BIOREP®
TECHNOLOGIES

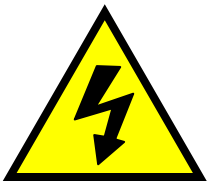
PERIFUSION SYSTEM
MODEL No: PERI-LITE-115/230

USER MANUAL
REV 2.0

BIOREP®
TECHNOLOGIES

TABLE OF CONTENTS

1 Getting Started



WARNING:

Please, read this section before unpacking, installing, or operating this machine.

1.1 Symbols Used in this Manual

The lightning flash with arrowhead symbol, within an equilateral triangle, is intended to alert the user to the presence of dangerous voltage within the machine's enclosure that may be of sufficient magnitude to constitute a risk of electric shock.

The exclamation point within an equilateral triangle is intended to alert the user to the presence of important information in the literature that accompanies the device.

This symbol warns of moving parts that may cause personal injury.

This symbol is used when two people are needed to do the task.

1.2 Machine Information

In the spaces provided below, record the Model and Serial No. located on the rear panel of your machine.

Model No. _____ Serial No._____

RETAIN THIS INFORMATION FOR FUTURE REFERENCE.

1.3 Contact Information

Biorep Technologies, Inc.
15804 NW 57th Avenue
Miami Lakes, FL 33014
info@biorep.com
www.biorep.com
Tel: 305-330-4449

1.4 Safety Information

PLEASE READ AND OBSERVE ALL WARNINGS AND INSTRUCTIONS GIVEN IN THIS USER'S MANUAL AND THOSE MARKED ON THE UNIT.
RETAIN THIS BOOKLET FOR FUTURE REFERENCE.

DO NOT REMOVE THE MACHINE'S COVER OR YOU MAY BE EXPOSED TO DANGEROUS VOLTAGE. REFER SERVICING TO QUALIFIED PERSONNEL ONLY.

TO REDUCE THE RISK OF FIRE OR ELECTRIC SHOCK, DO NOT EXPOSE THIS DEVICE TO RAIN OR MOISTURE. DANGEROUS HIGH VOLTAGES ARE PRESENT INSIDE THE ENCLOSURE.

READ AND UNDERSTAND HOW MOVING PARTS ARE CONTROLLED BEFORE OPERATING THE MACHINE. KEEP YOUR FINGERS AWAY FROM MOVING PARTS; NEGLECTING TO DO SO MAY CAUSE PERSONAL INJURY.

READ AND FOLLOW THESE INSTRUCTIONS:

1. Keep these instructions for future reference and heed all warnings stated in this manual.

2. Do not block any ventilation openings. Install the machine in accordance with the manufacturer's instructions. See Site Preparation in section **Error! Reference source not found.** for details.

3. Do not defeat the safety purpose of the polarized or grounding-type plug. A polarized plug has two blades with one wider than the other. A grounding-type plug has two blades and a third grounding prong, which is provided for your safety. If the provided plug does not fit into your outlet, consult an electrician for replacement of the obsolete outlet or contact Biorep Technologies, Inc. at (305) 330-4449.

4. Protect the power cord, the power entry module, and the plug from being walked on or pinched to avoid damaging them.

5. Refer all servicing of the machine to qualified personnel. Servicing is required when the apparatus has been damaged in any way, for example: the power-supply cord or plug is damaged, liquid has been spilled or objects have fallen into the apparatus, or if the apparatus has been exposed to rain or moisture, has been dropped, or does not operate normally for any reason.

2 Introduction

Congratulations on your purchase of the Biorep Perifusion System. This is the newest generation of a product that has received countless hours of development to include all the features necessary to perform the most controlled and precise stimulated cell secretion experiments in the field.

The Perifusion System accurately stimulates and collects the secretions of pancreatic islets in-vitro. Its use has been extended to include the measurement of secretions of differentiated cells derived from pancreatic progenitor stem cells and pancreas slices. Coupled to a tandem mass spectrometer, the Perifusion System can help identify molecules secreted during the stimulation of pancreatic islets.

The machine accurately controls temperature, flow, stimulus source and sample collection in up to 4 channels in parallel. The Perifusion System is designed to execute pre-programmed protocols that can last for hours and require several sample trays.

This User Manual is intended to be used by scientists, researchers, and technicians who have received training in cell perifusion methods and technologies. This document contains the necessary information for installing and operating the machine. However, it is not meant to provide scientific guidance on perifusion protocols. Any perifusion protocols encountered in this manual will be solely for the purpose of illustration of machine operation and should not be interpreted otherwise.

1 - Donor pancreas
2 - Organ transport
3 - Perfusion
4 - Isolation
5 - Purification
6 - Islet Counter
7 - Perifusion
8 - Islet shipping
9 - Islet transplant

Figure 1: Perifusion System in the islet isolation process

3 System Description and Terminology

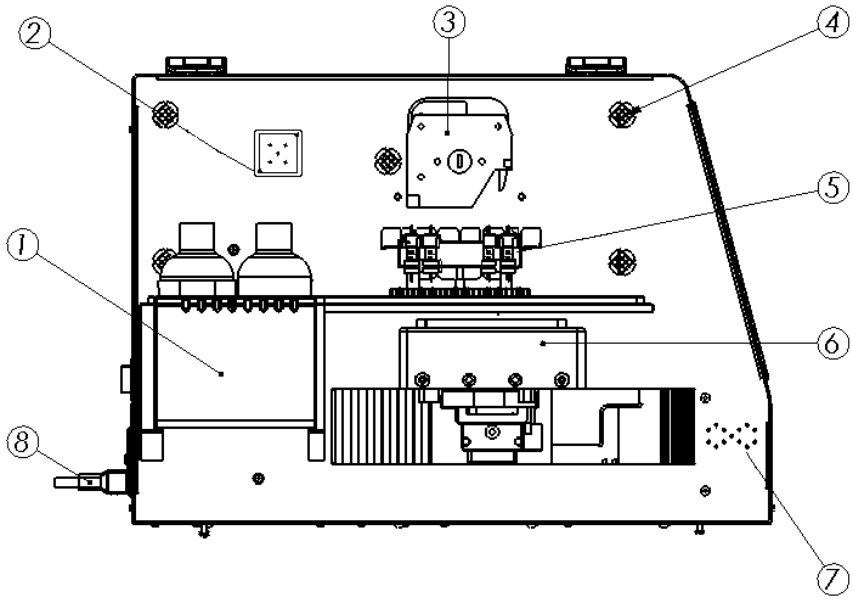


Figure 2: Perifusion System, front view

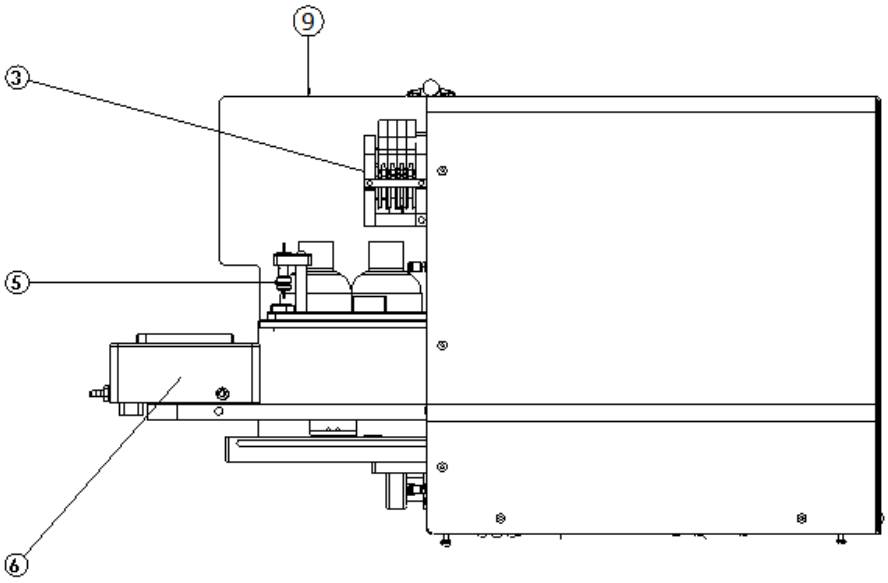


Figure 3: Perifusion System, right side view

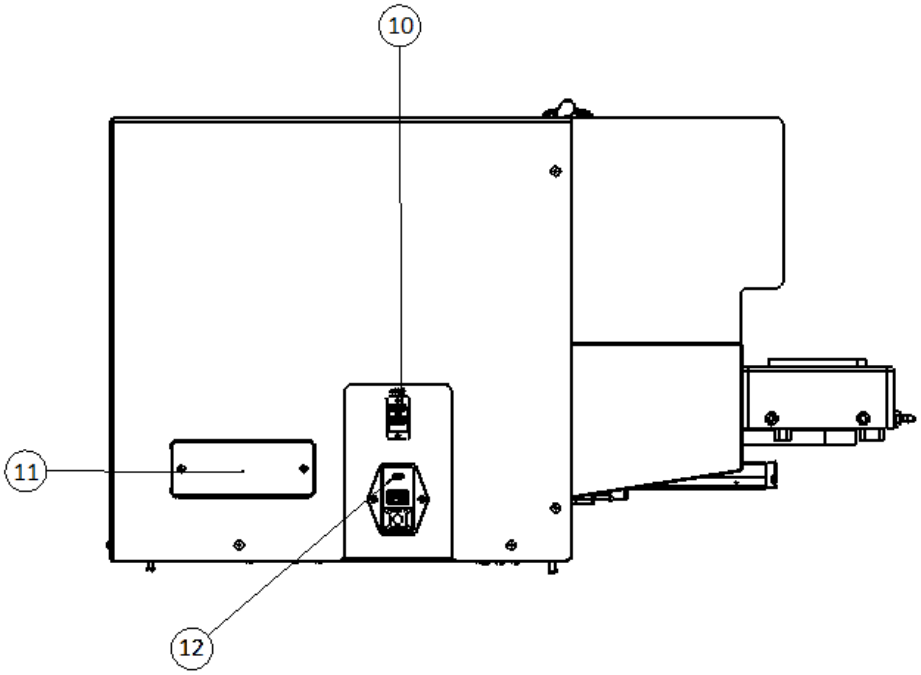


Figure 4: Perifusion System, left side view

1. Removable source solution holder
2. 4 x 1 valve manifold for source solution handling
3. 4 Channel peristaltic pump (Main pump)
4. Temperature control inlet/outlets (x6)
5. Perifusion chamber (x4)
6. Sample collection tray
7. Alarm buzzer
8. Power cable
9. Incubator cover
10. Ethernet port
11. Serial Number Plate
12. Power Entry Module

4 Accessories

A complete set of accessories is included with the machine. For ordering additional or replacement accessories please consult our webpage **www.biorep.com** or contact one of our sales representatives at (305) 330-4449.

4.1 Perifusion Accessories

The following accessories are included with the purchase of the PERI-Lite:

Qty	Reference	Description
1	12885T21	Single Hole Punch – 1/4" (6.4mm)
25	SC040-0750-SO	Manifold Fittings Caps
1	5298A15	Miniature Precision Screwdriver
1	5709A44	Black-oxide Metric L-Key Set
4	75165A551	16 Ga x 1/2" L Dispensing Needle with Luer Lock
1	86610610	Power Cord
1	C3307	Syringe with Luer Lock , 10 mL
1	FTLLSB004-1	Female Luer to Barb – 1/16” ID Tubing
1	PERI-BEADS-20	Bio-Gel P-4 Gel (45-90um), (20 g)
4	PERI-CHAMBER	Perifusion Chamber, Acrylic
12	PERI-CLIPS	Mini Binder Clips
10	PERI-FILTER	Perifusion Chamber Filter Paper
4	PERI-FITTING-1	Straight Through Tube Fitting (1/16” ID Tubing)
4	PERI-FITTING-2	Four-Port Cross Style Manifold Fitting (1/16” ID Tubing)
4	PERI-FITTING-3	Y Connector Fitting (1/16” ID Tubing)
1	PERI5-CELL LOADING RACK	Perifusion Cell Loading Rack
5	PERI-NOZZLE	Perifusion System Dispensing Nozzle
50	PERI-ORING	O-rings for Perifusion Chamber
1	PERI-STD-WELL	Nunc 96 Well-Plate, 0.5 mL
1	PERI-TUB-040	Silicone Tubing, 0.04" ID x 0.085" OD x 50' L
4	IS 3510	Click n’ Go Peristaltic Pump Cassette
1	PERI5-TUBSET-PVC	PERI5 2-Stop Tubing with Connectors (12/pk)
1	5007	Perifusion Accessories Box

Table 1: Packing List

4.2 Tubing Set

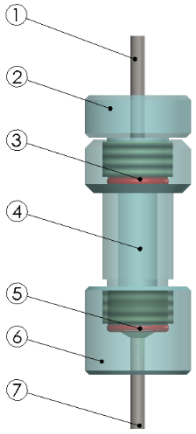
The Perifusion tubing set delivers the stimulus to the cell chamber while minimizing dead volume. The perifusion tubing set is made out of 2 types of tubing: A - Silicone tubing, which is used to connect the source solutions to the manifold and B- the 2-stop peristaltic pump tubing, which provides the precise 0.38mm inner diameter to minimize dead volume. A set of each type is included with this machine.

Item	Description	Reference
A	Silicone Tubing, 0.04" ID X 0.085" OD x 50'	PERI-TUB-040
B	PERI5 2-Stop Tubing with Connectors (12/pk)	PERI5-TUBSET-PVC

Table 2: Perifusion Tubing Sets

4.3 Perifusion Islet/Cell & Pancreas Slice Chambers

The Perifusion chamber is where all cell reactions will take place. Substances will be delivered through the inlet tube, cells contained in the chamber will react to the substances, and secretions will come out the outlet tube for collection.



1- Inlet tube

2- Top cap

3- Top O-Ring

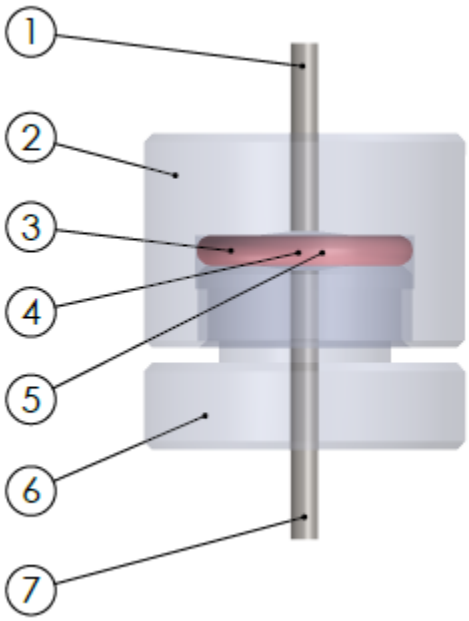
4- Cell chamber

5- Bottom O-Ring + Filter

6- Bottom cap

7- Outlet tube

Figure 5: Perifusion Chamber – Islets/Cells



1 -Inlet Tube

2- Top Cap

3- O-ring

4- Pancreas Slice

5- Perforated Support Plate

6- Bottom Cap

7- Outlet Tube

Figure 6: Pancreas Slice Chamber

4.4 Perifusion Cell Loading Rack

The auxiliary rack is included with every Perifusion System to assist in the preparation of the Perifusion Chambers. The rack can hold up to 12 completely disassembled chambers at a time for cell loading outside the machine. This allows the scientist to do the preparation away from the machine in a more comfortable position.

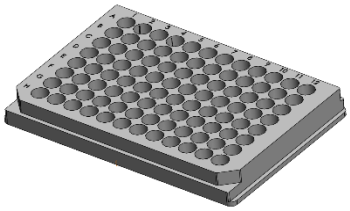
Figure 7: Perifusion Cell Loading Rack

4.5 Perifusion Nozzles

The Biorep® Perifusion Nozzle reduces the droplet size dispensed into the 96 well-plate. Small droplets (~ 12µL) ensure optimal resolution of the dynamic insulin response.

Figure 8: Perifusion Nozzle

4.6 Collection well-plates



The collection 96 well-plate is where the cell secretions and the perfusate are collected for analysis. The Perifusion System was designed to work with 0.5mL standard 96 well-plates and 2.0 mL deep well plates.

Figure 9: 96-well Collection Well-plate

4.7 Bio-Gel P-4

Bio-Gel P-4 polyacrylamide beads (45 – 90 µm) are used at the time of Perifusion Chamber setup. They are used to homogenously distribute the cells and prevent clogging of the filter.
*See appendix A for beads solution preparation.

Figure 10: Polyacrylamide beads

4.8 Fiberglass pre-filters

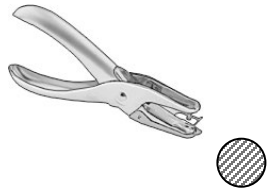


Figure 11: Hole Punch

A fiberglass filter sheet is included with every Perifusion System. A hole puncher (included) is used to make round cut-outs of the filter sheet to place inside the Perifusion Chamber during its set-up. The purpose of this filter is to retain the chamber contents while allowing the flow of the perfusate.

5 Perifusion System overview

5.1 Perifusion schematic

The following schematic shows the basic functional elements and stages of the Perifusion System. Potential users should become familiar with all the components of the system before attempting to operate the machine.

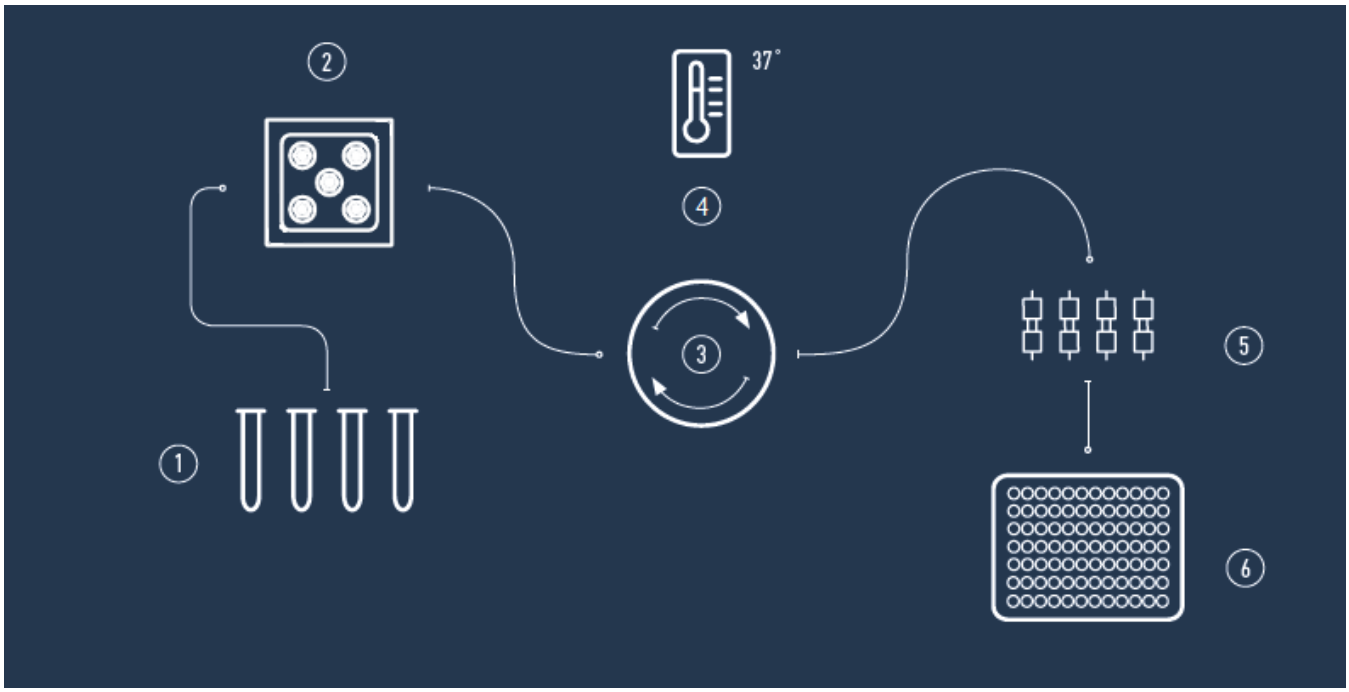


Figure 12: Perifusion Schematic

1. **SOURCES**: Up to four different test solutions can be used as sources in a single experiment. These sources can be administered either manually by the user or automatically by the machine through the valve manifold. Sources are contained inside the enclosure's temperature-controlled environment.

2. **VALVE MANIFOLD**: An automated valve manifold handles the source selection for each channel. The manifold has 4 input channels (1, 2, 3, 4) and one output channel (A). A detailed description can be found in section 5.2. The output channel can be split to feed up to four chambers in parallel.

3. MAIN PUMP: A high precision, 4-channel peristaltic pump moves the test compounds from the source, through the manifold, to the Perifusion Chambers and dispense into the 96 well-plate.

4. TEMPERATURE CONTROL: All the test sources are housed in the same incubator enclosure as the Perifusion Chambers to provide a controlled temperature environment for the entire process. A multi-point circulation design ensures temperature uniformity within +/- 1ºC around the Perifusion Chambers.

5. PERIFUSION CHAMBER or PANCREAS SLICE CHAMBER: The Perifusion Chambers hold the cells or pancreas slices to be stimulated. The input to each chamber is the source selected by the manifold for its respective channel. The output will contain the secretions and metabolites to be collected on the 96 well-plate. Up to four Perifusion Chambers can be used in parallel.

6. COLLECTION: The perfusate is collected in a 96 well-plate for further analysis. The collection tray moves automatically accordingly to the number of active columns (Perifusion Chambers) and the pre-set sampling rate. The collection tray can hold standard (0.5mL) and deep (2.0mL) 96 well-plates; be sure to place the included spacer adapter inside the tray to use it with 0.5mL standard plates.

5.2 Valve Manifold

The valve manifold was designed to automate the selection of the stimulus. Manual solution changing is not only time consuming for the user, but it also increases the potential for artifacts on the results. Manual solution changing requires the user to pause the machine to avoid drawing air into the system, but cell metabolism cannot be paused! Cells will continue to metabolize and, when flow is resumed, a “spike” of metabolites will be generated at the output.

Minimizing this problem was a priority at the time of developing the new Perifusion System. The result was a self-contained, 4x1 manifold (4-in to 1-out). The manifold can change the solution for every channel “on-the-fly” (milliseconds), eliminating the need for pausing the system. It also eliminates the need for input solutions requiring a separate heater; the solutions can be housed inside the machine, allowing for better temperature control.

In conjunction with the ability to perform automated protocols, the valve manifold provides a hands-off solution from start to finish of a Perifusion experiment. The user can now leave the machine unattended and have the certainty that solution changes will be made flawlessly every time.

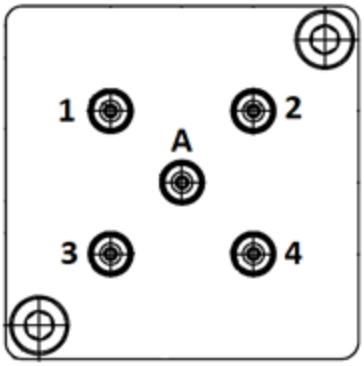


Figure 13: 4x1 Liquid Manifold

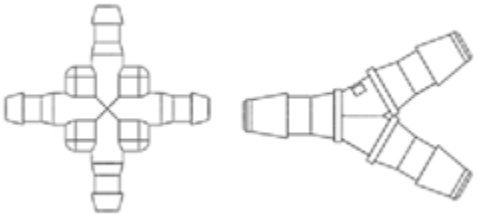


Figure 14: Tubing splitters

Understanding the valve manifold is key for a proper experiment setup. Inputs are counted from left to right, and top to bottom, like words on a page, starting with input **1** at the top left (see F*ig. 13*). Sources should be connected to the input ports according to this numeration.

There is only one output channel (**A**) located in the center of the manifold. It can be split into 2 channels using a "Y" fitting or split into 3 channels using a cross "X" fitting. To Split **A** into 4 channels, connect one "Y" fitting to the output, then connect one more "Y" fitting to each output of the first "Y" fitting.

A detailed description of protocol creation can be found in section 7.2.

5.3 Peristaltic pump

The sources are pumped from their containers through the open valves of the manifold by the main pump. The main pump is a 4 roller, high precision peristaltic pump, capable of handling up to 4 channels simultaneously, providing accurate and consistent dispensing across channels.

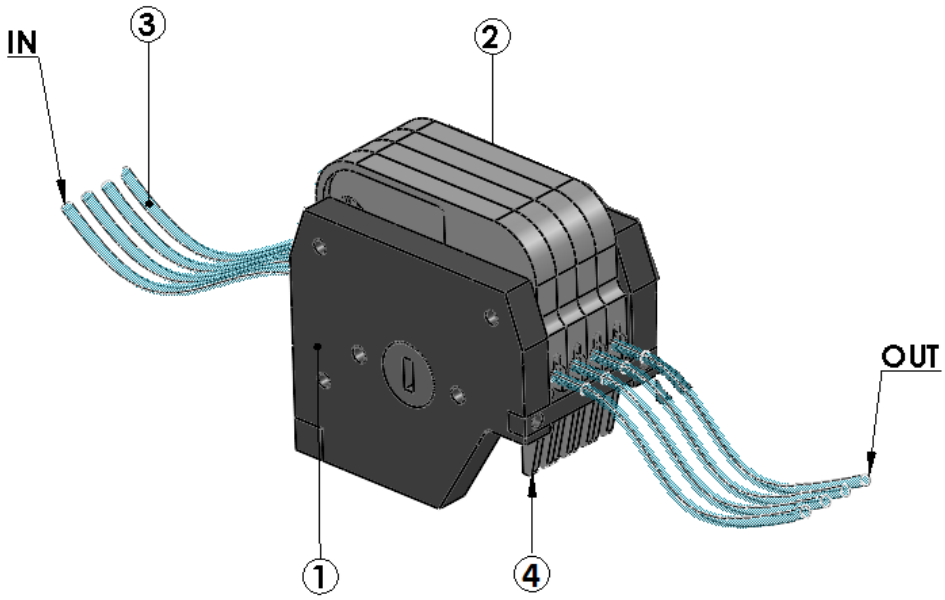
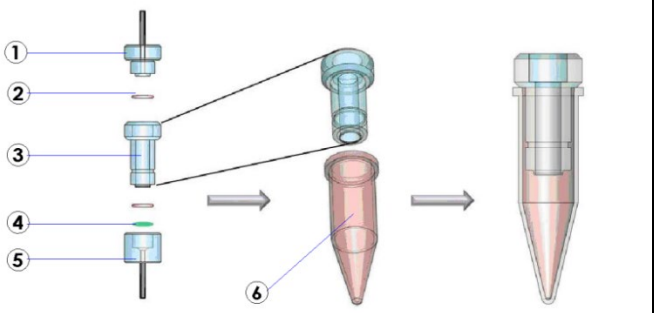


Figure 15: Four-Channel Peristaltic Pump

The main body of the pump (1), is fixed to the machine chassis. The 2-stop Perifusion Tubing (3) setup is installed on the individual cassettes (2). The cassettes snap in and out of the pump by pressing on the release lever (4). A detailed description of tubing setup can be found in section 7.5.

5.4 Perifusion Chamber

Sources delivered by the pump will react with the cells inside the Perifusion Chamber. The cells are contained inside the chamber within a gel bead matrix. A fiberglass filter is used as a bottom layer before the output. Secretions will come out the outlet tube for collection in the well-plate. The internal volume is 275 µL. The chamber is transparent to allow visualization of its contents. It is designed to fit a micro-centrifuge tube for easy cell recovery. This is important for DNA normalization. A detailed description of the chamber setup for an experiment can be found in section 7.4.



1. Top cap
2. O-ring
3. Cell chamber
4. Fiberglass filter
5. Bottom cap
6. Micro-centrifuge tube

Figure 16: Perifusion Chamber and collection tube

The Pancreas Slice Chamber has the ability to be extended to test multiple pancreas slices by using the Pancreas Slice Chamber Extender Kit.

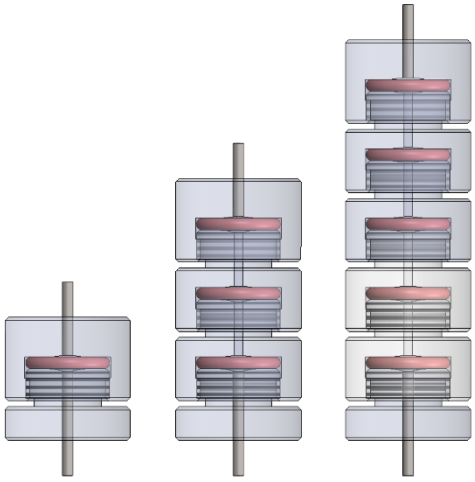


Figure 17: Pancreas Slice Chamber (1 Slice, 3 Slice, 5 Slice Configurations)

5.5 Collection tray

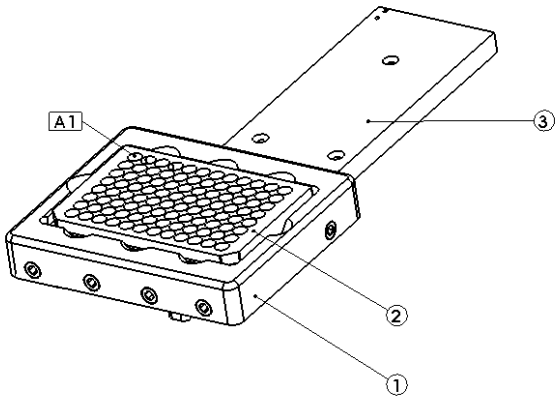


Figure 18: Collection Tray

The collection tray (1) holds and positions the 96 well-plate (2) for automated collection of the perfusate and cell secretions according to programmed time intervals. The collection tray's location is adjusted during experiments by the positioning mechanism arm (3).

The Perifusion System allows for the use of both 0.5mL and 2.0mL 96 well-plates. To use standard 0.5mL well plate insert the included spacer adapter into the tray.

When inserting the well-plate on the collection tray, make sure that well A1 is in the position shown on Figure 18. This will ensure that the dispensing sequence will start at this point, avoiding any confusion at the time of analysis when transferring to ELISA plate.

6 Installation

This machine is intended to work in a controlled laboratory environment. To properly install the machine, please refer to the figure below. The machine has a ventilation opening in the rear, which should not be blocked. A 12.7cm (5.0 in) clearance is recommended between the back of the machine and any obstacle. Bench-space required is 52.4cm (20.62 in) x 38.3cm (15.06 in).

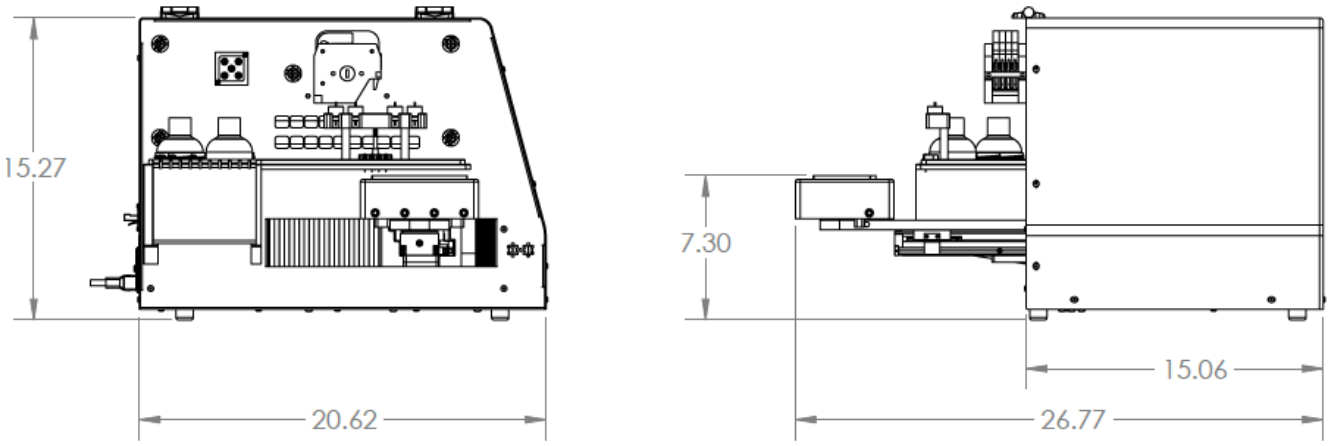


Figure 19: Site Preparation (Note: Units are in inches)

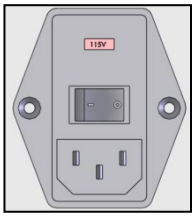
7 Perifusion System Basics

Control and operation of the Perifusion system is done through a Perifusion System application running on a separate PC computer connected via Ethernet to the Peri-Lite system. The operator should familiarize with the different screens, features, and controls of the interface before attempting to operate the machine during an actual perifusion experiment.

Anyone who will operate and/or supervise the use of this machine should read and understand this section in its entirety.

This machine has been designed with alarm features that will help the user identify and correct potential problems. Alarm messages will be displayed at the bottom of the screen. A detailed description of the different alarms can be found later in this manual.

7.1 Power-Up



The machine's main power switch and fuse-box are located on the back of the machine, above the power entry module. Connect the power cord (provided in the accessory box) to the module and then plug it into a 115/230 VAC outlet depending on your location. The red box in the module displays the voltage rating intended for your machine.

Figure 20: Power Entry Module

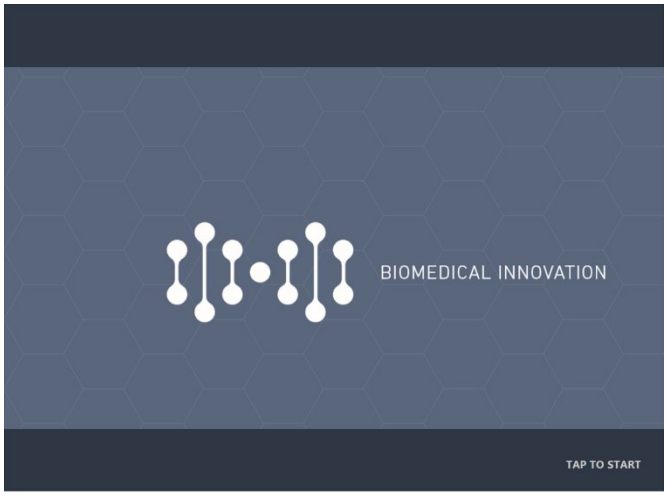


Figure 21: Power-up screen

A screen will with the Biorep logo will appear after a successful system boot-up. Click anywhere on the screen to continue.

7.2 User Login

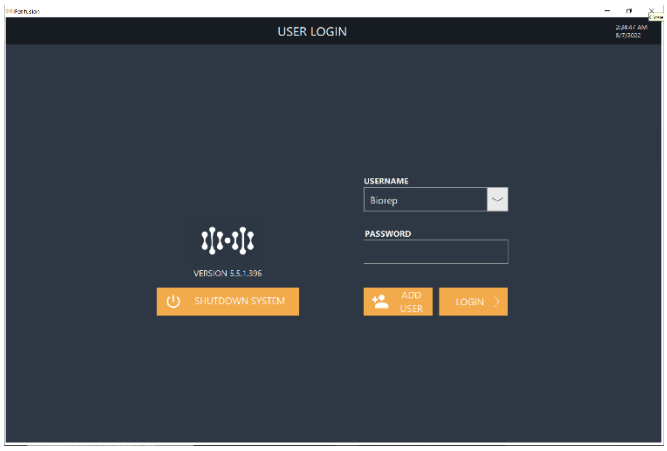


Figure 22: Login page

The User Login Page allows you to Log into your password-protected user account. The user account saves your protocols, reports, and the preferences of your experiments; it also saves time by pre-filling some frequently used fields during the experiment process.

In this page you will also see the Software Version installed in your perfusion machine and the SHUTDOWN SYSTEM button,

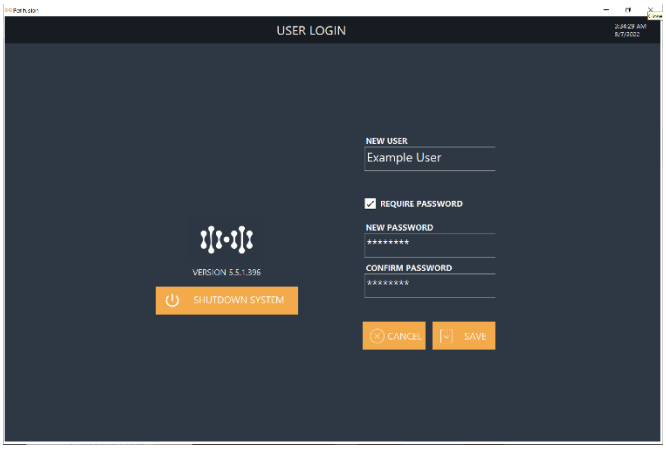


Figure 23: Add User

The first step is creating a user account. To create a new user account, press the **Add User** Button.

When the “Add User” button is pressed, the “New User” fields on the right of the screen will appear. Upon clicking on any field a pop-up keyboard will appear. Enter a unique username and password using the keyboard and press “Accept”. You may also choose to not use a password by unchecking REQUIRE PASSWORD. Click Save to create the user.

Now you will be able to find your Username on the drop-down list at the top right. The next time you do a Perifusion experiment, just select your user from the list, enter your password, and press “Login”.

7.3 Main Menu

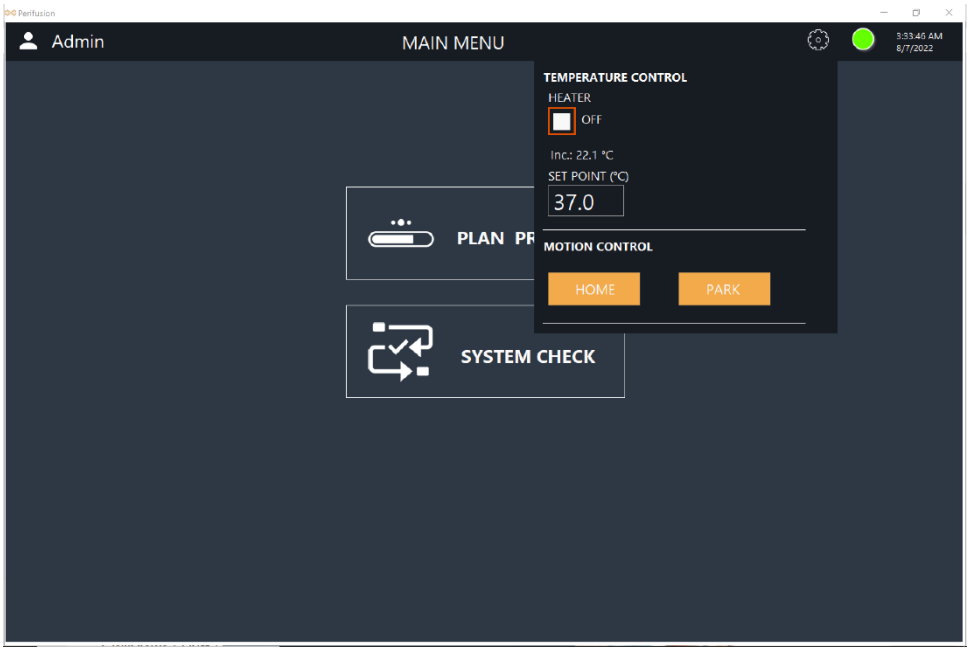


Figure 24: Manual Control via “Gear Icon”

Once logged in, you will arrive at the Main Menu page. On this page you can start an experiment, via “PLAN PROTOCOL” or manually control all the different sub-systems of the machine via the “System Check”.

On this page, and on every page of the software, you also have access to critical manual controls by clicking on the “Gear” icon on the top right and the color-coded “Status Light” next to it, here shown green. The Gear icon allows you to turn the Heater ON/OFF, change the heater set point, and either HOME or PARK the 96 well-plate tray.

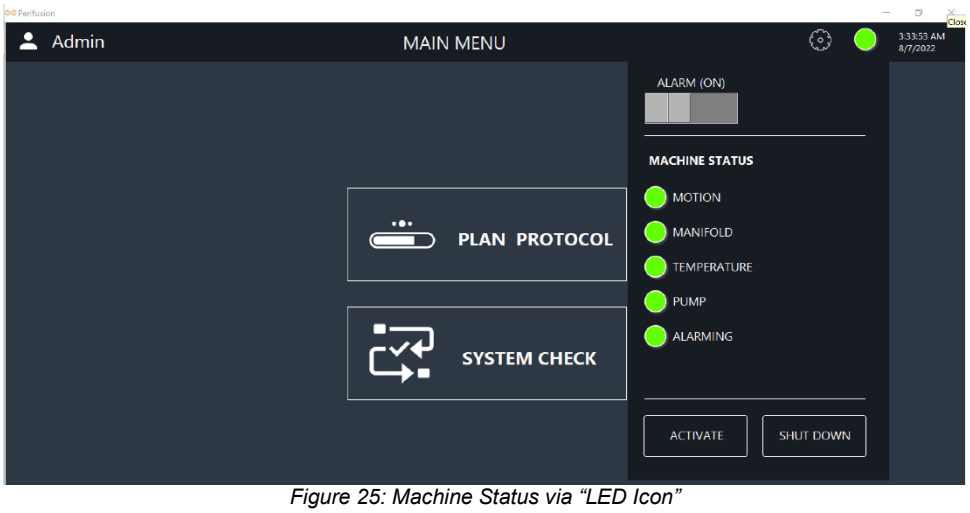


Figure 25: Machine Status via "LED Icon"

Clicking on the Status Light allows you to mute the machine's alarm, see the status of critical machine components (green means functioning or nominal, red means malfunctioning or out of safe range), and SHUT DOWN button to power off system.

The ACTIVATE button should only be pressed to reset the communications between the software and the machine controller.

You can also end the session from this page either by logging out of your user account (clicking your username on the top left) or by turning the system off. You can also change your user's password or delete the user completely by clicking on your username on the top left.

Please do not turn the machine off from the power entry module until the Perifusion System application has completely closed.

7.4 System Check

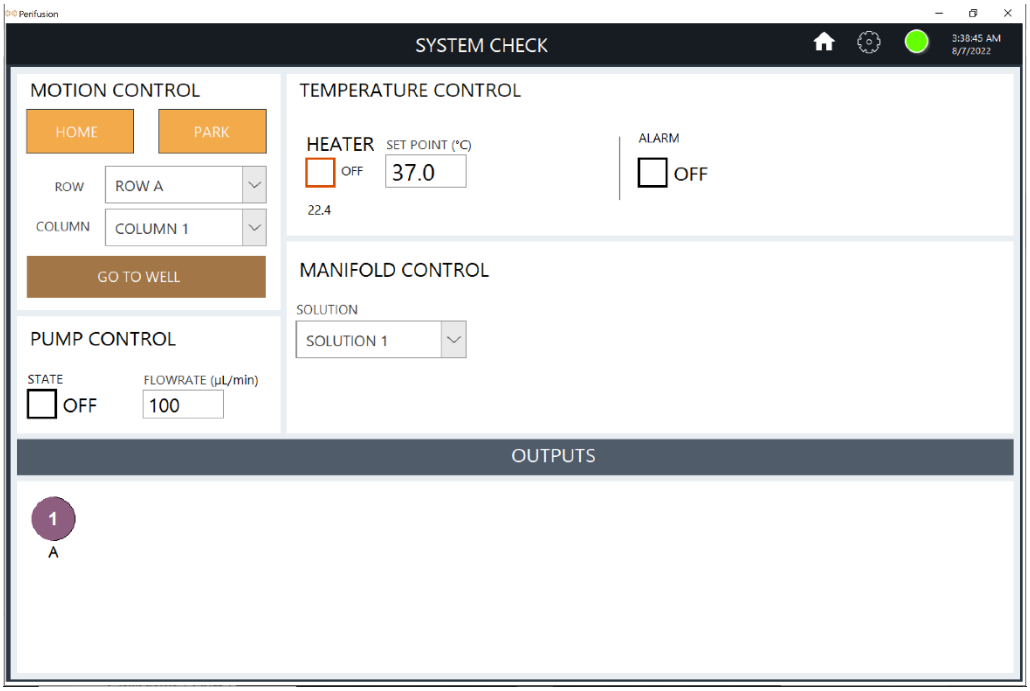
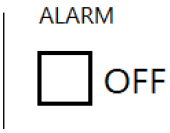


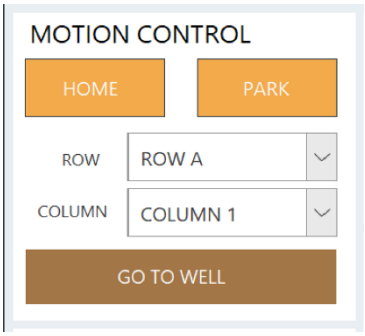
Figure 26: SYSTEM CHECK Page

To get acquainted with the different sub-systems of the Perifusion machine use the SYSTEM CHECK page.



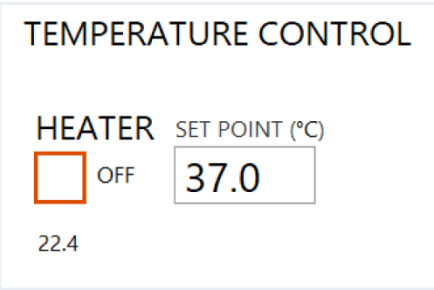
You can test the alarm buzzer by clicking the “Alarm” check box.

Figure 27: Alarm Control



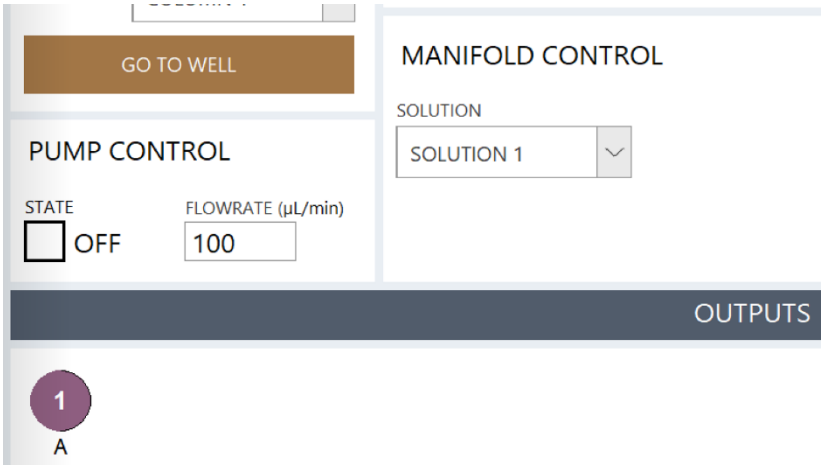
MOTION CONTROL: The motion controls manipulate the position of the tray by enabling access to the 96 positions on a well plate. Simply select a row and column and click GO TO WELL and the tray will move to align that well location with the rightmost nozzle holder. Press HOME to return to the starting position, and PARK to stow away the tray.

Figure 28: Motion Controls



TEMPERATURE CONTORL: The temperature systems can be tested by these controls. The “HEATER” checkbox enables the heater and the convection fans. The incubator SET POINT will be displayed to the right, and the actual temperature below (22.4 C in this image).

Figure 29: Heater controls



FLUIDICS: The fluid handling manifold is used for automating a Perifusion experiment. It is composed by the precision peristaltic pump and the 4x1 manifold. The pump can be tested by assigning a value to the FLOWRATE box and using the STATE checkbox. The state of the manifold can be manually changed by selecting the desired solution in the SOLUTION drop-down. The selected solution is represented by its corresponding color and number under the OUTPUTS section of the screen.

Figure 30: Valve controls

At any point, press the “Home” symbol on the top right to return to the MAIN MENU

Figure 31: Return to Main Menu

8 Perifusion Operation (Plan Protocol)

Continuing our effort to make Perifusion experiments more streamlined, and reduce the chance of error during setup, we have developed a “Wizard” type interface to set-up and run your experiments. The wizard will guide you step by step through the setup of the machine, setup of the sample, execution of your protocol, reporting of the results, and even post-experiment maintenance of the machine.

Start creating your protocol from the Main Menu screen by clicking the **Plan Protocol** button (see Section 7.3).

8.1 Protocol Browser

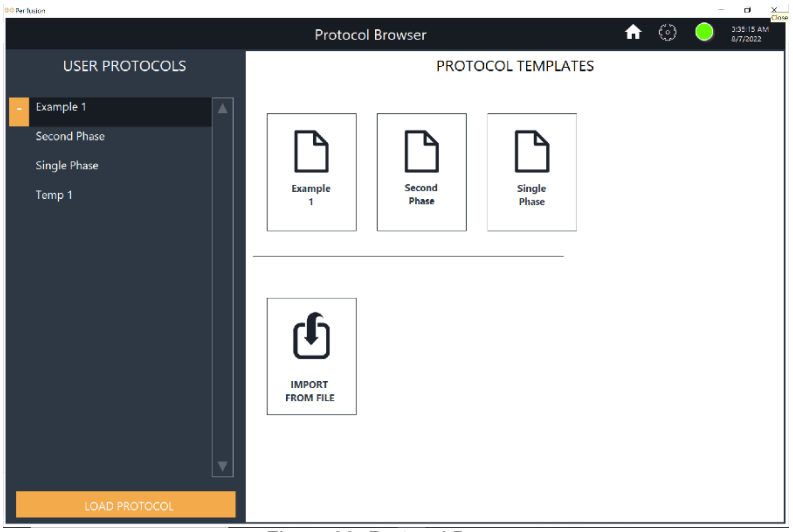


Figure 32: Protocol Browser

Before starting to design your perifusion experiment you have the option of selecting an existing USER PROTOCOL from a list or selecting a PROTOCOL TEMPLATE to work from. These templates are set by the Admin user that can be created when first using the machine. The last 3 protocols created on that user will show up on all the users on the machine but can only be edited from the Admin user (see Figure 32 for the view of the Protocol Browser from the Admin user).

IMPORT FROM FILE: This option allows you to import protocol files from other users.

USER PROTOCOLS: Select from the existing list of saved protocols. To delete a protocol, click it twice so the orange delete button with a “ – “ symbol appears on the left. Click it to delete the profile. Click a profile once and the click LOAD PROTOCOL to open it.

PROTOCOL TEMPLATES: Click any one of the three templates to automatically start building a protocol based off of it.

8.2 Protocol-Setup

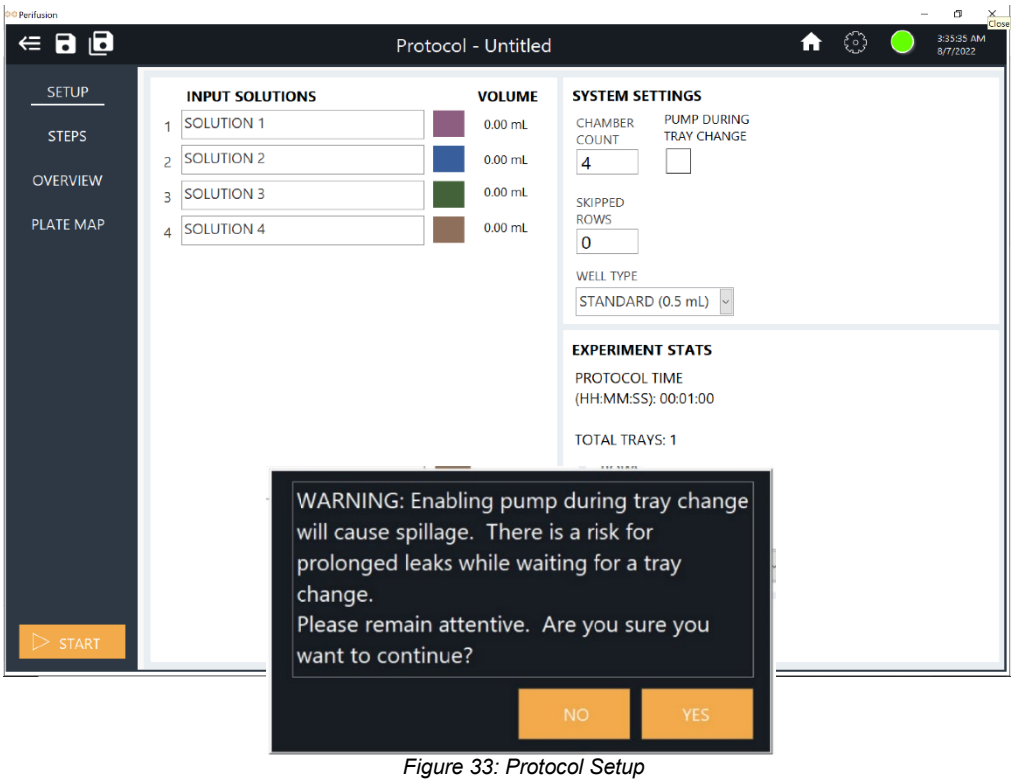


Figure 33: Protocol Setup

On the first screen of the Protocol section, enter how many chambers you plan to use on your experiment. You can use any number from 1 to 4. Select the chambers to be used by changing the value on the CHAMBER COUNT box. Chambers should be ordered starting from the right-most position and adding more chambers to the left. Chamber number one (1) will always be the left-most chamber.

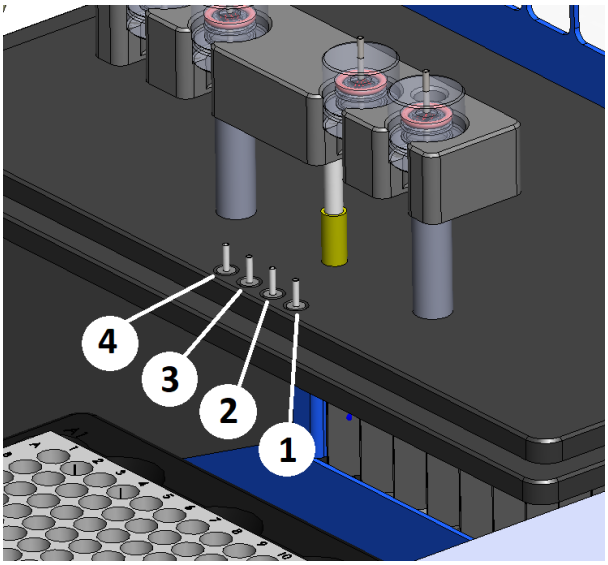


Figure 34: Perifusion Nozzle Placement

Misplacing the chambers will cause the collection sequence to fail. Please make sure the chambers are numbered and located on the rack correctly.

SKIPPED ROWS: When this option is enabled, the machine will automatically start in row 2, 3, 4, etc., of every tray used in a protocol. This option is used when the first rows of the tray will be post-filled with a control sample by the user.

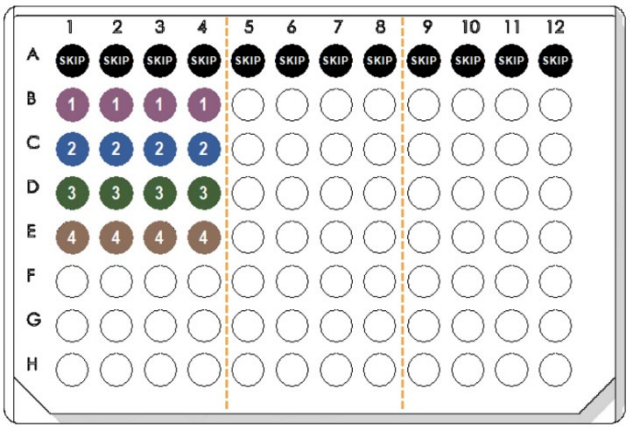


Figure 35: SKIP First Row Example

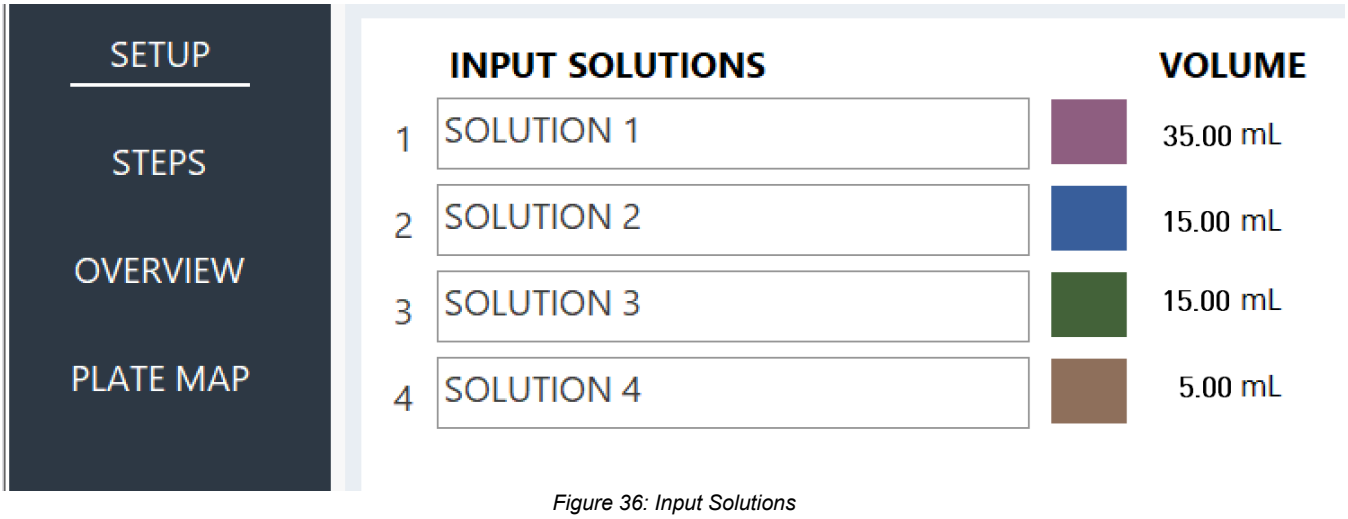


Figure 36: Input Solutions

INPUT SOLUTIONS: Here you can name your input solutions. You can also check how much volume of each solution will be required based on the entered protocol.

PUMP DURING TRAY CHANGE: This option allows the machine to continue pumping solution while trays are being switched out. This ensures that islets do not sit in glucose without secreting solution, which depending on how long

the tray change lasts, can cause a spike of hormone secretion that will show up in the first wells of the next tray when analyzed. It is recommended to enable this option.

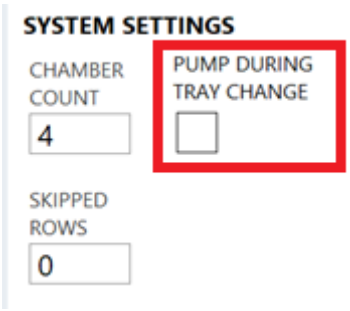


Figure 37: Pump During Tray Change Option

WELL TYPE: Select whether you'll be using standard (0.5 mL) or deep (2.0 mL) well plates. This determines the max well volume that is used by the system to trigger an alarm if the combination of dispensing time and flow rate exceed the capacity of the well plate.

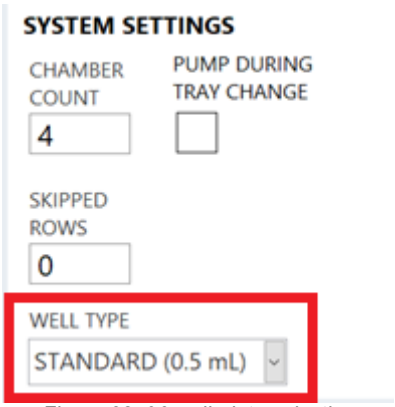


Figure 38: 96 well-plate selection

TOTAL TRAYS: This number will tell you the number of well plates required to complete the protocol. This will help you have all the plates in hand to avoid unnecessary delays.

8.3 Protocol Creation

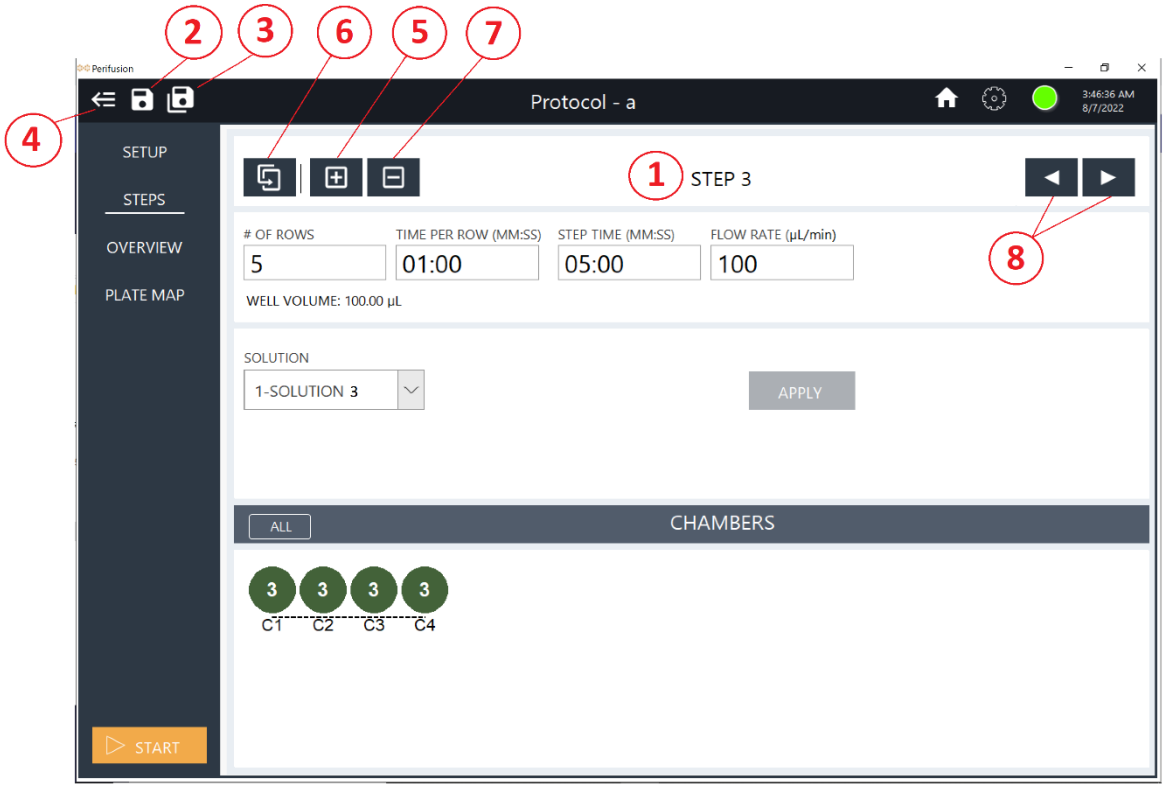


Figure 39: Protocol Creation

On this screen you will be able to select or create a protocol for the machine to run automatically. A protocol is divided into a series of steps. The step number being currently programmed is displayed at the top center (1).

You can save your protocol by clicking the floppy drive icon on the top left (2). The double floppy icon (3) allows you to save the protocol with a new name. The hamburger menu with the left arrow (4) to the left of the save buttons serves to return to the previous screen.

Each **STEP** has the following attributes: **# OF ROWS** is the times the step is consecutively repeated before changing to the next step. **TIME PER ROW** determines the duration of each repetition of the step. **STEP TIME** counts the total time the step will take. **FLOW RATE** in µL/min determines the flow rate at which the solutions are supplied to the cells during the step. Given it's a single pump with multiple channels, all channels will have the same flow rate.

The **CHAMBERS** section relates to the automatic fluid handling system (see section 5.2). In these fields you can assign the solution (1 to 4) for each of the channels by selecting it from the **SOLUTION** dropdown.

For example, in Figure 39:

 # OF ROWS = 5
TIME PER ROW = 1:00
STEP TIME (MM:SS) = 5:00
CHAMBERS = SOLUTION # 3

FLOW RATE (µL/min) = 100

Therefore, **# of ROWS** x **TIME PER ROW (MM:SS)** = 5:00.

Based on the data entry above, the machine will execute as follows:

Solution # 3 will flow out of the liquid manifold output for 1:00 minute at a flow rate of 100 µL/min and dispense in the first row of the 96 well-plate. The collection tray will then move to the next row of wells and continue dispensing Solution # 3 for another 1:00 minute, and so on for 5 consecutive well rows. The total **STEP TIME** would be 5:00 minutes.

Once the entry of the first step of the protocol is complete, press the Plus (+) symbol (5) near the top left to add a new blank step, or press the Copy symbol (6) to copy the parameters of the current step to the next one. The Minus symbol (7) deletes the current step and the arrow buttons (8) change the step you are adjusting.

8.4 Protocol Overview

This page displays a summary of the protocol in a table format. Using the buttons along the top, you adjust and re-organize the steps of your protocol.

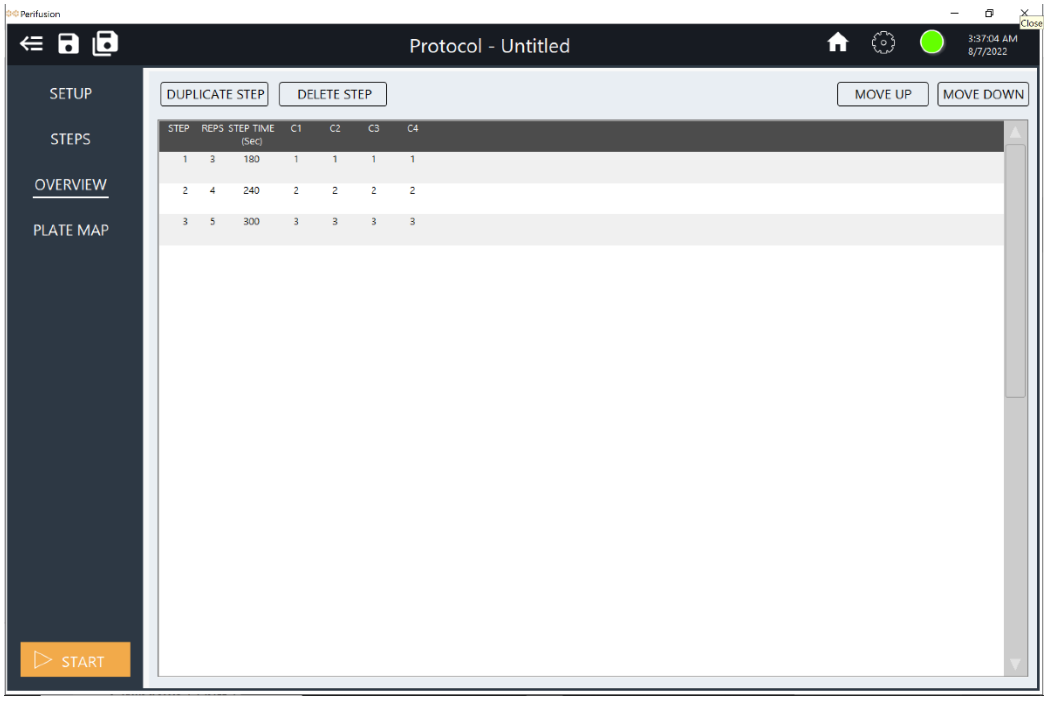


Figure 40: Protocol Overview – Table Format

8.5 Protocol Plate Map

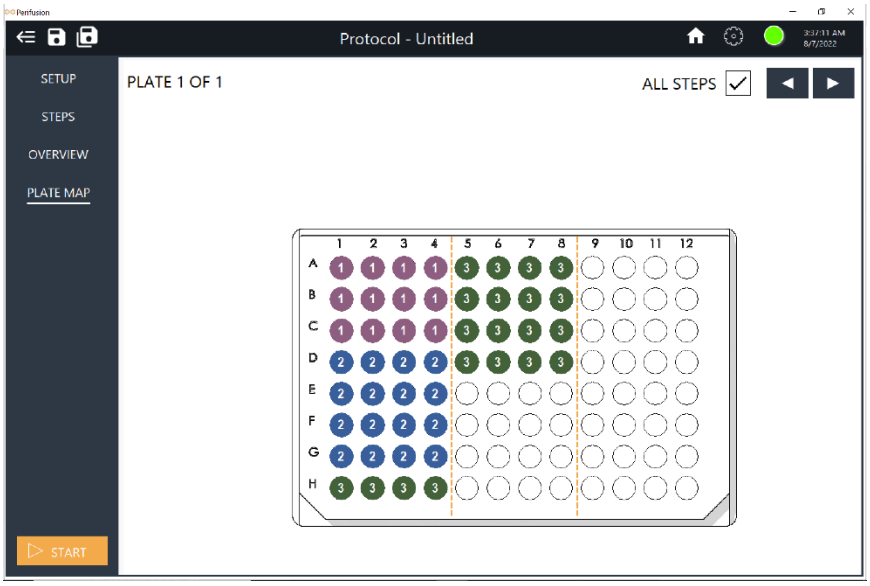


Figure 41: Protocol Overview – Plate Map Format

The Plate Map lets you confirm that the solutions are being dispensed in the correct wells. You can scroll through all the 96-well plates of the experiment by clicking the forward and back arrows on the top right. Uncheck ALL STEPS in the right hand corner to have the software indicate which wells will be filled during which Step.

8.6 System setup

Before an experiment can be performed, the machine has to be properly set-up and prepared. The basic steps to complete are:

- Stock solutions and preparation of the Perifusion Buffer (PB)
- Chamber setup
- Tubing setup
- Pre-Priming
- Source Priming
- Perifusion chamber cell loading

Please read the entire setup instructions carefully and perform a practice experiment before using cells. For further assistance please contact customer support.

8.7 Stock solutions and preparation of the Perifusion Buffer (PB):

It is practical to purchase premade stock solutions; they are inexpensive and last a long time. Suitable stock solutions are indicated in the table below. The pH of the PB, after mixing the stock solutions in the order indicated in the table below, is suitable without any adjustment. However, if using another source of HEPES, a pH adjustment might be needed. It is also recommended to use ultrapure water, like Milli-Q or similar. It is good practice to prepare fresh PB to preserve the properties of $NaHCO_3$.

When making custom solutions that do not follow the recipe below, avoid using phosphates and sulfates, particularly Na2HPO4, KH2PO4, and MgSO4. These compounds can react with the beads and filters in the chambers and cause them to become clogged over the course of an experiment.

Reagent	Cat. No/Vendor	PB (mM)	Stock solutions	To 500 mL	To 1000 mL
Ultrapure water	N/A	N/A	N/A	480	960
$NaHCO_3$	S6014-500g/Sigma	24	Added as powder	1.0 g	2.0 g
NaCl	71386-1L/Sigma	120	5M NaCl	12.0 mL	24.0 mL
KCl	60135-250mL/Sigma	4.8	3M KCl	0.8 mL	1.6 mL
$CaCl_2$ $2H_2O$	21114-1L/Sigma	2.5	1M $CaCl_2$ $2H_2O$	1.25 mL	2.5 mL
$MgCl_2$ $6H_2O$	M1028-100mL/Sigma	1.2	1M $MgCl_2$ $6H_2O$	0.6 mL	1.2 mL
HEPES	15630/Life Technologies	10	1M HEPES	5.0 mL	10 mL
Gas	95%O_2/5%CO_2	N/A	N/A	N/A	N/A
BSA	A7888-58g/Sigma	0.25%	Added as powder	1.25 g	2.5 g

Table 3: Perifusion Buffer composition

8.8 Preparation of the bead suspension:

1. Add dry beads to the 5mL mark of a 50mL conical tube.
2. Add 40mL of PB without glucose or BSA. Mix well by inverting the tube.
3. Prepare the bead suspension at least one day before the experiment to allow it to hydrate fully.
4. The bead suspension can be kept refrigerated (4℃) for 4 weeks or longer. Discard if there are any signs of microbial growth such as bad smell.
5. Warm up the bead suspension at RT or 37°C before using.

8.9 Cleaning and storing the nozzles

The nozzles are ready to use when new. After the first use, rinse them well with tap water and then ultrapure water. If there is a reason to use soap, rinse them well with ultrapure water before using them again. Let them dry on top of paper towel after cleaning. The nozzles will not drip properly if they have dirt or salt residues in their surface. In fact, they might not drip at all because the perfusate will climb backward and accumulate in the nozzle holder and eventually in the enclosure; Avoid accumulation of perfusate in the nozzle holder. Store clean and dry nozzles in their respective nozzle holder below the chamber holders. Attach the tubing that will connect the chambers to the nozzles (see Figure 1). This tubing can be reused several times, but it needs to be rinsed with ultrapure water after each use, otherwise the salt in the BP will dry out and clog them.

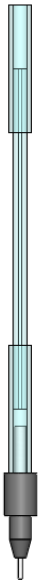


Figure 42: PERI-NOZZLE with Tubing Extension

8.1 Cleaning and storing the Perifusion Chambers (islet containers)

The Perifusion Chambers are ready to use when new. After the first use, rinse them well with tap water and then ultrapure water. If there is a reason to use soap, ensure it is fully rinsed with ultrapure water before using them again. Let them dry on top of paper towel after cleaning.

8.2 Chamber Setup

It is time efficient to prepare the chambers in advance of the day of the experiment.

1. Use the included hole-puncher to cut the necessary number of fiberglass disk filters (use one filter per chamber)

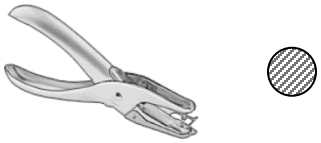


Figure 43: 1/4" Diameter Fiberglass Disk Filters

2. Place a fiberglass disk filter (2) centered inside the bottom cap (1) bore.
3. Place an o-ring (3) on top of the filter. Ensure no wrinkles are present on the filter.
4. Tighten the bottom lid (1) on the chamber body (4).
5. Place the top o-ring (5) inside the chamber body bore (4).

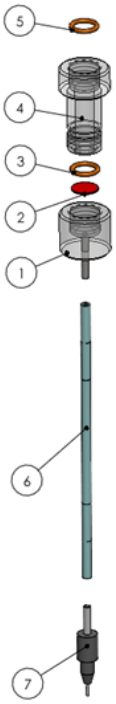


Figure 44: PERI-CHAMBER setup

6. Connect the short section of the silicone tubing set (6) to the outlet tube of the bottom cap (1). This tubing segment will help start the flow going when the beads are added.

7. Place the above assembly on the Cell Loading Rack.

8. Place the above assembly on the Cell Loading Rack.

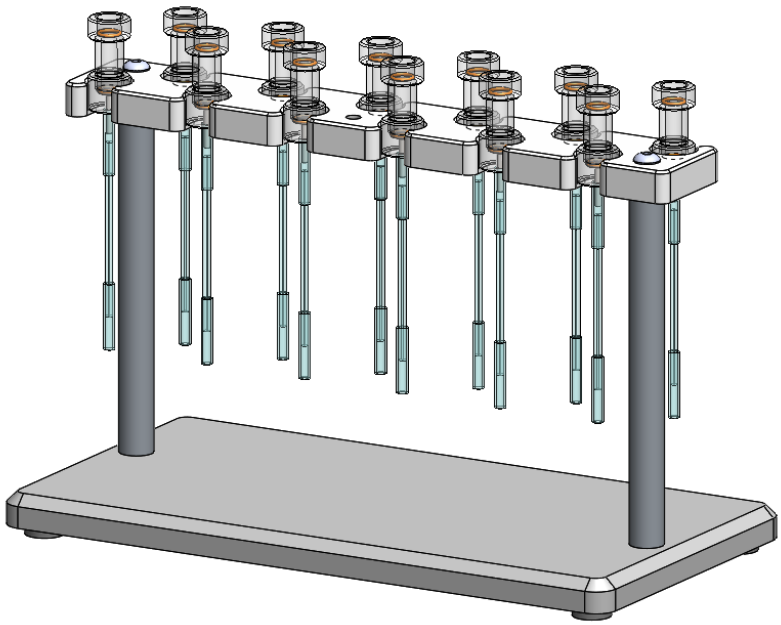


Figure 45: Cell Loading Rack (PERI-LITE can only use 4 chambers)

9. Cover chambers with paper towel to avoid accumulation of dust if not using immediately.

You can proceed to the tubing setup, priming and cell loading stages. The top half will be used in the tubing setup and the bottom half will be prepared for cell loading. Once both stages are complete and the system is primed, the cells can be loaded, the two parts of the chamber re-connected, and the experiment can begin.

8.3 Perifusion Tubing Setup

It is time efficient to setup the tubing in advance. However, clamping the tubing on the pump roller for a prolongued period will damage them. Therefore, if setup of the tubing is done in advance, simply let the tubing sit inside the enclosure without fully engaging the pump cassetes.

The tubing setup can be divided in two main parts: The source side and the chamber side. This section will assume that the chamber has been set up as shown in section 7.4. Prepare the tubing as described below:

Source Side- Number your input source(s) from 1 to 4 and connect them to the corresponding port on the liquid manifold with a 12” section of 0.040” ID silicone tubing. The silicone tubings relatively easy to tear/puncture. **Avoid tearing the tubing by all means**, as this will become a source of bubbles and may ruin your experiment. Tubing

lengths are not critical on this side of the manifold if they are long enough to reach the bottom of your source solution container.

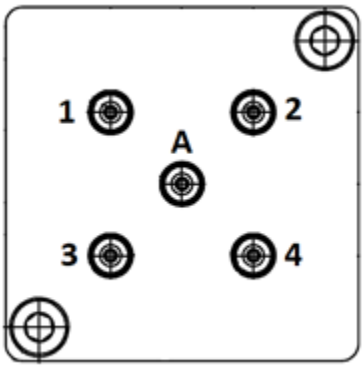


Figure 46: 4x1 Liquid Manifold

Attach a dispensing needle with luer-lock connection to the other end of the input tubing. This ensures the end will sink to the bottom of the source solution container. If this end does not go all the way to the bottom, air can be aspirated and enter the system as the solution is spent. If this happens, air will reach the chamber with the islets and the experiment may fail. **Avoid the entrance of air into the system as it will eventually reach the chambers containing the islets.**

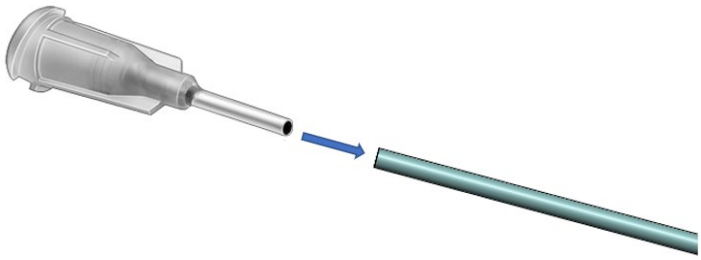


Figure 47: Insert dispensing needle into silicone tubing

Chamber Side- Insert the three-stop tubing in the pump as follows:

1. The tubing is inserted in the cassette by sliding it through the groove.
2. The orange detent on the manifold side should mate with the notch in the back of the cassette. Make sure the green detent does the same with the notch of the front.
3. When the tubing is secure, align the guide onto the pump shaft and push the cassette until the lever locks in place **Note:** Ensure to minimize the amount of time the tubing is clamped by the cassette when not pumping. This flattens and decreases the flow rate accuracy of the tubes.
4. Make sure the chamber cap is on the right side of the pump cassette. Repeat for the number of chambers used in the experiment.
5. Skip this step if performing a 1 chamber experiment. Connect a 3/4" long segment of 0.040” ID tubing to the output of a Y (for 2 or 4 chambers) or Cross splitter (for 3 chambers).
6. For 2 and 3 chambers: connect the end of the pump tubing to the open ends on the splitters.
7. For 4 chambers: Connect two more 3/4" long segment of 0.040” ID tubing to the open ends of the Y splitter. You should now have a Y split into 2 more Ys. Connect the pump tubing to the four open ends on the Y splitters.

IMPORTANT: Ensure all lines have the same tubing section lengths on the chamber side (after the manifold These sections make up the “dead volume” of the line. For best results, tubing length should be minimized and kept as consistent as possible between lines.

8.4 Pre-Priming

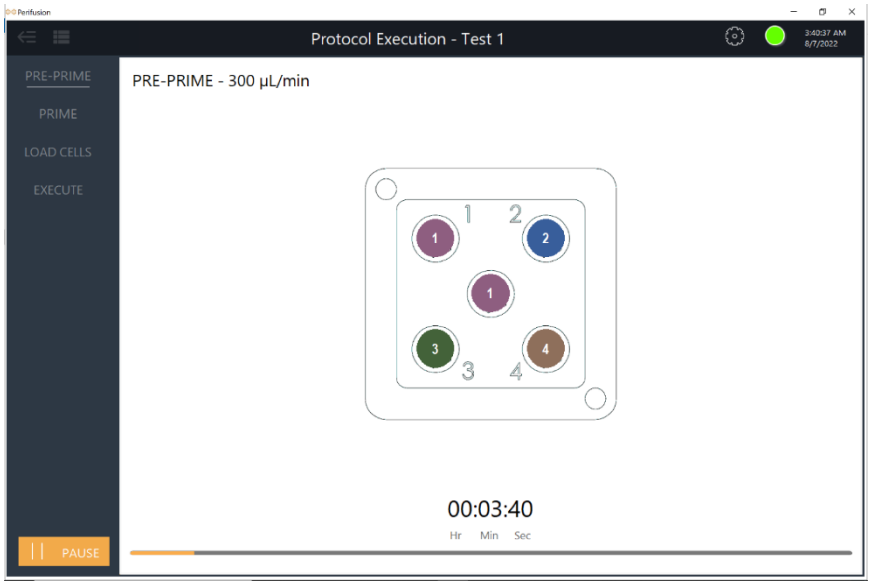


Figure 48: *Pre-Priming page*

After the tubing is properly setup and the solution sources have been prepared, the system should be primed to remove any air in the system. The machine automatically performs the priming sequence depending on the active protocol. The progress will be displayed on the bottom bar.

Before starting, place all 4 input tubes into buffer solution. The solution number displayed on the center/ output channel corresponds to the solution currently being pumped through the manifold. In Figure 48 it is Solution 1.

8.5 Priming

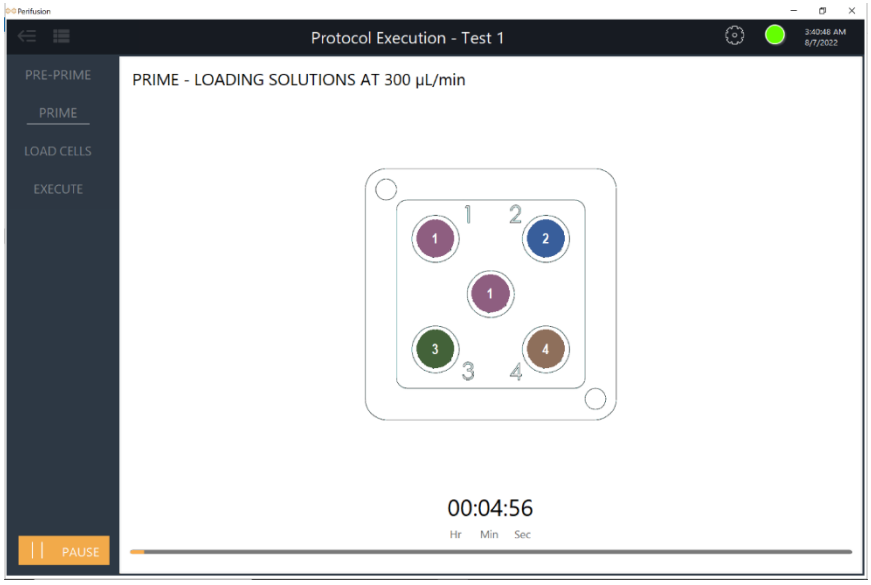


Figure 49: *Priming page*

After the system is Pre-Primed, it must be primed with the appropriate test solutions.

Place the input tubes into their corresponsing input solutions
Click on START to initiate PRIMING sequence.

By this step, nearly all bubbles should be gone from pump tubing and solution should be dripping from the ends of the tubes. If this is not the case, double-check all connections and the level of solution in their containers.

8.6 Cell loading

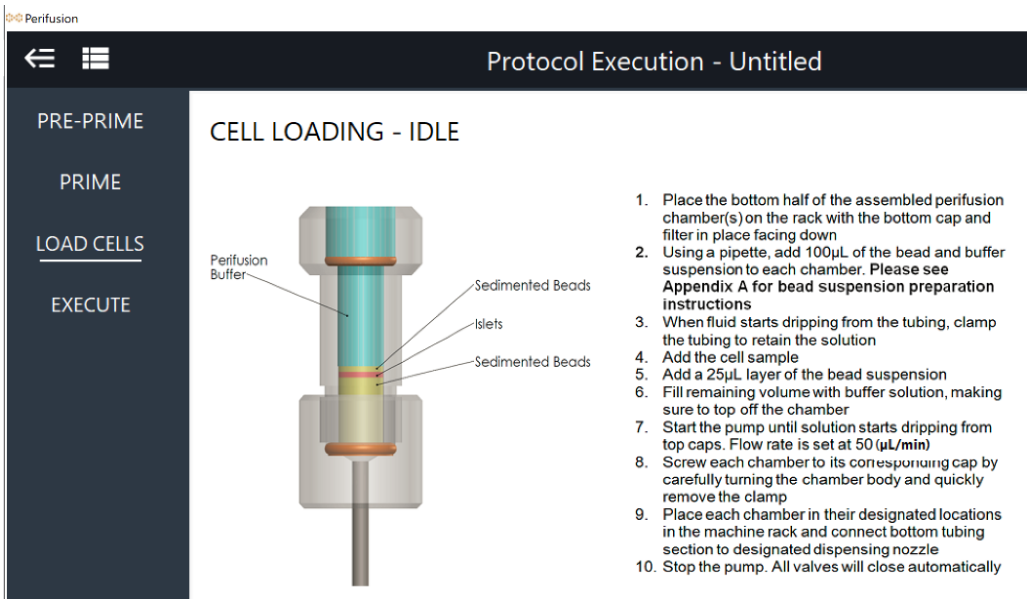


Figure 50: *Cell Loading*

When the system is completely primed and ready for the chamber to be attached:

1. Place the assembled perifusion chamber(s) on the rack (see Figure 3).
2. Add 100µL of PB to each chamber to wet the fiberglass filter.
3. Add 100 - 150µL of premixed bead suspension to each chamber (**Please see Appendix A for bead suspension preparation instructions**). Do not add more than ¼ chamber of sedimented beads (Figure 50). It is important to have a column of PB in the chamber to trap any bubble floating. If necessary, remove any excess of beads with a pipette. If there is no PB on top of the sedimented beads, adding more PB to the chamber will facilitate the removal of excess beads.
4. When liquid drips from the bottom of the tube, clamp the silicone tubing with a binder clip/paper clamp to avoid the beads drying.
5. Add the islets in 150µL of perifusion buffer solution. If you need to add more islets remove the clamp from the silicone tubing to release some buffer from the chamber and clamp it again. Do not let the islets dry out.
6. Add a couple drops (with a micropipette and 100µL tip) of bead suspension on top of the islets. This step is optional, but it prevents losing the islets if you drop the chamber or other unexpected events happen.
7. Fill the chamber with perifusion buffer; make sure to top off the chamber.
8. Start the CELL LOADING step of the protocol execution until the perifusion buffer starts dripping from the top caps.
9. Screw the body to the cap of the chamber while trapping as little air as possible. (Air bubbles can cause the experiment to fail if they reach the cells inside the chamber.) Twist the body and not the cap to prevent the tubes from twisting.
10. Remove the binder clip/paper clamp immediately after screwing on the body to prevent pressure from building up inside the chamber.

11. Place the nozzle in the nozzle holder that corresponds to it. Do this quickly and smoothly, trying not to touch the sides of the holder with the tip of the nozzle. If too much liquid collects in the nozzle holder, adhesion and capillary forces will prevent the nozzle from dripping properly and a lage drop will collect around the nozzle. If this happens, remove the nozzle, thoroughly dry it and the holder, and try again.
12. Place the chamber on its corresponding location on the rack.
13. Ensure that solution is dripping from the nozzle. If it is not, this is the time to fix any problem as it is not advisable to open the incubator enclosure once the experiment has started. **Temperature variations inside the enclosure will change the amount of hormones released from the islets and adversely affect results.**
14. Repeat the process for every chamber.

8.7 Chamber positioning

Care must be taken when positioning the perifusion chambers on the perifusion chamber rack inside the incubator. Chamber placement starts from the right-most position with the last chamber, and then subsequently filling positions to the left until the first chamber is placed. The first chamber should be the leftmost chamber. This is of critical importance to the proper functioning of the experiments; a misplaced chamber will deliver in the wrong well or even outside the tray!

Please see the following example:

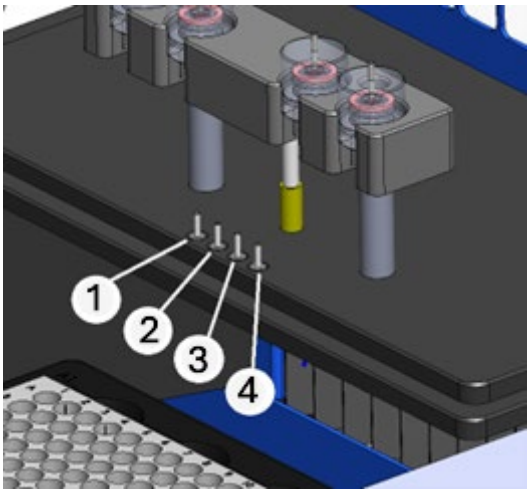


Figure 51: Four active chambers

Temperature control

Once the system has been prepared, the incubator must be heated up to the desired temperature.

Heating

1. Close the incubator cover.
2. Press the "HEATER" button to enable the heater and turn on the fans.
3. For better results and better temperature distribution, do not open the incubator cover during the experiment. To re-fill the sources, run a tubing section from the source to the outside of the incubator through the channels of the removable source tray.
4. An incubation/stabilization step of approximately 60 minutes is recommended for islet secretions to stabilize. This time also allows for temperature conditions inside the incubator to stabilize.

IMPORTANT: If the incubator cover needs to be opened for any reason, turn the heater OFF. This will ensure that when the incubator cover is closed, there is no temperature overshoot.

8.8 Protocol execution

When you reach this screen, the cells should be in the chambers, the manifold and should be primed with the input solutions and the temperature of the incubator and tray should be at their set points. On this screen you can start your Perifusion experiment by hitting the START button. The flow rate, manifold state, and movement of the tray will be automatically handled by the machine. The machine will only require your input when the machine requires manual change of the sample tray. This screen is primarily used to monitor experiment progress.

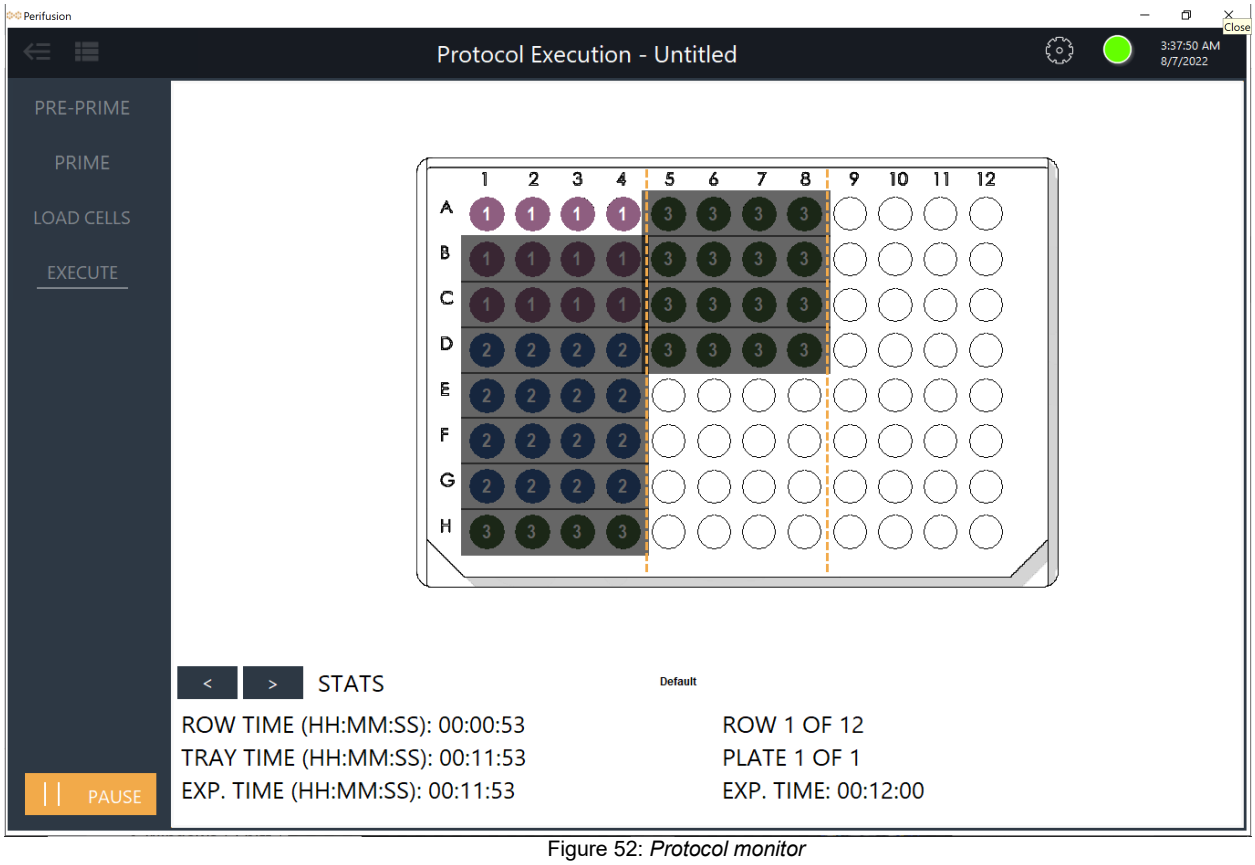


Figure 52: *Protocol monitor*

Remember that the heater can be controlled by clicking on the Gear icon in the top right hand corner

PLATE MAP

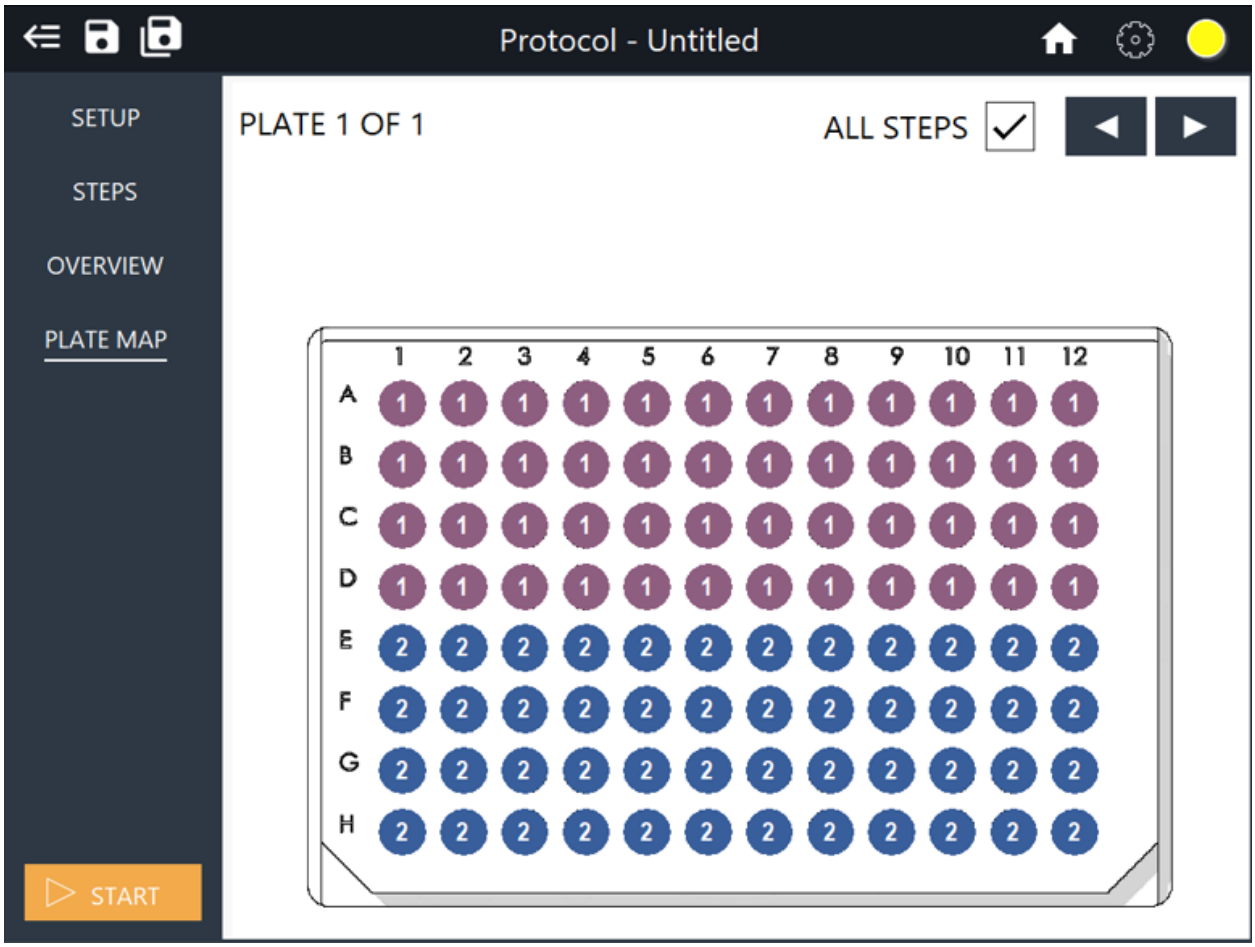


Figure 53: *Plate Map*

The changes in the fluid handling systems can be monitored on-screen, by means of a plate map. The entire plate will be greyed out except the actual row being dispensed into. The solution # being dispensed will be shown inside each well.

TIMING STATS

These indicators will provide information related to the timing and progression of the experiment. Since the experiment has many chronological “levels”, these indicators will help you identify where you are in your protocol.

Click on EXECUTE to start the protocol execution.

8.9 Protocol Examples

The following are some example protocols for use with the Perifusion system and a typical insulin release profile.

Protocol to measure only the first phase of insulin release

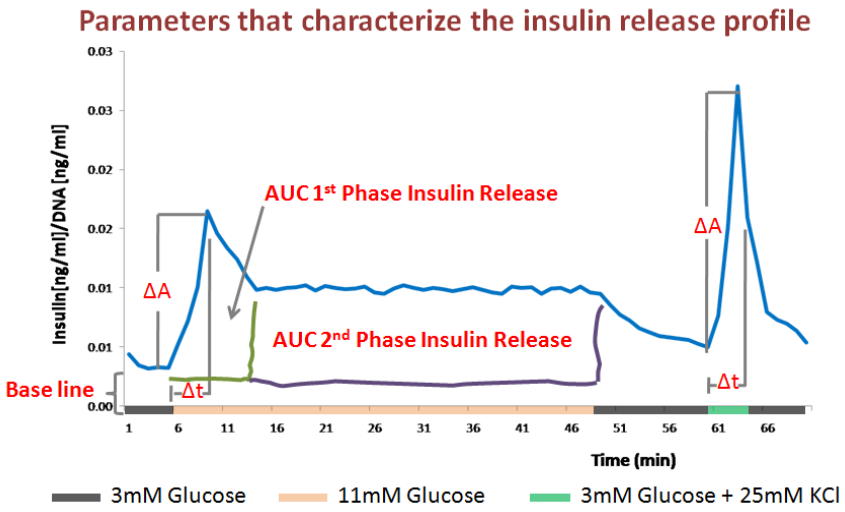
Solution No.	Stimulus	Reps	Time SV	Duration (min)
1	3mM Glucose	5	60	5
2	11mM Glucose	10	60	10
1	3mM Glucose	15	60	15
3	25mM KCl	5	60	5
1	3mM Glucose	5	60	5

Flow rate at 100µl/min

Protocol to measure the first and second phase of insulin release

Solution No.	Stimulus	Reps	Time SV	Duration (min)
1	3mM Glucose	5	60	5
2	11mM Glucose	40	60	40
1	3mM Glucose	15	60	15
3	25mM KCl	5	60	5
1	3mM Glucose	5	60	5

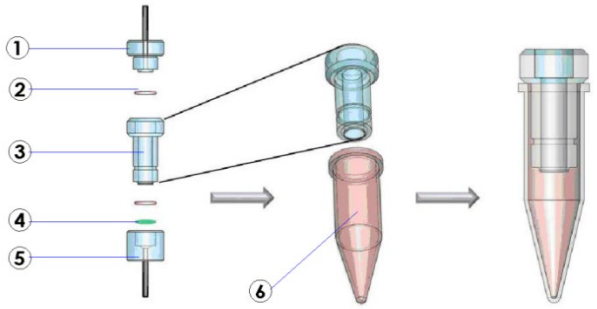
Flow rate at 100µl/min



AUC= Area Under Curve

9 Finalizing the Experiment

9.1 Collecting the cells



1. Top cap
2. O-ring
3. Cell chamber
4. Fiberglass filter
5. Bottom cap
6. Micro-centrifuge tube

Figure 54: *Perifusion Chamber and collection tube*

1. Disconnect the tubing attached to the bottom cap of the chamber.
2. Remove the bottom cap and introduce the body of the chamber into a micro-centrifuge tube. If the filter remains attached to the body, remove with a pair of forceps.
3. Remove the top cap.
4. Add 1 mL of Hank's balanced salt solution (HBSS) from the top to flush down beads and islets.
5. Remove the body of the chamber from the micro-centrifuge tube.
6. Close the lid of the micro-centrifuge tube.
7. Centrifuge at 300g for 5 minutes.
8. Aspirate the supernatant leaving beads and islets behind. At this point, the samples can be frozen for later processing or proceed with the following steps.
9. Add 180 µL of ATL buffer.

10. A recommended protocol for DNA purification is the QIAamp DNA Mini and Blood Mini Handbook (QIAGEN® , Third edition, 2012, pg 32-35) Follow the steps in "**DNA Purification from Tissues**". You can use the QIAshredder, Cat. No. 79656 to breakdown the tissue or simply proceed with Proteinase K digestion (step 3). Both procedures should work, but QIAshredder use minimizes the digestion time.
    Quantify the DNA using the NanoDrop (Thermo Scientific) or your method of choice.

9.2 Normalizing the insulin output by islets DNA content

Even though the islets can be counted and size matched, it is often a good practice to normalize the insulin output by the DNA content of the islets, which will account for small differences in islets mass among the chambers.

1. A recommended protocol for DNA purification is the QIAamp DNA Mini and Blood Mini Handbook (QIAGEN®, Third edition, 2012, pg 32-35) Follow the steps in "**DNA Purification from Tissues**". You can use the QIAshredder, Cat. No. 79656 to breakdown the tissue or simply proceed with Proteinase K digestion (step 3). Both procedures should work, but QIAshredder minimizes the digestion time.
2. Add 180 µL of ATL buffer.
3. Quantify the DNA using the NanoDrop (Thermo Scientific) or your method of choice.

9.3 Measuring the hormones released by the islets into the perfusate

There are many suitable assays in the market, but the following two assays have been tested by Perifusion users with good results.

Meso Scale Diagnostic (MSD), offers a variety of assays, which have a larger dynamic range that other technologies like RIA and ELISA. This is very convenient because often the sample doesn't need to be diluted after the perifusion, or the dilutions are easier to perform. See the vendor website for further details on how to use these assays (https://www.mesoscale.com/).

Mercodia, inc. offer a variated of well validated assays, which are calibrated based on international standards. This feature is a must for some experiments, like estimating insulin resistance from in vivo samples. See this vendor website for ample and detailed resources (https://www.mercodia.com/).

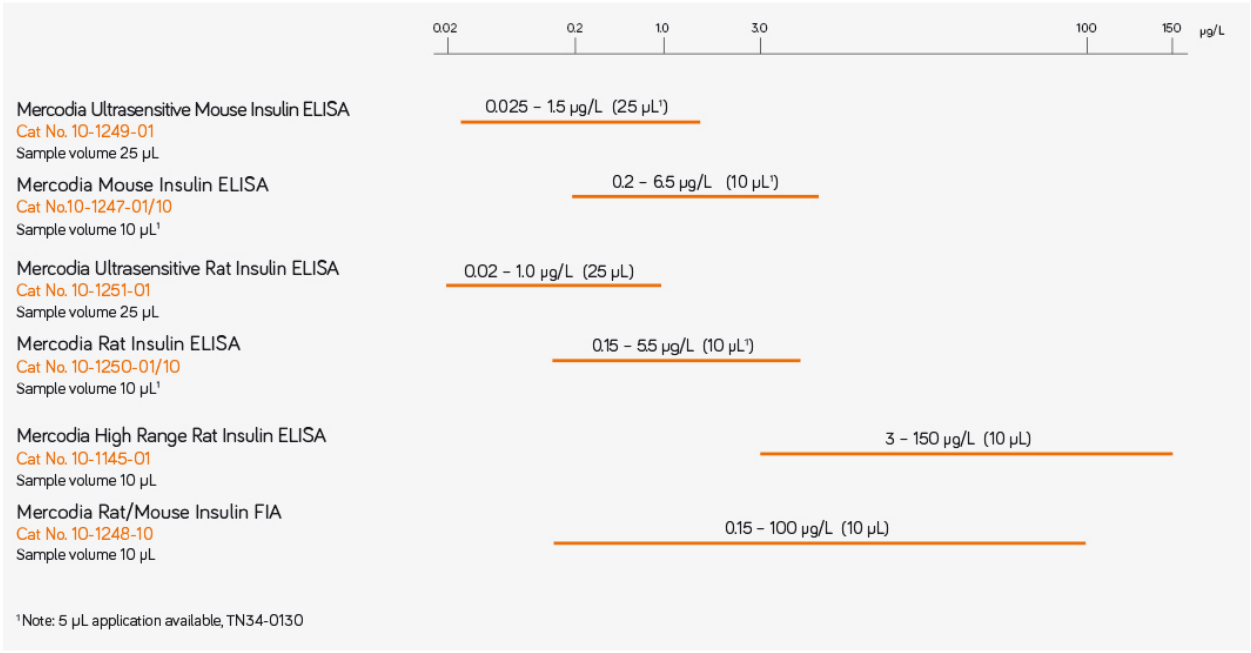


Figure 55: *Insulin assay concentration ranges*

9.4 Cleaning the system

WARNING: The chambers and manifold must NOT come into contact with any organic solvent including but not limited to: methyl, ethyl and isopropyl alcohol, acetone, and toluene. We recommend cleaning the chambers with soap).

After the protocol has been executed successfully, the system must be thoroughly cleaned, not only to ensure your system lasts longer, but most importantly, to eliminate all residues from the fluidic system, that may cause the system to malfunction, or inadvertently affect the results of future experiments.

The most efficient way of doing this is by returning to the pre-prime screen and running this cycle 4 times as follows:

Cleaning Sequence:

1 - Flush with de-ionized water. To do this, take all input tubing to the manifold and change the source to draw up from a de-ionized water container. Start Pre-prime.

2 - Sanitize with a mixture of 9 parts distilled water and 1 part bleach based disinfectant*. Start Pre-prime.

3 - Rinse once more with de-ionized water. Start Pre-prime.

4 - Dry by drawing air (remove input tubing from water container). Start Pre-prime.

Make sure that all parts you wish to re-use that come in contact with test solution/perifusion buffer are cleaned in this manner. Peri chambers should be cleaned of cells, filter residue and beads before running them through the cleaning sequence. The nozzles should be also be pre-cleaned so there is no dirt or salt residues on their outside surface. Nozzles might not drip at all because the perfusate will climb backward and accumulate in the nozzle holder and eventually in the enclosure if they are dirty. Avoid accumulation of perfusate in the nozzle holder.

Once cleaned, set aside the chambers and nozzles on a paper towel to dry.

*Example: Current Technologies Bleach-Rite™ Disinfecting Spray with Bleach

10 Report

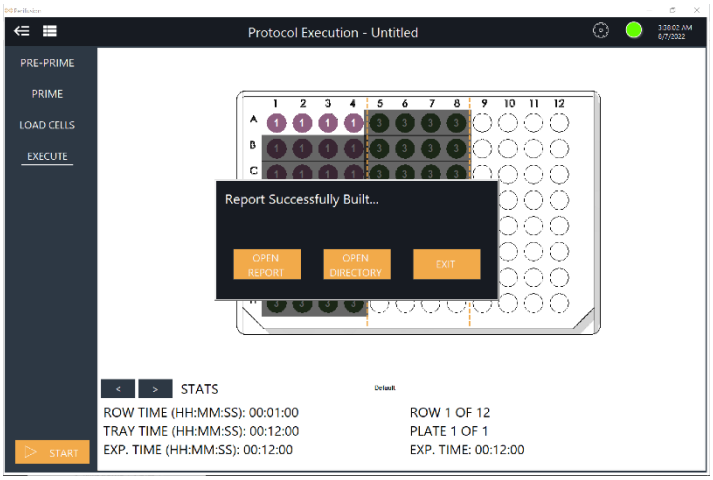


Figure 56: *Report page*

Any time during the Execution steps (Pre-Priming to Execution) you can click the hamburger menu.

This report will have a plate map (this is useful when cross-referencing the data to subsequent processing such as ELISA), solution information, date, length, time of the experiment, and a table detailing every step of the protocol.

11 Maintenance

Hazard - Only qualified personnel, trained to service Biorep machines, should perform all internal maintenance requirements.

Biorep machines must operate within stringent specifications. In order to keep the system working within the stated specifications, a professionally trained and qualified technician must perform most maintenance procedures. If unqualified personnel perform any maintenance procedures not described in this manual, the machine may not perform to its stated specifications.

Hazard - Do not remove covers to any component of your system, unless it is specified in a procedure.

There are dangerous voltages and moving parts inside the machine that may cause bodily injury or damage to equipment.

11.1 Preventive Maintenance

Preventive maintenance is the periodic inspection, cleaning, and lubrication of the test system. The following sections provide guidelines for preventive maintenance.

To ensure that the Perifusion System continues working at its optimal performance, it is recommended that the machine receive an annual service check. Biorep's Service department can perform this annual service, and replace any damaged or worn parts to ensure that your machine operates to its stated specifications.

Biorep offers many service agreements that provide a variety of services, including annual service visits. Contact Biorep for details on a service agreement or contract that best matches your needs.

11.2 Daily Maintenance Checks

Before operating the system each day, ensure:

• All cable connections are tight and secure.
• All accessories are free of dirt, damage and deformation.
• • Signal and power cables have adequate slack to prevent excessive strain on connectors.
• All cables are free of wear and chafing. Re-route the cables if necessary, and replace any damaged cables.
• After turning on the system, make sure that power is adequately supplied to the electronics.

Correct any problems before you operate the system. If you require assistance contact the Biorep Service department.

11.3 Periodic Inspections

Every twelve months, perform the following inspections:

• Visually inspect the machine for any loose parts. Check the cable connections, and connections for any accessories that are attached to the machine. Tighten any loose connections that you may find.

• Operate the 96 well plate through the full range of motion. It should move smoothly with no unusual noise, or erratic motion.

• Test the end of travel limit stops to ensure they are in working order.

• Verify the tray alignment is square with sheet metal enclosure

• Verify the tray aligns properly below the first dispensing position.

• Verify peristaltic pump flow rate

• Verify incubator temperature

• Verify functioning of temperature alarms

• Verify function of each channel in manifold

• Visually inspect the dispensing nozzles for dents or damage (replace if necessary)

• Visually inspect the Perifusion chambers for damage or leaks (replace if necessary)

Every 5 years:

• Replace 4x1 manifold

If you notice any problems resulting from these inspections, contact Biorep's Services department for immediate assistance.

11.4 Cleaning

Do not clean with solvents or abrasive cleaners. Some household or commercial cleaners can react with painted surfaces or panel markings.

Do not apply excessive amounts of detergent cleaner. It may seep into electrical circuits within the base and cause equipment failure.

Do not use too much oil. It attracts abrasive particles that may accelerate wear.

Use low pressure air to blow dust. Do not direct the air stream directly at sensitive components.

It is recommended that the machine be cleaned weekly, or more often if it is operating in a dusty or dirty environment.

To clean sheet metal enclosure wipe exterior surfaces with a moist cloth.

11.5 Lubrication

No lubrication is necessary

12 Customer Service

If you encounter any problems, please contact customer support at:

Biorep Technologies, Inc.
15804 NW 57th Ave
Miami Lakes, FL 33014
info@biorep.com
www.biorep.com
Tel: 305-330-4449
Fax: 305-330-4402


BIOREP®
TECHNOLOGIES

13 APPENDIX A

13.1 Stock solutions and preparation of the Perifusion Buffer (PB):

It is practical to purchase premade stock solutions; they are inexpensive and last a long time. Suitable stock solutions are indicated in the table below. The pH of the PB, after mixing the stock solutions in the order indicated in the table below, is suitable without any adjustment. However, if using another source of HEPES, a pH adjustment might be needed. It is also recommended to use ultrapure water, like Milli-Q or similar. It is good practice to prepare fresh PB to preserve the properties of $NaHCO_3$.

Reagent	Cat. No/Vendor	PB (mM)	Stock solutions	To 500 mL	To 1000 mL
Ultrapure water	N/A	N/A	N/A	480	960
$NaHCO_3$	S6014-500g/Sigma	24	Added as powder	1.0 g	2.0 g
NaCl	71386-1L/Sigma	120	5M NaCl	12.0 mL	24.0 mL
KCl	60135-250mL/Sigma	4.8	3M KCl	0.8 mL	1.6 mL
$CaCl_2$ $2H_2O$	21114-1L/Sigma	2.5	1M $CaCl_2$ $2H_2O$	1.25 mL	2.5 mL
$MgCl_2$ $6H_2O$	M1028-100mL/Sigma	1.2	1M $MgCl_2$ $6H_2O$	0.6 mL	1.2 mL
HEPES	15630/Life Technologies	10	1M HEPES	5.0 mL	10 mL
Gas	95%O_2/5%CO_2	N/A	N/A	N/A	N/A
BSA	A7888-58g/Sigma	0.25%	Added as powder	1.25 g	2.5 g

Table 4: Perifusion Buffer composition

To prepare 200ml of KRB (prepare right before use):

1. Add 20mL of Solution 1 to 100mL of ddH2O
2. Bubble gently with 95% O2/5% CO2 for 10 minutes
3. Add 20mL of Solution 2
4. Repeat bubbling as above
5. Add 20mL of Solution 3
6. Add 1.2mL (6mM) of 1M HEPES
7. Adjust pH=7.4 with NaOH. After adding the above solutions, the pH ≈ 7.08
8. Bring volume to 200mL with ddH2O
9. Add 0.4g (0.2%) of BSA (Sigma Cat. A7888 OR A6003)
10. Filter Sterilize through 0.22μm filter

Notes:

a. When making Solution 2 dissolve CaCl2 first
b. Solution 1 and 2 should be stable for several weeks at 4℃
c. Solution 3 is made fresh every time. Prepared 50ml 10X stock solution dissolving 1g of NaHCO3 in 50mL of ddH2O

d. Gassing times may be overkill, but this minimizes the risk of precipitate formation
e. The working solution should last for 12-24h; beyond this, there is risk of precipitate formation.
f. KRBH= KRB containing HEPES

Bead Suspension Preparation

1. Add dry beads to the 5mL mark of a 50mL conical tube.
2. Add 40mL of PB without glucose and BSA. Mix well by inverting the tube.
3. Prepare the bead suspension at least one day before the experiment to allow them to hydrate fully.
4. The bead suspension can be kept refrigerated (4℃) for 4 weeks or longer. Discard if any sign of microbial growth or bad smell.
5. Warm up the bead suspension at RT or 37°C before using them.

Bead Suspension Use

Follow these instructions before using the bead suspension in a perifusion chamber preparation:

- Warm bead suspension to 37℃ using an incubator or water bath. **Do not add cold bead suspension to cells, since this may adversely affect your experiment**.
- Agitate conical thoroughly before pipetting bead suspension to add into perifusion columns. This ensures that approximately an even number of beads are present in each column.