliliter of freshly prepared DTZ solution.

2.4. Islet Isolation

1. C. Ricordi's stainless steel chamber (500 mL for a 100-g pancreas) with lid and triple lock, medium inlet, medium outlet, sample outlet, temperature and pH controls, four stainless steel marbles (2 cm in diameter), sieve with 500-μm mesh size, and O-ring (all items from Sauer Feinmechanik, Wuerzburg, Germany).
2. Sterile hood HS 18/2, approx. 185 cm wide (Heraeus Instruments, Hanau, Germany). The sterile hood should be wide enough to allow two team members to work side by side.
3. Scales EW 600-2 M (Kern, Albstadt, Germany) max. 600 g; fine scales ALJ 220-4 (Kern & Sohn, Balingen, Germany) max. 220 g.
4. Vortex MR 3001 (Heidolph, Schwabach, Germany).
5. Microscope Axiovert 25 CLF (Carl Zeiss, Jena, Germany).
6. pH meter 315i (WTW, Weilheim, Germany).
7. Water bath E200 (Lauda, Lauda-Koenigshofen).
8. Centrifuge Rotana 46 R (Hettich, Tuttlingen, Germany) with four plastic centrifuge tubes (600 mL).
9. Peristaltic pump ISM 404B with pump head model 7015-20 and Masterflex precision tubing (IsmatecSA, Glattbrugg-Zuerich, Switzerland).
10. Digital temperature control ama-digit ad 15th (Amarell, Koethen, Germany).
11. Laboratory stopwatch, sockets, and clamps.
12. Dimroth condenser, about 50 cm long (Hartenstein).
13. Stainless steel kidney trays, surgical scissors and forceps, small stainless steel clips, pipette set of range 10–1,000 μL, 50-mL Perfusor® syringe (Braun), Omnifix-F 1-mL Tuberculin syringes with injection needles (Braun).
14. Fresh DTZ solution.
15. Standard 24-well plastic plates.
16. Masterflex silicon tubes, 60–70-cm-long pieces with 0.5 cm inner diameter (Hartenstein; the tube ends are covered with

aluminum to keep them sterile); plastic T-junction stopcocks to connect silicon tubes (Hartenstein).

17. A 500-mL glass flask with HBSS-13% stopping medium.
18. A 500-mL glass flask with HBSS-10% washing medium.
19. Two 500-mL glass flasks to collect digest.
20. Sterile plastic ware, e.g., pipette tips, Falcon tubes, Petri dishes.
21. Disinfectant and cotton swabs.
22. Plastic basin with chipped ice.

2.5. Islet Purification

1. Sterile hood (see above).
2. COBE 2991 cell processor model 1 without cooling system (COBE BCT, Inc., Lakewood, CO, USA), 2991™ blood cell processing set (Gambro BCT, Inc., Lakewood, CO, USA).
3. Peristaltic pump ISM 404B with pump head model 7015-20 and Masterflex precision tubing.
4. 50-mL Plastic tubes, four glass beakers (250 mL).
5. Centrifuge Rotana 46 R.
6. Standard 24-well plastic plates.
7. Nuaire™ US auto flow water-jacketed incubator (IBS Integra Biosciences GmbH, Fernwald, Germany; adjusted to 22–24°C low temperature and 5% CO_2 in air, humidified).
8. Fresh DTZ solution.
9. Axiovert 25 microscope.
10. UW-1× solution, WS, and LDS stored on ice.
11. Pipettes, sterile glass, plastic ware.

2.6. Viability Test

1. Darkroom.
2. Standard glass and cover slides for histology.
3. Fluorescence microscope BX50 with ×10, 20, and 40 magnification and digital camera ColorView12 (Olympus, Hamburg, Germany).
4. Fresh FDA, PI, and DTZ solutions (see above).
5. IS mounting medium (Dianova, Hamburg, Germany).
6. Pipette set and cotton swabs.

2.7. Determination of Islet Purity and Islet Yield

1. Standard 24-well plastic plate.
2. Fresh DTZ solution.
3. Axiovert 25 microscope with ×50 magnification and a measuring eye piece (1 cm with 100 graduation lines, Carl Zeiss).
4. Manual cell counter (Hartenstein).
5. Pipette set and cotton swabs.

2.8. In Vitro Culture of Islets

1. Sterile hood.
2. Nuaire™ US auto flow water-jacketed incubator.
3. Axiovert 25 microscope.
4. Standard culture plastic flasks (250 mL).
5. HAM's F12 islet culture medium (see above).
6. Fresh FDA, PI, and DTZ solutions.
7. Pipette set, sterile glass, and plastic ware.

2.9. In Vitro Function of Islets

1. Sterile hood.
2. Nuaire™ US auto flow water-jacketed incubator.
3. EASIA or ELISA kit for insulin determinations (see Note 1).
4. Pipette set, sterile glass, and plastic ware.
5. ELISA reader (Thermo Max Microplate Reader, MWG Biotech, Ebersberg, Germany) and computer software MikroWin Version 3.0 (Mikrotek Laborsysteme GmbH, Overath, Germany).

2.10. Diabetes Induction and Islet Transplantation

1. A license to perform animal experiments.
2. Facilities to keep small and large animals, including intensive care units.
3. Fully equipped operation theaters for small and large animals.
4. Specially trained surgeons.
5. Tools for macro- and microsurgery.
6. Inbred rat or mouse strains (Harlan Laboratories, AN Venray, the Netherlands) and/or Goettingen minipigs (Ellegaard, Dalmose, Denmark).
7. STZ dissolved in "citrate buffer" to induce diabetes. To induce diabetes in rodents, dissolve STZ in this 0.1 M solution C and inject it intraperitoneally within 10 min. Give 200 mg/kg STZ for mice and 35 mg/kg for rats, adjusting injection volume precisely to the body weight (take into account recommended injection volumes for mice and rats).
8. Appropriate suture materials (Braun).
9. Light microscope (Wild M650, Heerbrugg, Switzerland, with ×6, 10, 16, 25, and 40 magnification) to magnify operation field.
10. Special medication for anesthesia, analgesia, hyper- and hypoglycemia.
11. Three blood glucose test systems: (a) Accu-Chek Sensor with Sensor Comfort Pro test sticks (Roche Diagnostics) for rats and pigs requiring 4-µL blood samples, (b) Ascensia Elite with test sticks (Bayer Diagnostics Europe Ltd., Dublin, Ireland) for mice requiring 2-µL blood samples, (c) CGMS® (Medtronic MiniMed, Northridge, USA) for pigs to measure blood glucose real time.

3. Methods

3.1. Media, Solutions, and Supplements

1. Most media can be prepared well before the day of isolation (day 0).
2. Total media volumes should be calculated for a number of subsequent isolations. Exceptions are the enzymatic digestion solution containing collagenase and neutral protease, DTZ solution to discriminate between endocrine and exocrine cells, and FDA medium and STZ in citrate buffer to induce diabetes. These solutions must be always prepared freshly.
3. Solutions and media should not be stored for more than 1 month.
4. Cleanliness and sterility of all devices, solvents, and supplements are essential.
5. Double-check each step carefully, as the time between media preparation and islet transplantation may span many weeks.

3.2. Harvesting Donor Organs at Slaughterhouse or Farm

Two team members are needed for this procedure as one member collects the organ packages and assists the second member with preparation and storage of the pancreata. Remember to take extra materials in case of unforeseen events if the slaughterhouse or farm is far from the laboratory.

1. Cover preparation area or desk at the slaughterhouse/farm with large sterile cotton sheets and use 70% ethanol for on-site disinfection.
2. Collect at least four donor pancreata following the procedure described below to identify the most suitable organ by light microscopy prior to isolation. The best donors may be retired female breeder pigs of the local landrace (1–2 years old; 150–300 kg body weight) (2). Microscopic histological prescreening of four potential donor organs prior to isolation saves disappointment, money, time, and man power. If you wish, you can collect more or fewer organs.
3. Transfer abdominal organs in toto from a brain-dead healthy pig to the clean transportation basin. To avoid bacterial and fungal contamination of the pancreas (and the isolated islets), make sure that all organs are intact, with no leakage from stomach and bowels.
4. Identify the pancreas and prepare it locally under the half-sterile conditions (see Note 2). To this end, touch and squeeze the organ as little as possible; remove fat, lymph nodes, and connective tissue with scissors and forceps while the organ is still warm; and avoid any cuts of the pancreas capsule. Carefully inspect the organ after preparation and exclude organs of

unusual anatomy and color, e.g., grey, dark red, or speckled organs or organs that contain excessive fat or a hematoma.

5. Cut the splenic lobe off the pancreas (it contains body and tail of ~100 g) and transfer it into a stainless steel kidney tray on ice containing 100 mL HBSS.
6. Cut off two tissue blocks of 1 cm^3 each from the pancreas body for histology and transfer them to a Falcon tube with ice-cold HBSS.
7. Identify the main pancreatic duct holding the organ upright and cannulate the duct with the Cavafix® catheter while the organ is still warm. Be gentle and do not use force when cannulating approximately 2/3 of the duct. The organ preparation and cannulation must be quick to avoid unnecessary (warm) ischemic time (cannulation of the pancreatic duct can also be performed later in the laboratory).
8. Fix catheter with suture material at the duct entrance to avoid its slipping during transportation.
9. Store pancreata in ice-cold HBSS inside the double plastic bags. Close bags with metal clips, put them in the polystyrene transportation box, and cover them with ice.
10. Make sure that each Falcon tube and double bag is correctly labeled to avoid mixing tissue samples and donor organs.
11. Transport donor organs immediately to the isolation laboratory (see Note 3). Warm and cold ischemic time should be as short as possible.

3.3. Microscopic Prescreening for a Suitable Organ

1. Swab the tissue blocks from the Falcon tubes with cotton wool, transfer to cryotubes containing the cryoprotection medium Tissue Tek, and then quick-freeze in liquid nitrogen.
2. Immediately, with the cryostat at –20°C, cut 10-µm-thick tissue sections from four frozen tissue blocks representing the four donor pancreata.
3. Place sections on glass slides, immediately stain with 100 µL fresh DTZ solution, and inspect under the microscope at ×10 and 20 magnification. Islets of Langerhans stain red within seconds, whereas acinar tissue remains unstained. The number, size, and morphology of islets in the DTZ-stained tissue sections correlate with the number, size, and morphology of islets in situ and after isolation. Only pancreata containing sufficient numbers of large islets (200–250 µm in diameter), showing nice morphology and evenly distributed strong red staining, should be considered as donor organs for subsequent isolation (3). A sufficient number on a 1-cm^2 pancreatic tissue section would be a minimum of five islets. No red staining or only slight red staining (always compared to a positive control)

indicates a lack of insulin vesicles in the islets, which may be due to trauma, stress, or inadequate conditions of feeding and/or keeping of the donor animals. Such pancreata should be excluded as donor organs, as it is not yet clear if the function of such poorly stained islets can be restored.

3.4. Islet Isolation

The basic isolation methodology is described in great detail and schematically outlined by its developer, Ricordi (4, 5). The methodology is used with great success by many groups around the globe. Fully automated, it can be used by beginners to avoid unnecessary costs. Details of the automated method for pancreatic islet separation, as designed by C. Ricordi, can be viewed on the Internet (6). The automation may save time and labor, but has no particular influence on final isolation results.

3.4.1. Assembly of Equipment on Day –1

1. Assemble all isolation equipment on day –1 under the sterile hood, ensuring that all parts are clean, dry, and sterile. Have enough isolation equipment parts in reserve in case of unforeseen events during isolation as isolations can be saved if sterile, clean spare parts are ready at hand. The isolation chamber can be made of stainless steel, transparent plastic, or glass. A plastic or glass chamber allows viewing of the tissue disintegration, but we use the stainless steel chamber with great success. The size of the Ricordi chamber must correlate with the size of the donor organ. For a 100-g pancreas, we use a 500-mL chamber.
2. Set up the connections, without media, in preparation for day 0. The Masterflex silicon tubes connect the glass flask containing the recirculation medium (enzymatic digestion solution) to the peristaltic pump, water bath, Dimroth condenser, chamber inlet, and chamber outlet, which is then connected to the glass flask containing the recirculation medium. Silicon tubes should be as short as possible to avoid unnecessary use of expensive enzymatic digestion solution. In the elution phase, silicon tubes are reconnected so that the stopping medium and then the washing medium elute the digest from the Ricordi chamber into the collection flask.
3. After assembly, double-check all parts, cover isolation equipment with sterile cotton sheets, and close the hood. All procedures concerning the handling of the equipment during isolation should be well learned in advance, as the viability of islets also depends on the total time between transfer of the pancreas pieces to the chamber and the moment the digest with the islets is put on ice prior to purification.

3.4.2. Islet Isolation on Day 0

1. First, turn on the water bath, as a digestion temperature of 37°C is required in the chamber when digestion begins.
2. Fill 2/3 of the chamber with UW-1× solution and start pumping the medium through the system to preheat the chamber.

3. Transfer the previously selected donor pancreas (see above) into an empty sterile kidney tray on ice inside the hood, and remove residual fat (see Note 4), connective tissue, and lymph nodes with small scissors and forceps. Do not damage the pancreas capsule to avoid leakage.
4. Weigh the organ.
5. Fill the 50-mL Perfusor® syringe with the enzymatic digestion solution, connect it to the catheter in the pancreatic duct, and inject the solution carefully, putting very little pressure on the syringe, via the duct into the organ. All parts of the 100-g pancreas must expand well as organ parts that do not expand will not digest and will not release islets. Use small stainless steel clips to fix capsule leakages. A 100-g pancreas takes as much as 200 mL of enzymatic digestion solution to be sufficiently expanded (blown up) (see Note 5).
6. Cut the organ into 3–4 pieces, put them into the chamber together with the four marbles, fill the chamber with the rest of the enzymatic digestion solution, put the sieve and O-ring into position, and close and lock the lid carefully.
7. Begin the recirculation phase by starting the peristaltic pump (85 rpm: flow rate 75 mL/min at inner tubing diameter of 0.5 cm). This step is t_0 of digestion. During digestion, shake the chamber by hand (10 hubs/10 s, 5 s pause, 10 hubs/10 s, etc.) to guarantee good mixture of its contents. An automatic shaker may be preferable, but shaking by hand leads to equally good results. Normal digestion time ranges from 15 to 25 min, depending on the activity and the amount of collagenase/neutral protease used, and the time to achieve the temperature optimum inside the chamber (range 35–37°C). If necessary, use prewarmed UW-1× solution to extend the recirculating medium volumes in order to remove air bubbles.
8. When the enzymatic digestion solution becomes cloudy (circulating tissue particles), take a 300 µL tissue/digest sample from the chamber every minute with a sterile Omnifix 1 mL Tuberculin syringe and transfer it to the first well of the 24-well plastic plate containing 300 µL fresh DTZ staining solution per well.
9. Quickly inspect the digestion status under the Axiovert 25 microscope (see Note 6).
10. Carefully check and document the temperature and pH value inside the chamber at various digestion times, as the pH tends to decrease during digestion from pH 7.45 (starting point) to about pH 7.3 (elution point). If it drops below pH 7.2—for reasons that are not yet well understood—the final islet preparation will have very poor viability. Thus, pH 7.2 is our cutoff point. This steep drop in pH during digestion is never seen with organs that are explanted in the operation theatre.

Therefore, we think a prolonged warm ischemic time during organ harvest leads to this otherwise unexplained drop of pH. We tested a number of different buffers and found that the KH_2PO_4 buffer gave the best results.

11. As soon as the sample shows 1–2 well-isolated normal-sized red-stained islets without an exocrine rim, halt recirculation of the medium and elute the digest from the chamber with ice-cold HBSS-13% (stop solution) (see Note 7). Then, collect the digest in the centrifuge tubes and wash three times with HBSS-10% at 250 × *g* in the centrifuge (slow acceleration, 3 min, 4°C, no brake).
12. Resuspend gently and pool the sediments before transferring to a 500-mL glass flask. Then, add the UW-1× solution up to a volume of 200 mL. Three samples of 100 µL each are transferred to the 300 µL DTZ-containing wells in the 24-well plastic plate to count islets within the digest prior to purification.
13. At this point, add 15 mL nicotinamide solution and 50 mL FCS to the pancreas digest plus UW-1× solution to bring the digest volume to a final 500 mL.
14. The pancreas digest is then put on ice for 1 h.
15. The undigested pancreatic tissue is collected from the Ricordi chamber and weighed (see Note 8).

3.5. Islet Purification

1. Place the COBE cell processor close to sterile hood and prepare it for islet purification. To this end, insert the 2991™ blood cell processing set very accurately (folds in the plastic material will interfere with the developing gradient), arrange and fix colored tubing according to processor function, and connect tubing of processing set with tubing of peristaltic pump (inside sterile hood). If you use a COBE cell processor that has no built-in cooling system, keep all solutions and gradients on ice.
2. Centrifuge at 200 × *g* the 500 mL digest that was rested for 1 h, discard the supernatant, and resuspend the digest carefully with 200 mL of ice-cold UW-1× solution and 120 mL of WS.
3. Calibrate the COBE cell processor as follows: 1,500 rpm, supernatant flow rate at 100 mL/min, minimal mixing time 60 s, supernatant volume 600 mL, valve selection V 1.
4. Pump the cold digest suspension into the processor at 150 rpm (peristaltic pump) and start centrifugation. Then, pump 96 mL of ice-cold LDS at 45 rpm into processor, followed by 120 mL of ice-cold UW-1× solution, also at 45 rpm. Centrifuge digest for 1–2 min to allow gradient build. The tissue separation inside the processing set can be viewed macroscopically.
5. Operating the processor buttons manually, discard the first 50 mL of the gradient, elute the subsequent gradient, and collect

samples of 30 mL each in seven carefully numbered 50-mL Falcon tubes (nos. 1, 2, …, 7). Keep tubes with contents on ice.

6. Transfer three 100-μL samples from each tube to a new 24-well plastic plate containing fresh DTZ solution in 7 × 3 carefully numbered wells (nos. 1.1, 1.2, 1.3, …, 7.3).
7. Select tubes containing the most pure islets (usually nos. 3, 4, and 5). These 3 islet fractions are then pooled and resuspended in 100 mL of ice-cold UW-1× solution. Then, three 100-μL samples are transferred to DTZ-containing wells to count the purified islets.
8. Transfer the main islet suspension to two Falcon tubes and centrifuge (200 × *g*, slow acceleration, 3 min, 4°C, no brake). Then, discard the supernatant and resuspend the sediment with 32 mL of HAM's F12 islet-culture medium (with supplements).
9. Transfer 30 mL of islet suspension to a 250-mL culture plastic flask and put into the water-jacket incubator for low-temperature (24°C) in vitro culture. The final 2-mL islet suspension is used for subsequent testing of viability and purity, and for quantification of the islet yield.

3.6. Viability Test

The viability tests are performed in the darkroom (see Note 9).

1. Add 1 μL of FDA solution to 50 μL of islet suspension (the reaction time of this mixture is 15 min at 37°C in the dark).
2. Then, add 10 μL of PI solution to the suspension, transfer 100 μL of this mixture to a glass slide, add 25 μL of mounting medium to stabilize the fluorescence, and cover the mixture with a cover slide.
3. Remove superfluous solution with a cotton swab.
4. Immediately analyze islets under the fluorescence microscope at 488 nm wavelength with a 530-nm filter at ×10, 20, or 40 magnification. Document the result with the ColorView12 digital camera. Viable islets show a bright green color, whereas dead cells display a red nucleus. Count viable and dead islets and indicate as percentage of the total cell number (see Note 10).

3.7. Determination of Islet Purity and Islet Yield

1. Transfer 100 μL of the final islet suspension to each of the five wells of a 24-well plastic plate, each well containing 100 μL of fresh DTZ solution.
2. To determine islet purity, inspect and measure the red-stained islets and unstained residual acinar tissue fragments (preferably by two team members independently) under the Axiovert 25 microscope at ×50 magnification using the measuring eyepiece. Then, calculate mathematically the percentage of endocrine tissue in relation to the total amount of tissue. This calculation is performed for each well.

Table 1
The number of islet equivalents (IEQ = an islet with a diameter of 150 μm) in an islet preparation is estimated by measuring the islet diameters in a representative sample observed under the light microscope and calculating their volumes mathematically. The islet volumes are converted with the help of the conversion factor

Islet diameter, range (μm)	Mean volume (μm^3)	Conversion factor (counted islet number × conversion factor)
50–99	294.525	0.16
100–149	1,145.373	0.66
150–199	2,977.968	1.7
200–249	6,185.010	3.5
250–299	11,159.198	6.3
300–349	18,293.231	10.4
≥350	27,979.808	15.8

This table was first published by Bretzel et al. (9) and is reproduced here with kind permission of Springer Science and Business Media

3. To determine the islet yield, measure the size of each single islet in the five wells with the eyepiece and grade it according to Table 1. Depending on the type of pancreas selected for isolation, smaller or bigger islets may predominate. To harmonize differing results among various research groups, count islets of each size group (see Table 1) according to an imaginary islet 150 μm in diameter. Such an ideal islet is called an "islet equivalent" or IEQ (see Note 11).

3.8. In Vitro Culture of Islets

1. Inspect in vitro-cultured islets every day under the light microscope at sufficient magnification to identify possible bacterial and fungal contamination. Do not mistake the smaller fragments of dying islet cells (e.g., as a consequence of mechanical stress during isolation) as bacterial contamination; again, sterility of all materials is essential (see Note 12). Islets should not aggregate or disintegrate.
2. Perform FDA/PI viability testing according to research aims. DTZ staining is not necessary to inspect islets.
3. Change half of the HAM's F12 culture medium (with supplements) every second day to put islets under minimal stress. Use sedimentation instead of centrifugation to separate islets and medium. We have also cultured porcine islets in the (very expensive) human islet cell-specific hCELL medium—exactly as produced by the manufacturer (hCELL technologies Inc.,

Reno, USA)—with very good success for up to 6 weeks (islet viability and function in vitro were excellent).

3.9. Testing In Vitro Function of Islets

In vitro assessment of the function of isolated islets of various species is a routine procedure in all laboratories that deal with islet isolation/transplantation on a regular basis. The physiology of insulin secretion in standard assays for testing the dynamic insulin response upon a glucose challenge is extensively described by Luzi et al. (7) and is not repeated here in detail. Such assays are easy to perform, either by static incubation or perifusion of the islets. Assays for the rat (8) and the human system (9) are basically also applicable to porcine islets, although the islet stimulus "glucose" must be dissolved in culture medium specific for porcine islets.

1. Set up assay with pig islets as desired (see Note 13).
2. Collect pig insulin-containing culture supernatants at predetermined time points and store at –20°C before being used in the commercial EASIA/ELISA kit exactly as described in the manual.
3. After conducting the immunodetection, results are analyzed at two wavelengths (450 and 490 nm) against a reference filter (650 nm) with the ELISA reader.
4. Determine final insulin levels following the standard curve and show in μU/mL.

3.10. Diabetes Induction and Islet Transplantation

In principle, the technique of transplanting pig islets into specific recipients, e.g., diabetic mice or rats, does not differ from transplanting rodent or human islets into these diabetic recipients. However, whether it is biologically sensible to transplant them into an STZ diabetic recipient or an animal whose diabetes was induced by pancreatectomy depends on the particular research problem. Mice and rats usually become diabetic within 2–3 days after intraperitoneal STZ injection (in case they do not, the full dosage of STZ is injected a second time) (see Note 14). As single beta cells can survive the STZ treatment, residual insulin production may be observed. Pancreatectomy requires the skills of an experienced surgeon or microsurgeon and results in complete elimination of the insulin-producing cells. It is, thus, the technique of choice for diabetes induction when the commercially available insulin detection assay does not discriminate between recipient and donor insulin. Our own group recently described an STZ and a pancreatectomy diabetes model in miniature pigs (10). Details of both methods and their pros and cons are presented in the publication.

Appropriate in vivo testing of isolated pig islets requires their transfer into small diabetic animals, e.g., mice and rats, or, to mimic the clinical situation, into much larger diabetic animals, e.g., diabetic pigs or nonhuman primates. Like in vitro assays, in vivo assays are a

standard procedure in most if not all "islet laboratories" around the world for testing the function of isolated pig islets in living animals. All materials and microsurgical techniques for transplanting xenogeneic islets into diabetic rodents are identical to those used in syngeneic or allogeneic transplantation. They are described at length in the book *Experimental Transplantation Models in Small Animals* (11). Porcine islets can also be used in these models; however, since these islets are xenogeneic to the recipient, specific modulation of the recipient's immune system is required after transplantation (see below). Many "classical" immunosuppressive drugs are applied with great success in small animal transplantation. Regarding larger animal models, transplantation of porcine islets into nonhuman primates is usually restricted to those few institutions performing biomedical research in rhesus monkeys or baboons. Transplantation of porcine islets into nonhuman primates was recently reviewed by Hering and Walawalkar (12). Details on the equipment, surgical procedures, immunosuppressive regimes, etc. can be found in this review and in many original publications cited there. In addition, an increasing number of universities provide the specific facilities to perform islet transplantation in diabetic pigs. Pig-to-pig transplantation is allogeneic by nature but can be used to test the in vivo function of islets, different transplantation sites, and techniques and to prepare all the logistics step by step for future clinical application. In this setting, blood glucose concentrations are monitored real time with the CGMS® as previously described by our group (10) or conventionally with the Ascensia Contour® blood glucose monitoring system using capillary blood harvested from the pig ear. In each case, it is advisable to train the pig for compliance well in advance of the blood glucose assays. To test the functional capacity of the transplanted islets, it is necessary to perform the intravenous glucose tolerance test and the hyperglycemic clamp; both were successfully performed in normal, diabetic, and transplanted pigs as described (10).

There is an ongoing debate as to which is the best transplantation site: for small animals, the portal system of the liver, the subrenal capsule, the subcutis, the spleen, the omentum, or the peritoneal cavity (11); for large animals and humans, the portal system of the liver (13), the omentum, or the peritoneal cavity (14). Microencapsulated islets, particularly double capsules, may be too large in diameter for the portal system of the liver. If grafted to the omentum, this site can be removed in case of an emergency, e.g., an infection or non-function. It may be impossible to remove every single islet or microcapsule from the pig's peritoneal cavity.

3.11. Immunomodulation of Islets

This section contains only theoretical consideration, as there is a wide range of concepts that have to be dealt with regarding this important subject. The strong immunological barrier between donor islets and the recipient's immune system in the xenogeneic pig-to-human or pig-to-nonhuman primate situations requires

manipulation of the donor islets' immunogenicity prior to transplantation, as well as of the recipient's immune system after transplantation. The book *Xenotransplantation* (15) has the subtitle *The Transplantation of Organs and Tissues between Species*, which gives an excellent overview of this type of transplantation and its specific immunology. There are multiple successful approaches to reduce the immunogenicity of isolated islets, as well as regimes to induce immunosuppression; these are extensively described in the book *Pancreatic Islet Cell Transplantation* (16). Our group favors microencapsulation of the isolated pig islets with highly purified alginates. There are already a few companies worldwide providing products and services in this fascinating field (17) (see Note 15). Knowledge and training are also provided by *The Bioencapsulation Research Group* (18). The different methods of encapsulation, e.g., micro- and macroencapsulation, and encapsulation materials are splendidly discussed in the book *Cell Encapsulation Technology and Therapeutics* (19). In addition, the promise and progress of this fascinating concept are critically discussed in an excellent review paper (20). As the field of bioencapsulation rapidly develops and expands, each group has to find its own type of encapsulation material and technique tailored to its particular research aims.

4. Notes

1. Always ensure that the antibodies for commercial insulin react in a species-specific manner and that the C-peptide ELISAs cross-react appropriately. Sophisticated antibodies and detection reagents are readily available for mouse, rat, and human experimentation, but rarely for pig; thus, cross-reaction should be tested in advance. Read the company's instructions carefully, but do not rely on them—do your own testing.
2. Sufficient test runs should be performed at the slaughterhouse or farm to learn sterile explantation, preparation, and storage to perfection before organs are shipped to the laboratory for isolation. A visceral surgeon with knowledge of pancreas anatomy/morphology may be helpful in this learning phase.
3. Warm ischemic time is detrimental to the viability of isolated islets—autolysis of the pancreas begins immediately in the brain-dead animal. Optimal organ harvesting is essential for successful islet isolation. In fact, warm and cold ischemic time of the donor organ can be completely avoided if it is explanted with expertise in the operation theatre of the same hospital, where the islets will be isolated.

4. Never use a pancreas containing fat that cannot be removed as fat dissolves during digestion at 37°C and clogs sieve pores and digestion equipment.
5. Prepare the enzymatic digestion solution *after* the pancreas is prepared, well cannulated, and weighed. The expensive enzymatic digestion solution would be wasted if cannulation of the pancreatic duct fails, which can happen due to unusual duct anatomy. Moreover, pancreas tissue that does not distend when the enzymatic digestion solution is injected via the pancreatic duct will not release islets! To proceed with such tissue is a waste of time, labor, and money.
6. Should the DTZ solution fail to stain the islets, do not panic. Isolated islets can be easily identified under the light microscope because they look like beige or brown potatoes; acinar tissue is grey/dark grey.
7. Determining the optimal digestion time point, i.e., the time point when islets are optimally digested in the recirculation phase, requires great experience (and intuition!). The optimal digestion time varies with each type of collagenase, each batch of the same collagenase, collagenase concentration, and temperature inside the chamber. We prefer collagenase NB8 for porcine islet isolation as collagenase NB1 produces much lower islet yields.
8. If you want to perform two isolations within 24–48 h, have a complete second isolation set ready for use. In case of unforeseen events, do not panic, stay calm, and rethink every step; always try to rescue the isolation by being inventive and using unconventional measures.
9. A parallel DTZ-stained control may help to discriminate between islets and rare small lymph nodes; an inexperienced eye may mistake small lymph nodes for islets that also stain green with FDA/PI. Photographic documentation should be carried out first thing as FDA/PI staining is very light sensitive and the preparation with viable cells cannot be preserved.
10. It is best if the number of dead cells is determined independently by two team members. The viability of "slaughterhouse islets" that have suffered up to a 20-min warm ischemic time—due to the slaughtering process—ranges from 80 to 95%, whereas islets isolated from organs explanted in the operation theatre without warm ischemic time have 95–98% viability.
11. Thus, an islet 300 μm in diameter equals 2 IEQ and an islet 0.75 μm diameter equals 0.5 IEQ. A very good donor organ may have a final islet yield of 400,000 IEQ, a yield of 5,714 IEQ/g of organ (100 g of the pancreas were put into the chamber, 30 g remained undigested, thus 70 g of digested pancreas released the 400,000 IEQ). Using a much smaller

minipig pancreas for isolation may result in similar IEQ/g of organ, but in much smaller total IEQ numbers.

12. Sterility and appropriate staff training are the highest priority in islet in vitro culture. It is extremely expensive and very frustrating to have to repeat a culture experiment with isolated islets because of neglect or lack of concentration.
13. Islet numbers and the total medium volume per individual test have to be adapted to the total number of islets available for such assays. In order to save sufficient islets for transplantation, rat and pig assays may require different islet numbers and need to be designed accordingly.
14. The sensitivity of inbred mouse and rat strains to STZ differs greatly; therefore, dosage, timing (single or repeated injections), and diabetes status should be predetermined in each single strain. Do not rely too much on specifications given in publications, as the same inbred rat or mouse strain/substrain may react differently according to where and when it was generated.
15. Cooperation with appropriate physicists, biotechnologists, material researchers, and companies that produce encapsulation devices can be extremely helpful.

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References

1. Latif ZA, Noel J, Alejandro R (1988) A simple method of staining fresh and cultured islets. Transplantation 45:827–830
2. Krickhahn M, Meyer T, Bühler C, Thiede A, Ulrichs K (2001) Highly efficient isolation of porcine islets of Langerhans for xenotransplantation: numbers, purity, yield and in vitro function. Ann Transplant 6:48–54
3. Krickhahn M, Bühler C, Meyer T, Thiede A, Ulrichs K (2002) The morphology of islets within the porcine donor pancreas determines the isolation result: successful isolation of pancreatic islets can now be achieved. Cell Transplant 11:827–838
4. Ricordi C, Socci C, Davalli AM et al (1990) Isolation of the elusive pig islet. Surgery 107:688–694
5. Ricordi C, Rastellini C (1995) Automated method for pancreatic islet separation. In: Ricordi C (ed) Methods in cell transplantation. RG Landes, Austin, pp 433–438
6. http://www.biorep.com/products-diabetes.aspx

7. Luzi L, Secci A, Pozza G (1992) Metabolic assessment of posttransplant islet function in humans. Methodological considerations and possible pitfalls: a lesson from pancreas transplantation. In: Ricordi C (ed) Pancreatic islet cell transplantation. RG Landes, Austin, pp 361–382
8. Lacy PE, Finke EH, Conant S, Naber S (1976) Long-term perfusion of isolated rat islets in vitro. Diabetes 25:484–493
9. Bretzel RG, Hering BJ, Federlin KF (1995) Assessment of adult islet preparations. In: Ricordi C (ed) Methods in cell transplantation. RG Landes, Austin, pp 455–463
10. Strauss A, Moskalenko V, Tiurbe C, Chodnevskaja I, Germer CT, Ulrichs K (2010) Goettingen minipigs (GMP): comparison of two different models for inducing diabetes. Diabetol Metab Syndr. 2012, 4:7
11. Leow CK (1995) Pancreatic islet transplantation in rodents. In: Green MK, Mandel TE (eds) Experimental transplantation models in small animals. Harwood Academic, Chur, pp 85–105
12. Hering BJ, Walawalkar N (2009) Pig-to-nonhuman primate islet xenotransplantation. Transpl Immunol 21:81–86
13. Bühler L, Deng S, O'Neil J et al (2002) Adult porcine islet transplantation in baboons treated with conventional immunosuppression or a non-myeloablative regimen and CD154 blockade. Xenotransplantation 9:3–13
14. Calafiore R, Basta G, Luca G et al (2006) Microencapsulated pancreatic islet allografts into nonimmunosuppressed patients with type 1 diabetes. Diab Care 29:137–138
15. Cooper DKC, Kemp E, Platt JL, White DJG (1997) Xenotransplantation. Springer, Berlin
16. Ricordi C (1992) Pancreatic islet cell transplantation. RG Landes Company, Austin
17. http://www.lctglobal.com
18. http://impascience.eu/bioencapsulation
19. Kühtreiber WM, Lanza RP, Chick WL (1999) Cell encapsulation technology and therapeutics. Birkhäuser, Boston
20. Orive G, Hernández RM, Gascón AR et al (2003) Cell encapsulation: promise and progress. Nat Med 9:104–107