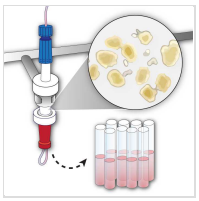

Nov 01, 2022

Mouse Islet Perifusion (3-stimuli protocol)

Islet and Pancreas Analysis Core[1]

[1]Vanderbilt Diabetes Research Center

dx.doi.org/10.17504/protocols.io.n2bvj6yenlk5/v1

Vanderbilt Diabetes Research Center

Islet and Pancreas Analysis Core
Vanderbilt Diabetes Research Center

ABSTRACT

Our perifusion setup consists of 4 independently-driven channels, which allows for up to 4 different groups of islets to be studied simultaneously. Each channel has an intake catheter, a peristaltic pump, a chamber to hold the islets, and a fraction collector.

This protocol details stimulation of mouse islets with 3 secretagogues (high glucose, IBMX, and KCl). The flow rate for the perifusion is 1 mL/minute, and fractions are collected every 3 minutes. The animation below illustrates the protocol.

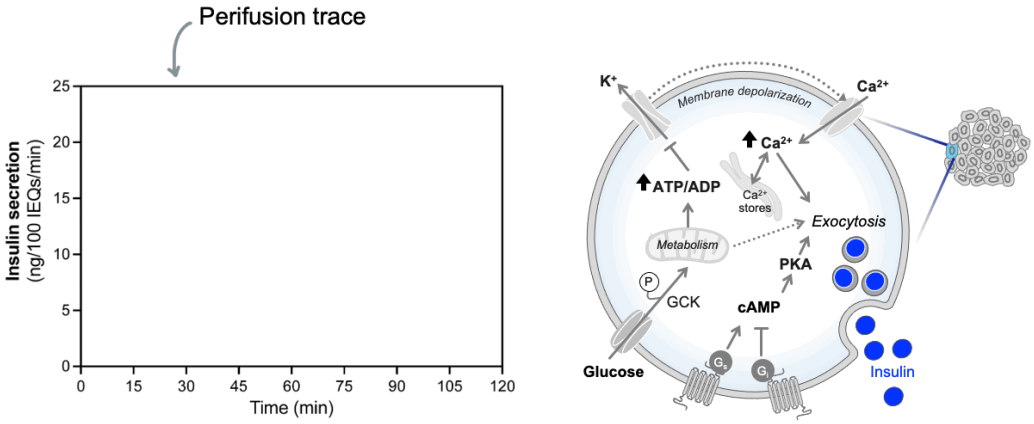

Figure 1. Insulin secretion (left) and cell schematic (right) showing response to stimuli during islet perifusion.

DOI

dx.doi.org/10.17504/protocols.io.n2bvj6yenlk5/v1

PROTOCOL CITATION

Islet and Pancreas Analysis Core 2022. Mouse Islet Perifusion (3-stimuli protocol). **protocols.io** https://dx.doi.org/10.17504/protocols.io.n2bvj6yenlk5/v1

CREATED

Jan 12, 2022

LAST MODIFIED

Nov 01, 2022

PROTOCOL INTEGER ID

56839

MATERIALS TEXT

Equipment

Analytical balance
Micropipettes (10-100 µL, 20-200 µL, and 100-1000 µL ranges)
Drummond Pipette Aid automatic pipettor or equivalent
Inverted microscope with 10X magnification eyepieces and 4X magnification objective
Eyepiece with calibrated reticle, 1 mm
Magnetic stir plate
Olympus SZX12 stereomicroscope equipped with an Olympus DP-80 high-resolution digital camera
Olympus cellSens™ image acquisition and analysis software

Perifusion System

Circulating water bath
Fraction collector
Peristaltic pumps capable of pumping 1 mL/min
Glass columns with 2 fixed-end pieces (
Diba Omnifit® LC columns **Cole Parmer Catalog #006CC-10-05-FF**)

Supplies

6-cm untreated petri dish **Sarstedt Catalog #83.3901.500**

Hank's Balanced Salt Solution (HBSS) **Gibco - Thermo Fischer Catalog #14025**

Phosphate buffered saline (PBS) without Ca/Mg **Invitrogen Catalog #14190-144**

1.5-mL microcentrifuge tube **Fisher Scientific Catalog #05-408-129**

NaHCO3 **Sigma Aldrich Catalog #S6014-500G** Step 4

L-Glutamine **Sigma Aldrich Catalog #G8540-100G** Step 4

Sodium Pyruvate **Sigma Aldrich Catalog #P2256-25G** Step 4

DMEM **Corning Catalog #90113** Step 4

RIA-grade BSA **Sigma Aldrich Catalog #A7888** Step 4

Isobutylmethylxanthine (IBMX) **Sigma Aldrich Catalog #I5879** Step 8

L-Ascorbic acid **Sigma Aldrich Catalog #A5960** Step 4

200-µL (P-200 ART) and 1000-µL pipette tips (P-1250 ART)
0.22 µM vacuum filtration system (Millipore #S2GPU05RE)
5-luer caps (Western Analytical #BC-125)
25 µM frits, polyethylene (Cole Parmer #11945-04)
13 X 100 mm polyethylene tubes (Fisher Scientific #149567A)
Caps for 13 X 100 mm tubes (Fisher Scientific #02681204)
Glucometer (Bayer #9545C)
Glucose strips (Bayer #7097C)
Pressurized gas, 95% O_2, 5% CO_2

General Perifusion Startup

1 Fill perifusion water bath with deionized water to about 1 inch from the top and set the temperature to **37 °C** .

2 Label perifusion tubes with date, islet type, fraction number, and any other identifying information. Position the fraction collector trays for perifusion, and load the tubes into the fraction collector.

3 Rinse the tubing with deionized water at max pump speed for **00:15:00** , then place new frits

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into the islet chamber.

Preparation of Base Perifusion Media and Secretagogues

4 Prepare **base perifusion media** by combining reagents below in a 1-L Erlenmeyer flask. Add **1 L** of deionized water and mix on a magnetic stir plate for at least **00:15:00** until dissolved.

- NaHCO3 **Sigma** **3.2 g** **Aldrich Catalog #S6014-500G**
- L-Glutamine **Sigma** **0.58 g** **Aldrich Catalog #G8540-100G**
- Sodium Pyruvate **Sigma** **0.11 g** **Aldrich Catalog #P2256-25G**
- HEPES **Sigma** **1.11 g** **Aldrich Catalog #H7523**
- **8.28 g** DMEM **Corning Catalog #90113**
- RIA-grade BSA **Sigma** **1 g** **Aldrich Catalog #A7888**
- L-Ascorbic acid **Sigma** **70 mg** **Aldrich Catalog #A5960**

Base media can be made up to 24 hours before perifusion, and stored at **4 °C** . **All subsequent media must be made fresh.**

4.1 Use a vacuum-filtration system to filter media, transfer to a side-arm flask, and de-gas at **37 °C** for at least **00:30:00** .

5 Prepare **5.6 mM glucose media** by adding **0.5549 g** Glucose **Fisher Scientific Catalog #D16** to **550 mL** **base perifusion media** in a 500-mL bottle and mix until dissolved.

5.1 Wait **00:30:00** and check glucose levels using a glucose meter.

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5.2 Reserve **30 mL** **5.6 mM glucose media** in a 50-mL conical tube for islet loading and unloading.

6 Prepare **16.7 mM high glucose media** by adding **0.7522 g** Glucose **Fisher Scientific Catalog #D16** to **250 mL** **base perifusion media** in a 250-mL bottle and mix until dissolved.

6.1 Wait **00:30:00** and check glucose levels using a glucose meter.

7 Prepare **5.6 mM glucose with 20 mM KCl** by adding **0.149 g** Potassium chloride **Fisher Scientific Catalog #BP366-500** to **100 mL** **5.6 mM glucose media**.

8 Prepare **16.7 mM glucose with 100 µM IBMX** by first weighing approximately **10 mg** Isobutylmethylxanthine (IBMX) **Sigma Aldrich Catalog #I5879** in a microcentrifuge tube.

8.1 Make **200 millimolar (mM)** stock by adding the appropriate volume of DMSO (see **Table 1**).

A	B
IBMX weight (mg)	10
DMSO to add (µl)	45

Table 1. Copy and paste into Excel, enter IBMX weight in B1, and volume of DMSO will be automatically returned in B2.

DMSO readily penetrates skin and may carry other dissolved chemicals into the body. Wear appropriate PPE and avoid skin contact.

8.2 Dilute IBMX stock solution (prepared in step 8.1) to a final concentration of **100 micromolar (µM)** by adding **100 µL** IBMX stock solution to

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100 mL **16.7 mM glucose media**.

Setup of Secretagogues in Perifusion Water Bath

9 Place the bottles of media in the water bath to warm for at least **00:10:00** before beginning the perifusion.

10 Replace Pyrex orange caps with 4-Luer (+1) caps on every bottle of media to be used and tape over the holes so that gas cannot escape.

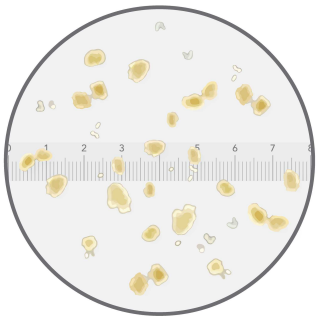
11 Turn on gas (**95 %** O_2, **5 %** % CO_2) and insert one gas line to each bottle cap. Block additional holes in caps with tape. Make sure that gas line is suspended above the media and not submerged in the liquid.

12 Place intake catheters into baseline media bottle, making sure that they rest on the bottom of the bottle. Run **baseline media** through the chambers at maximum pump speed for about **00:10:00** while islets are being aliquoted for perifusion. Discard flow-through.

Islet Preparation and Loading

13 Prepare **islet picking media (HBSS with 10% FBS)**: Remove and discard **50 mL** from a 500-mL bottle of Hank's Buffered Saline Solution (HBSS) **Gibco - Thermo Fischer Catalog #14025**, then add **50 mL** Fetal Bovine Serum (FBS) **Millipore Sigma Catalog #F0926**.

14 Prepare a 6-cm dish with **5 mL** **islet picking media** for each channel to be run. Place freshly-isolated mouse islets on the stage of inverted microscope and view the islets using the 4x objective. Using the reticle in the 10x eyepiece, size the islets according to the chart below.



Using reticle calibrated to 25-µm increments:

Islet size category	Number of tick marks	Islet diameter (µm)
Small	>4 and ≤6	>100 and ≤150
Medium	>6 and ≤8	>150 and ≤200
Large	>8 and ≤10	>200 and ≤250
Extra large	>10 and ≤12	>250 and ≤300

Figure 2. A reticle is used to IEQ islets.

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Handpick islets using a 200-µL micropipette and transfer to the 6-cm tissue culture dish containing **islet picking media**. Record islet number and size to calculate IEQ.

To measure insulin secretion (Mouse Insulin ELISA, Mercodia #10-1247-10), you will need between 50-70 IEQ. To measure glucagon secretion (Mouse Glucagon ELISA, Mercodia #10-1281-01), you will need between 150-200 IEQ.

15 Using a 200-µL micropipette, transfer all islets from the center of the dish to a clear 1.5-mL microcentrifuge tube for loading into perifusion chamber.

16 **Perform islet imaging:**

Place the dish containing clean islets on the stage of a stereomicroscope equipped with a high-resolution camera and swirl until all islets are in the camera field of view at 10x magnification. Using cellSens software, capture brightfield images at approximately 12 ms exposure and darkfield images at approximately 400 ms exposure, each at 10x magnification. Ensure all islets are present in image. Save all image files.

17 Ensure perifusion intake and air uptake pumps are off. Turn the stopcock on the air uptake lines so that the waste pathway is open, and close the stopcock on the outlet line at the fraction collector.

18 Remove the chamber from its mounting, turn it upside down, and remove the red end piece (inlet). Remove and discard two thirds of the media from the chamber.

19 Using a 1-mL micropipette, transfer the slurry of islets from the microcentrifuge tube to the perifusion chamber. Rinse the tube at least 3 times with **baseline media** and transfer to the chamber.

20 Place the chamber back onto the mounting, and fill it up with **baseline media** until there is a convex meniscus. Tap the sides of the column lightly to dislodge air bubbles from the walls, and collect and discard any bubbles from the top of the meniscus.

21 When all the bubbles have been removed, carefully replace the inlet plunger.

Make sure that no bubbles are introduced into the chamber during this process; if bubbles get in, remove the plunger, and repeat step 20.

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22 Turn the chamber right side up, and put it back on the mounting rack. Open the outlet line at the fraction collector and close the waste line on the air uptake line.

23 Turn on the intake and air uptake pumps, set the fraction collector to **00:03:00** and flip the collector arm so that it is over the first collection tube. Press “Start” on the collector to start the timer.

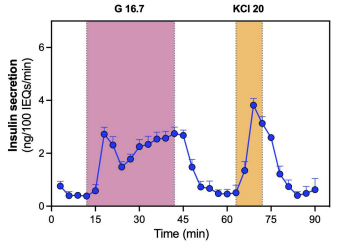
24 Tighten column end pieces, and end fittings, and make sure there are no leaks. Lower the column mounting rack into the water bath, and tighten the clamp to prevent wobbling.

Collection of Perifusate Fractions

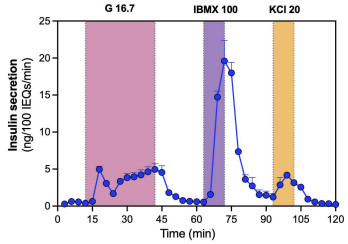
25 Collect 10 preliminary fractions using **5.6 mM glucose media** to rinse the islets and to synchronize intake pump speed to deliver 1 mL per minute fractions, collecting **3 mL** (3 minute) per fraction.

26 Record each intake pump speed on the perifusion worksheet in the perifusion logbook.

27 Begin to collect fractions, changing media/secretagogues at predetermined timepoint based on fraction collector timer (see sample protocol below).When the fraction collector moves at the desired timepoint, pause the intake pump.



Secretagogue	Purpose	Time start (min)	Time end (min)	Duration (min)
High glucose (16.7 mM)	Glucose-stimulated insulin secretion	12	42	30
Glucose (5.6 mM) + KCl (20 mM)	Membrane depolarization	63	72	9



Secretagogue	Purpose	Time start (min)	Time end (min)	Duration (min)
High glucose (16.7 mM)	Glucose-stimulated insulin secretion	12	42	30
High glucose (16.7 mM) + IBMX (100 µM)	cAMP signaling potentiation	63	72	9
Glucose (5.6 mM) + KCl (20 mM)	Membrane depolarization	93	102	9

Figure 3. Perifusion traces and 2- or 3-secretagogue protocols for measuring mouse insulin secretion.

28 Move the media intake catheters from one secretagogue to the next, making sure to not tangle the tubing and that the catheter is resting on the bottom of the bottle.

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29 Restart intake pump until next secretagogue change.

30 When perifusion is complete, cap the perifusion fractions and store at **-20 °C** .

Islet Recovery and System Cleanup

31 When perifusion is complete, stop intake and air uptakes pumps and close stopcocks to air uptake and media outlet lines.

32 Raise the mounting rack from the water bath and wait approximately **00:02:00** so that islets can settle to the bottom of the chamber.

33 Carefully remove the blue outlet end piece from the islet chamber. Pipet the islets and media from the chamber into a 6-cm untreated tissue culture dish and rinse the chamber and the blue column end piece with 5 times with **1 mL** each of **baseline media** into the dish.

34 Rinse the chamber with deionized water and remove frits from chamber.

35 Reassemble column and rinse all lines, including waste lines, with **10% bleach** at maximum pump speed for approximately **00:15:00** .

36 Rinse all lines, including waste lines, with deionized water at maximum pump speed for approximately **01:30:00** .

37 Turn off intake and air uptake pumps, drain the water bath, turn off gas, and record the perifusion date, details, and any errors or problems in the perifusion logbook.

Islet Hormone Extraction

38 Using 6-cm dish with recovered islets, quantify IEQ (**go to step #14**) and perform imaging (**go to step #17**) to calculate IEQ recovered.

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The average expected retrieval rate is approximately 93%. Lower islet recovery has been observed for islets showing hallmarks of disintegration.

39 Using a pipette, transfer all islets from the center of the dish to a clear 1.5-mL microcentrifuge tube for islet hormone extraction.

40 Centrifuge the tube at **200 rcf, 00:03:00** and aspirate the supernatant using a pipette, being careful not to disturb the islet pellet. Place tube **On ice** .

41 Prepare fresh acid alcohol for hormone extraction by adding **50 µL** 12M HCl to **5.5 mL** 95% ethanol.

41.1 Add **200 µL** **acid ethanol** (prepared in step 41) to islet pellet and incubate at **4 °C** **Overnight** or for up to **24:00:00** .

42 Centrifuge tube at **3000 rpm, 00:05:00** and transfer three **50 µL** aliquots of supernatant to 2-mL screwcap tubes. Store at **-80 °C** .

Data Collection and Analysis

43 Open the darkfield image in the cellSens software. Using the **manual HSV threshold** function, segment the islet tissue channel.

44 Use the custom **Count and Measure** algorithm to determine islet count and mean islet diameter. Split adjacent but discrete islets using the **Manually Split Objects** tool to get an accurate islet count and mean diameter measurements.

45 Use the mean diameter measurements to assign islets to a diameter group using the chart provided (**go to step #14**).

46 Perform insulin and/or glucagon assays on perifusion fractions and extracts.

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