Islet Isolation: Procedure, Laboratory, Costs

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Summary: Islet Isolation

Introduction — Roughly 1 million islets of Langerhans are scattered throughout the human pancreas. The diameter of these clusters of cells ranges from 0.05 to 0.5 mm. The total volume of all the islets together is only about 1% of the volume of the pancreas.
An islet is usually surrounded by a thin connective-tissue capsule embedded in the rest of the pancreatic tissue (the acinar tissue). In essence, the islets are freed ('isolated') from the surrounding tissue by digesting the (collagen-containing) connective tissue in the pancreas using a collagenase containing enzyme solution. After infusing a collagenase solution into the pancreas via the duct, the tissue is shaken for about 30 minutes at 37°C, during which the connective tissue is digested and the pancreas breaks down into fragments of acinar tissue and free ('isolated') islets. Procedure — The 'state-of-the-art' technique (the so-called automated Ricordi method) was recently studied by us during 6 human islet isolations at the Diabetes Research Institute (Miami) of Professor Ricordi. In this method, after the pancreas is infused with collagenase, it is digested in a stainless-steel perfusion chamber connected to a shaking device. The chamber is fitted with a thermometer and is incorporated, via inlet and outlet ports, into a system of tubing through which the isolation fluid is circulated by a pump via a heating element. Inside the chamber, a screen in front of the outlet port prevents large — not yet sufficiently digested — tissue fragments from leaving the chamber. Initially (first phase, about 25 minutes) digestion takes place at 37°C with recirculation of the isolation fluid. Once digestion is sufficient (2nd phase), fresh medium at a lower temperature (down to about 10°C) is continuously supplied to the chamber, and the suspension of isolated islets and fine acinar particles leaving the chamber is collected on ice. In this way, while digestion of the remaining pancreatic fragments in the chamber is being completed, damage to the islets already isolated is prevented. The resulting tissue suspension, approximately 8 liters, is reduced by centrifugation to about 250 ml for the next step: purification of the islets. The isolated islets are purified from the acinar particles by exploiting the difference in density (weight per unit volume, expressed in grams per ml) between the islets and the acinar particles. The tissue is taken up in a purification fluid of high density (e.g. 1.085 g/ml) in a special centrifuge: the Cobe cell processor. Over this high-density tissue suspension, several layers of pure purification fluid of lower density (e.g. 1.075, 1.070 and 1.045 g/ml respectively) are pumped on top. During subsequent centrifugation of this 'density gradient', the islets — owing to their relatively low density — migrate to the top of the gradient, while the acinar tissue remains almost entirely in the heavier bottom layer. After centrifugation, the gradient is pumped out of the centrifuge and the fraction containing the purified islets is collected separately. Finally, the purified islets are kept under tissue-culture conditions before being transplanted, if appropriate. Quality control — During and after the procedure, samples are taken to check for (an)aerobic contamination (sterility) and for microscopic determination (morphometry) of the yield of isolated islets and the purity of the preparation. The viability and function of the islets are examined: i) with a membrane-integrity test (uptake of acridine orange and propidium iodide), ii) by determining the survival percentage of islets under tissue-culture conditions, and iii) by transplanting a threshold dose of islets under the kidney capsule of diabetic nude mice.

Isolation: Procedure, Laboratory, Costs

Introduction

Purpose of the isolation procedure

Roughly 1 million islets of Langerhans are scattered throughout the human pancreas. These clusters of cells (which produce insulin and other hormones) have a diameter ranging from 0.05 to 0.5 mm. The total volume of all the islets in the human pancreas is about 1 ml; this represents about 1% of the volume of the pancreas (weight approx. 100 g). An islet is usually surrounded by a thin connective-tissue capsule embedded in the rest of the pancreatic tissue (the acinar tissue). For a successful islet transplant it is important that i) sufficient, viable islets are available, and ii) that the islet transplant is contaminated as little as possible with acinar tissue particles. The isolation procedure is therefore aimed — in two steps — first at freeing as many islets as possible, intact, from the surrounding pancreatic tissue (the actual 'isolation' of the islets), and subsequently at purifying the islets as much as possible from the acinar tissue.

General principles of the 'islet isolation' technique

In general, when one speaks briefly of 'islet isolation', this usually refers to a process consisting of two successive procedures: the actual isolation of the islets, followed by a purification procedure. In the first step — the isolation — a physiological solution of enzymes, mainly collagenase enzymes, is infused into the pancreas via the pancreatic duct (the duct that normally drains the digestive enzymes into the small intestine). The pancreas is then kept at about 37°C, during which the supporting tissue of the pancreas is digested and the islets and acini (or fragments of acinar tissue) are freed ('isolated') into suspension with minimal mechanical assistance, such as shaking. Good control of the digestion process (the correct duration, the enzyme mixture used, etc.) is important here, in order to prevent, as much as possible, damage to both the islets and the acinar tissue of the pancreas. The suspension of islets and acinar fragments that results from digestion of the pancreas ("digestion") is usually referred to by the term "digest". Because the islets of Langerhans make up only 1–2% of the total pancreatic tissue, the isolated islets still need to be purified after this digestion process. To separate the isolated islets from the rest of the tissue ("purification"), use is made of the difference in density (weight per unit volume, expressed in g/ml) between the islets and the acini. Purification takes place by centrifuging the tissue (digest) in a density gradient, a stack of solutions (for example in a centrifuge tube; see Figure 1) of decreasing density. In the density gradient, the relatively lighter islets float to the top, while the acinar tissue — which has a higher density — remains at the bottom of the gradient. Finally, the purified islets can be harvested from the top of the density gradient and, after brief incubation under tissue-culture conditions if needed (to allow recovery), transplanted.

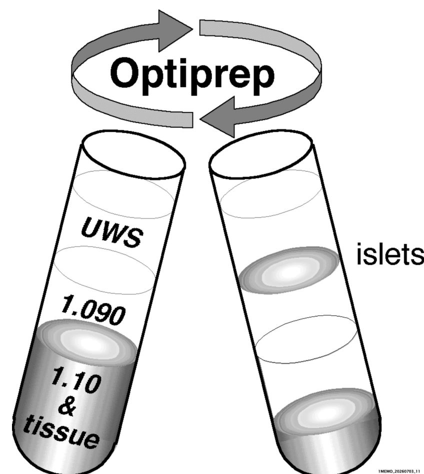

Figure 1. *Schematic representation of islet purification in density gradients, in this example using iodixanol (trade name OptiPrep) mixed in University of Wisconsin organ-preservation solution (UWS). The tissue is mixed into the bottom layer of the gradient (left in the figure). This bottom layer has a density of 1.10 g/ml. Layers of lower density are placed above it. During centrifugation the islets migrate to the top (right in the figure), while the rest of the pancreatic tissue remains at the bottom.*

Problems with isolation procedures

In addition to the many variables of the isolation procedure itself, there are numerous donor- and procurement-related variables, which means that the outcome of successive isolation procedures is not consistent. As a result, it depends, among other things, on the experience of an islet center whether about one in ten or one in three isolation procedures is successful enough to make a transplant possible. In general, it can therefore be said that obtaining sufficient purified (and viable) islets from a pancreas for transplantation remains a problem. The goal of the isolation procedure (and further developments) is therefore always: i) as high a yield of viable islets as possible, with ii) a high purity of the preparation — at least >50% — and iii) a technique offering the best guarantee for maintaining (and achieving) sterility of the preparation.

“State of the art” procedure, introduction

The procedure for the isolation and purification of islets of Langerhans from the human pancreas described below has, in essence, been used by virtually all centers elsewhere in the world for the past 5–8 years. The most important feature of this “state of the art” procedure is that the enzymatic digestion of the pancreas takes place using the so-called “automated Ricordi method” in a so-called “Ricordi chamber”. A description of this laboratory set-up follows below under “Description of the isolation procedure”. This procedure was recently studied by us in detail (working visit by Michel van der Burg over 3 weeks in April–May 1998) during 6 human islet isolations at the Diabetes Research Institute in Miami (head: Prof. Camillo Ricordi). During this visit, a new purification method recently developed by us (in a pig model) was also tested during the human isolations, with promising results (1). Prior to this visit, the details, variations, and recent developments surrounding this isolation procedure and transplantation were discussed during an informal workshop for 30 invited participants from all clinical islet-transplantation centers (Michel van der Burg; 1st Workshop on Clinical Islet Transplantation, Topic: Human Islet Preparation; New Orleans, April 17–19, 1998).

1. *Efficacy of the Novel Iodixanol-UWS Density Gradient for Human Islet Purification. Van der Burg MPM, Ranuncoli A, Molano R, Kirlew T, Ringers J, Bouwman E, and Ricordi C . Department of Surgery, Leiden University Medical Centre, Leiden, Netherlands, and Diabetes Research Institute, University of Miami, Miami, USA. Submitted: 18th Workshop of the AIDSPIT Study Group, Igls, Austria, Jan 24–26, 1999.*

Description of the isolation procedure

Equipment and set-up

The entire procedure (isolation and purification) can be carried out in a standard tissue-culture laboratory, equipped with at least 1, but preferably 2, biohazard flow cabinets. For isolating islets that will ultimately be transplanted into a human recipient, a laminar-flow cleanroom with temperature control and positive pressure is preferred, in order to minimise the risk of contamination. One, preferably two, high-capacity centrifuges are needed to process the large volumes of tissue suspension (about 8 liters). Purification of islets in a density gradient can, if necessary, also take place in this centrifuge using large centrifuge tubes; however, given the large number of tubes (about 20–40) usually required, purification in a Cobe 2991 cell processor (one run in the Cobe is generally sufficient for the whole preparation) offers many advantages.
The equipment for the "automated" isolation method can be set up at one of the flow cabinets. The other flow cabinet is used for further processing of the tissue suspension obtained from digestion of the pancreas, and for the subsequent purification procedure in the Cobe. The procedure is performed by 2 people. At the start of the procedure, a double-wrapped sterile isolation pack is opened in the flow cabinet. This pack contains: the base and lid of the Ricordi digestion chamber with accompanying sealing ring, screen, and marbles; two temperature sensors; a stand for the Ricordi chamber; a second stand with 3 clamps; a stainless-steel heating coil; a glass beaker or cylinder (as shown in the drawing); two stainless-steel trays and surgical instruments for trimming the pancreas and cannulating the duct.
The Ricordi chamber is the most important component, and consists of a 500 ml stainless-steel cylindrical chamber and a conical lid (Figure 2). Ports for flow through the chamber (from bottom to top) with isolation medium are present at the bottom of the chamber and at the top of the lid. A plastic sealing ring and a stainless-steel screen can be placed between the upper and lower parts of the chamber. The screen (with mesh openings of about 0.5 mm) serves to keep the pancreas and the larger, not-yet-digested fragments inside the chamber during digestion, while allowing the suspension of fine tissue particles to pass through so that it can be collected outside the chamber — protecting the already-isolated islets from further digestion and fragmentation. The side wall of the chamber contains an access port for a temperature sensor, so that the heating of the incoming isolation fluid can be regulated based on the measured temperature in the chamber.

Isolation procedure

After unpacking the sterile materials in the flow cabinet and setting up the Ricordi chamber, a large stainless-steel tray is filled with sterile cold saline and frozen saline. A smaller tray containing cold Hanks' solution is placed inside it. While the Ricordi chamber is pre-warmed to about 40°C by recirculating warmed Hanks' solution (see below), the pancreas is roughly trimmed free of adherent tissue (fat, connective tissue, and any duodenum or spleen) in the tray. After weighing, the pancreas is then divided at the neck (directly below the head). The tail is infused retrograde and the head of the pancreas anterograde, after cannulating the duct, with a collagenase (Liberase) solution in Hanks' solution — approximately 2 ml of solution is infused per gram of tissue. While the chamber is being emptied, the infused pancreas is further trimmed. The pancreas is then transferred, together with a few marbles (which serve to

prevent the pancreas from moving 'without delay' along with the chamber during shaking), into the chamber along with the remaining collagenase solution. The sealing ring and screen are fitted, the chamber is closed, and it is then suspended in a custom-made shaking machine (Figure 2).

The isolation process consists of two phases. During the first phase (recirculation phase), the medium circulates in a closed system: a roller pump pumps the medium from the "Recirculating cylinder" through a heating coil in a water bath ("Heating circuit") and through the Ricordi chamber ("Digestion chamber") containing the pancreas, back to the cylinder. At start-up, the system is filled entirely with Hanks' solution via the recirculation cylinder. At the outset the chamber temperature is about 30°C, and during recirculation with Hanks' solution (at a flow rate of 100 ml/min) the temperature is gradually raised to about 35–37°C. During this process the chamber is continuously shaken up and down at a rate of 1–1.5 times per second, and a sample is taken approximately every 5 minutes ("Sampling port"). When digestion is judged adequate (sufficient tissue and free isolated islets present in the suspension), the process moves to the 2nd phase (the dilution phase) by switching valves, as shown schematically in Figure 2. During the dilution phase, fresh cold medium ("Dilution solution") is continuously supplied to the chamber (the heating coil is then kept outside the water bath), and the tissue suspension is collected from the chamber in the "Collecting flask". Other laboratories sometimes also vary the temperature during this second phase to more precisely control the digestion process. When islets are no longer released from the chamber, the process is stopped.

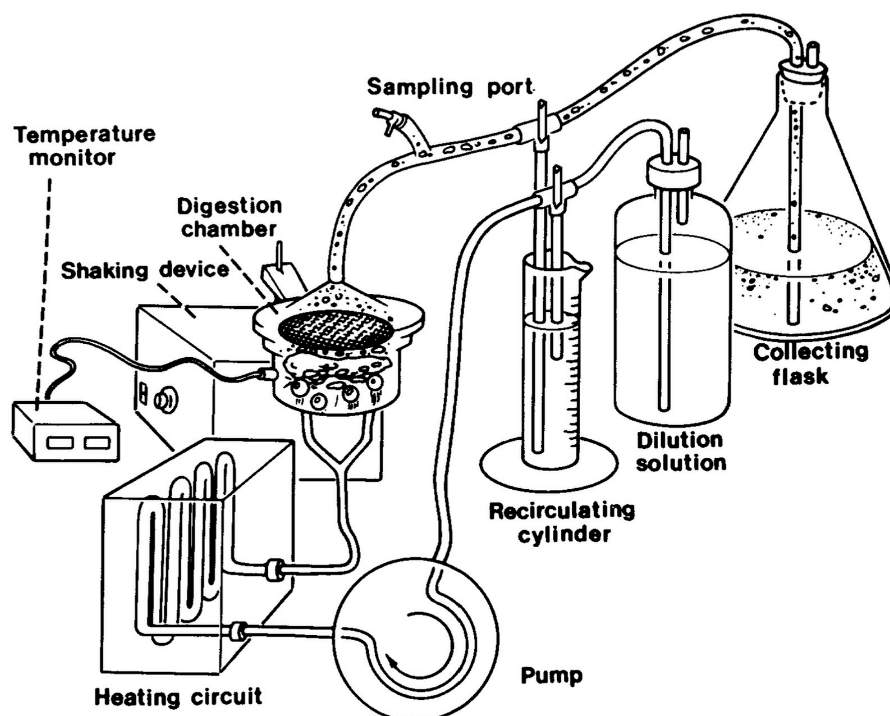

Figure 2. *The automated method — schematic of the set-up. During the first phase the fluid recirculates from the cylinder via the pump and a heating coil (in a water bath) through the chamber (containing the pancreas) and back to the cylinder. When the pancreas appears to have broken down sufficiently, valves are switched so that fresh medium ("dilution solution") is continuously supplied to the chamber, and the tissue suspension leaving the chamber is collected in a vessel.*

The tissue suspension, typically 6–8 liters, is centrifuged in 250-ml tubes. The pellets are pooled into a single tube and then incubated on ice for about 1 hour in an organ-preservation fluid — the University of Wisconsin solution (UWS) — prior to purification in an Iodixanol-UWS gradient ("IsletPrep"; see below) in the Cobe cell processor (Figure 3).

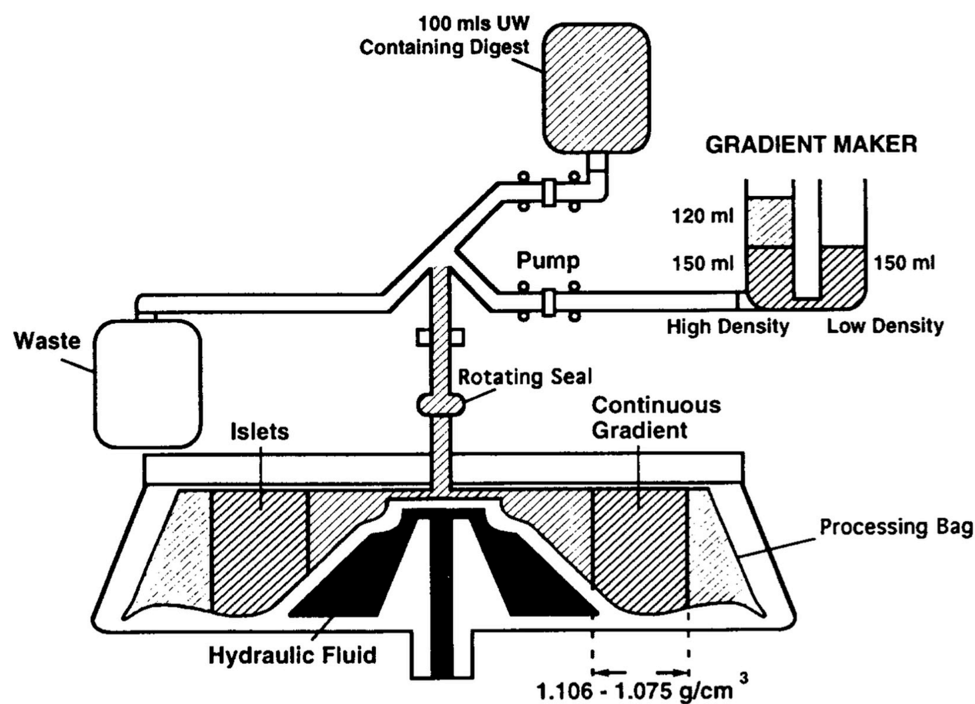

Figure 3. *Schematic representation of the use of the Cobe cell processor for purification of islets. The tissue suspension ("digest") is transferred to the Cobe centrifuge bag (Processing Bag), which lies over the hydraulically movable base of the centrifuge bowl. Via a second line, the rest of the density gradient is supplied using a pump. In this example, the construction of the density gradient differs slightly from the method described by us.*

The Cobe is essentially a centrifuge in which a (disposable) sterile 600-ml plastic bag is spun. This Cobe centrifuge bag forms a single unit with a system of supply lines leading outside the centrifuge. Through these supply lines — even while the Cobe centrifuge is running — the various density fluids can be supplied. First, the digest (the tissue suspension in UWS) is mixed with "IsletPrep" for the bottom layer (with a density of 1.086 g/ml) of the gradient. After the bottom layer has flowed into the centrifuge bag via one of the supply lines (using gravity), the centrifuge is started, and the subsequent density layers (1.075, 1.070, and UWS as the top layer) are then layered 'over' the bottom layer using a roller pump via another line. Finally, after 3 minutes of centrifugation, the gradient is forced back out by hydraulically pushing the Cobe base against the centrifuge bag, with the various fractions (top layer, the interface of the 1.075

and 1.070 layers, etc.) collected separately. Fractions containing highly purified islets are combined, and the islet yield and purity of the preparation are measured.

In Miami, incubation is currently performed in EuroCollins, and the islets are subsequently purified in a density gradient of Ficoll in EuroCollins ("EuroFicoll"). The results achieved with this density medium are still too often disappointing, so islet centers are searching for a better purification fluid.
The density medium "Iodixanol-UWS" that we recently developed (in a pig model) proved promising also when used to purify human islets in Miami (see above). Iodixanol-UWS is composed of iodixanol — which has been extensively tested for clinical use as a contrast agent — and the University of Wisconsin organ-preservation fluid (UWS). Recent collaboration with another major islet centre in Minneapolis (2) has led to this gradient currently being used there for all human islet purifications (personal communication). Collaborations are currently under way with several other centers (including Giessen, Leicester, Oxford) to also trial this purification method there, each in a somewhat different setting. Large centers elsewhere (including Los Angeles, Brussels) have recently shown interest. Furthermore, we are assisting with the planning of possible commercial production of this purification medium (which offers many advantages) under the — provisional — name "IsletPrep", by the manufacturer of iodixanol (Nycomed, Oslo, Norway).

***2.** Large Scale Isopycnic Islet Purification Utilizing Non-Toxic, Endotoxin-Free Media Facilitates Immediate Single-Donor Pig Islet Allograft Function. Matsumoto S, Zhang HJ, Gilmore T, Van der Burg MPM, Sutherland DER, Hering BJ. Department of Surgery, University of Minnesota; Department of Surgery, University Hospital, Leiden, Netherlands. 24th Annual Meeting of the American Society of Transplant Surgeons, May 13–15, 1998.*

Function / Quality control of the islet preparation

Contamination control

During the isolation procedure, samples of the media are taken to check for any (an)aerobic contamination. Samples are taken on arrival of the pancreas (of the UWS organ-preservation fluid) and at the end of the procedure (of the medium containing the purified islet preparation).

In vitro preservation and function

The viability of freshly isolated and/or cultured islets is assessed using an in vitro membrane-integrity test (fluorescence microscopy of a sample stained with acridine orange – propidium iodide). The islet preparation is stored at a low temperature (24°C) under culture conditions (in CMRL culture medium) for — at least for experimental purposes — 1 to 2 weeks. The recovery of islets at various time points is a good measure of viability.
Note: in vitro insulin-secretion test (pending).

In vivo function

During the recently held workshop in New Orleans (see above) it was determined that the in vivo function of the islet preparation after transplantation (at a threshold dose) under the kidney capsule of diabetic nude mice is currently the best parameter for functional quality control. As an alternative — it was agreed — an in vitro insulin-secretion test could be used if necessary. We have therefore initially chosen to test function and viability in vivo in STZ-diabetic nude mice, supplemented with histology.
Additional quality assessments are still under consideration.

Laboratory Layout and Equipment Overview

Layout of the isolation area: GMP facility

Management of the GMP facility takes into account (shared) occupancy of one of the five so-called 'cleanrooms', which will have 24-hour availability for the "clinical islet transplantation" project — from the start of the handover.

Storage space

Storage space in freezers (-20°C and -80°C, and liquid nitrogen) and warehouse space are part of the general facilities in the GMP complex.

Cleanroom

The cleanroom will, as standard (at no extra cost), have some of the equipment necessary for islet isolations:

- Biohazard flow cabinets, 2 units — the first cabinet is used for pancreas preparation and digestion; the second cabinet is used for processing the tissue suspension and purification of the preparation.
- Tissue-culture incubators, 2 units — one for low-temperature (24°C) culture and the other for 37°C culture.
- A refrigerator/freezer combination (for isolation, purification, and culture media, and supplements ready for immediate use).
- Standard light microscope (quantification of islet preparations).
- Inverted microscope (inspection of cultures).
- Bench space and 4 cabinets for disposables, etc.
- Mobile stainless-steel tables.
- A floor-model centrifuge (cost shared from the GMP facility budget) — for spinning down tissue suspensions, and for any alternative tube-based purification procedures.

  As standard, the cleanrooms are equipped with a smaller and cheaper bench-top model centrifuge. In the isolation cleanroom, this smaller centrifuge is replaced with a floor model. The additional costs associated with this must, for now, be included in our budget. (It may be possible to fund these additional costs later from the GMP budget instead.)

Overview of equipment still to be purchased (details below)

See above: additional cost for floor-model centrifuge instead of bench-top model.

Equipment for pancreas digestion:

- Two sets of surgical instruments, trays
- Two custom-made so-called ‘Ricordi digestion chambers’ (a stainless-steel chamber in which the pancreas is digested)

Peripheral equipment for digestion; a fixed set-up at one of the biohazard cabinets, consisting of:

- Mobile, lockable table (dimensions approx. 100–85 / 60–50 cm), on which a custom-made shaking device is mounted (for shaking the Ricordi chamber during digestion)
- Small (static) water bath, for warming the perfusion medium
- Thermometer
- Roller pump, for supplying solutions through the chamber

Shaking water bath (large; for thawing/bringing media to temperature)

General laboratory equipment:

- Top-loading balance
- Analytical balance
- Computer
- Magnetic stirrer
- pH meter
- Eppendorf pipettes
- Pipette controller

Special equipment:

- Densitometer (for checking density gradients before purification)
- Note: camera for the inverted microscope

Equipment for islet purification:

- Roller pump (for supplying density gradients to the COBE)
- Note: COBE 2991 blood cell processor (funding already committed by the Board)

Budget for the Isolation Project

Summary

**One-time total cost for Equipment, etc.: excl. VAT NLG 93,090; incl. VAT NLG 109,381.
This does not include the funding already committed by the Board of Directors for a Cobe cell processor (NLG 125,000).**

**Other costs (disposables, media, chemicals) per isolation (incl. controls): excl. VAT NLG 6,545; incl. VAT NLG 7,690.
For 20 isolations this amounts to (incl. VAT): NLG 153,808.**

Note: Share of the general cleanroom fit-out costs.

Details

One-Time Equipment Costs — Total NLG 93,090 (excl. VAT)

Item	Cost (NLG)
2x Pancreas-preparation instrument sets	3,590
Other instruments	550
Glassware	250
Custom-made: 2x Ricordi chamber; price: $1,500/unit	6,000
Custom-made: shaking machine for Ricordi chamber (1x) — price indication from the Physiology Department's Precision-mechanics Workshop	7,500
Settlement for additional cost of floor-model refrigerated centrifuge	38,500
Cost (incl. VAT) NLG 90,000, including rotor and a limited number of inserts (e.g. Hettich, capacity 6x2x250 ml tubes), including 250 ml inserts, including a limited number of 50 ml inserts	
Offset against originally planned small centrifuge for general GMP use: NLG 45,000	
Our net cost: NLG 45,000 = NLG 38,500 excl. VAT	
Thermometer	2,000
Water-bath thermostat	4,000
Shaking water bath	5,800
Densitometer	4,000
2x Masterflex peristaltic pump, model 7518-10, Cole-Parmer	8,000
General laboratory equipment (subtotal NLG 12,900):	
Top-loading balance	2,500
Analytical balance	5,000
Magnetic stirrer with heating	700
pH meter	2,500
Eppendorf pipettes	2,000
2x Pipette controller	200
COBE 2991 blood cell processor (funding already committed by the Board)	—
Note: computer	—
Note: camera for inverted microscope	—

Details

Other Costs per Isolation (incl. Culture, etc.) — Total NLG 6,545

Disposables (total NLG 795)

Item	Cost (NLG)
Drapes, waste, protection, clothing	50
General: pipettes, syringes	50
Ricordi chamber disposables	100
Duct cannulation	20
Filtering Liberase	15
Centrifuge tubes	200
Cobe disposables	60
Culture flasks	300
DTZ staining and islet counting	10
Pancreas preparation	30

Collagenase & Media

Item	Cost (NLG)
Liberase-HI 0.5 g (clinical grade)	1,650
Hanks' medium	10
RPMI medium	100
Fetal calf serum	1,000
Purification	700
Culture	1,000

Quality / function control (total NLG 550)

Item	Cost (NLG)
In vivo transplant test in nude mice (n=4 per isolation)	400
Contamination testing (pancreas and final preparation)	50
Histology / photography	25
Other	75
Note: in vitro insulin-secretion test	—

Estimated unforeseen costs: per isolation NLG 700