Guide to performing a successful experiment using the Biorep Perifusion System.

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				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				
BSA	A7888-58g/Sigma	0.25%	Added as powder	1.25 g	2.5 g

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 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°C) 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.

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. If 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. Place the clean and dry nozzles in the nozzle holder, ready for the next experiment. 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.

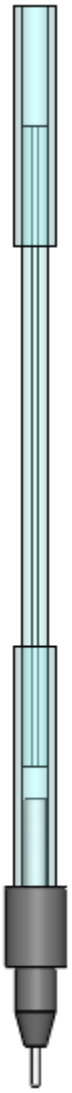


Figure 1. PERI-NOZZLE with Tubing Extension

Cleaning and storing the Perifusion Chambers (islets 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.

Preparing the chambers for the experiment

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)

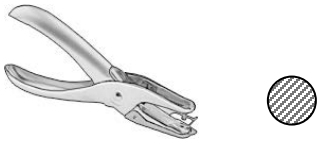
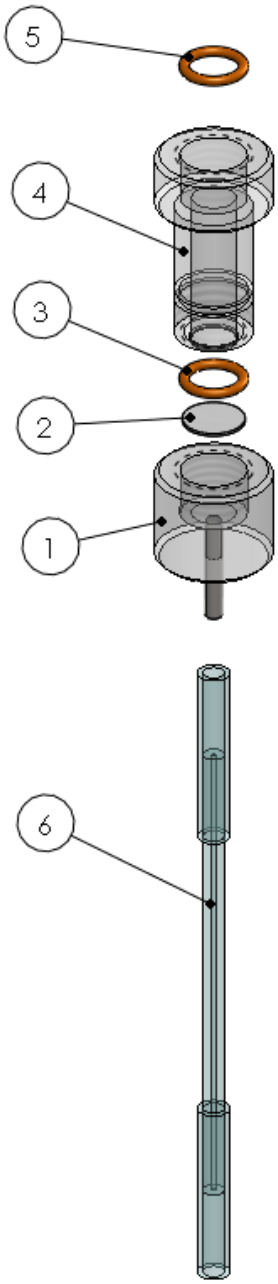


Figure 2. 1/4” Diameter Fiberglass Disk Filters



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).
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.

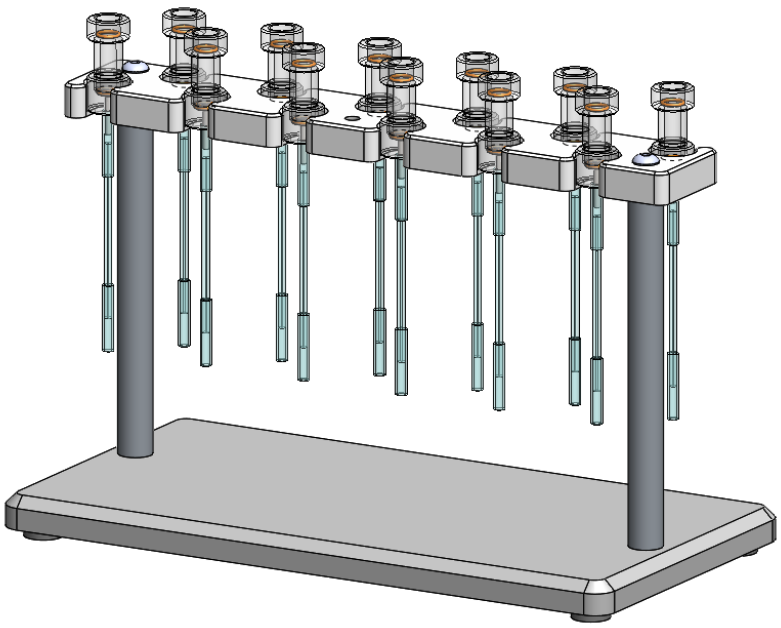


Figure 3. Cell Loading Rack

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

Setting up the instrument

Setting up the tubing

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.

1. Connecting the input tubing: connect one end of the silicone tubing (see Figure 4) to the top row of the manifold. Ensure that about 5mm of tubing is inserted over the fittings. The tubing is made of silicone, which is relatively easy to pierce. AVOID PIERCING THE TUBING BY ALL MEANS, AS THIS WILL BECOME A SOURCE OF BUBBLES AND MAY RUIN YOUR EXPERIMENT.

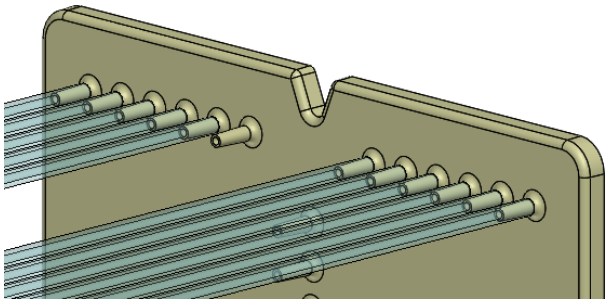


Figure 4. Input tubing connection

2. Attach a dispensing needle with luer-lock connection to the other end of the input tubing (see Figure 5). This ensures the end will sink to the bottom of the input 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 the later happens, air will reach the chamber with the islets and the experiment may fail. AVOID BY ALL MEANS THE ENTRANCE OF AIR INTO THE SYSTEM AS IT WILL EVENTUALLY REACH THE CHAMBERS CONTAINING THE ISLETS; AGAIN, BUBBLES ARE LIKELY YOUR MAJOR NIGHTMARE SO HEED ALL RECOMMENDATIONS TO MITIGATE THEM.

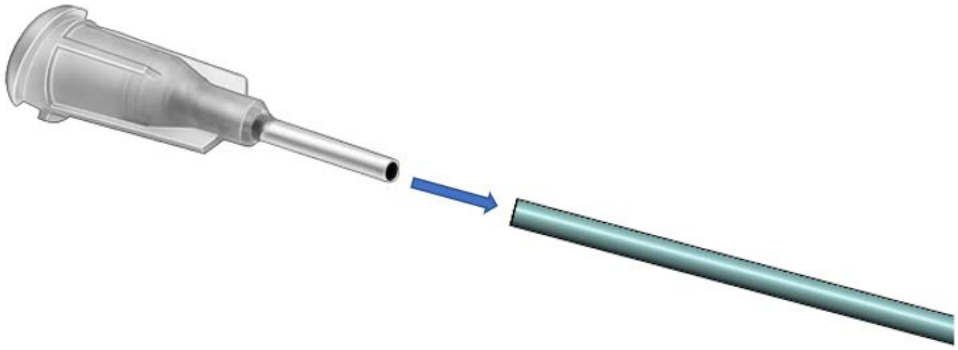


Figure 5. Insert dispensing needle into silicone tubing end

3. Repeat steps 1 and 2 for the remaining inputs.

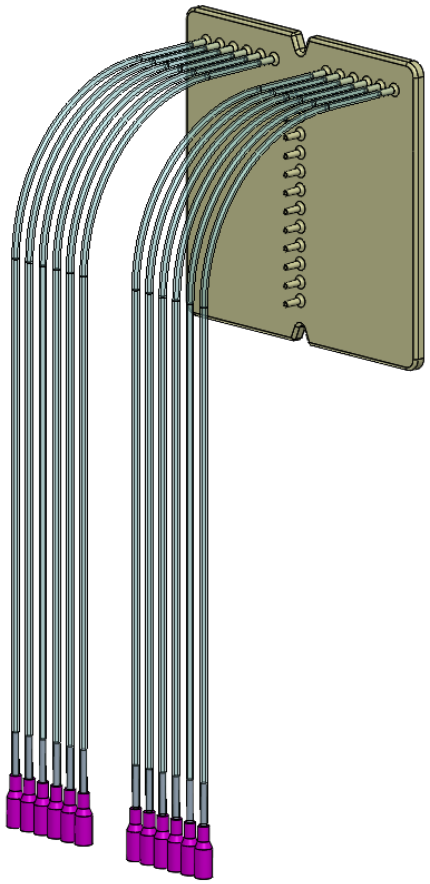


Figure 6. Input tubing connections

4. Connecting the pump tubing: connect the end terminated in a silicone tubing segment (see Figure 7) of the pump tubing to position 1 on the manifold. Ensure that about 5mm of tubing is inserted over the fitting. The tubing segment that connects to the manifold output is made of

silicone, which is relatively easy to pierce. AS STATED ABOVE, AVOID PIERCING THIS TUBING SEGMENT BY ALL MEANS AS THIS CAN BECOME A SOURCE OF BUBBLES.

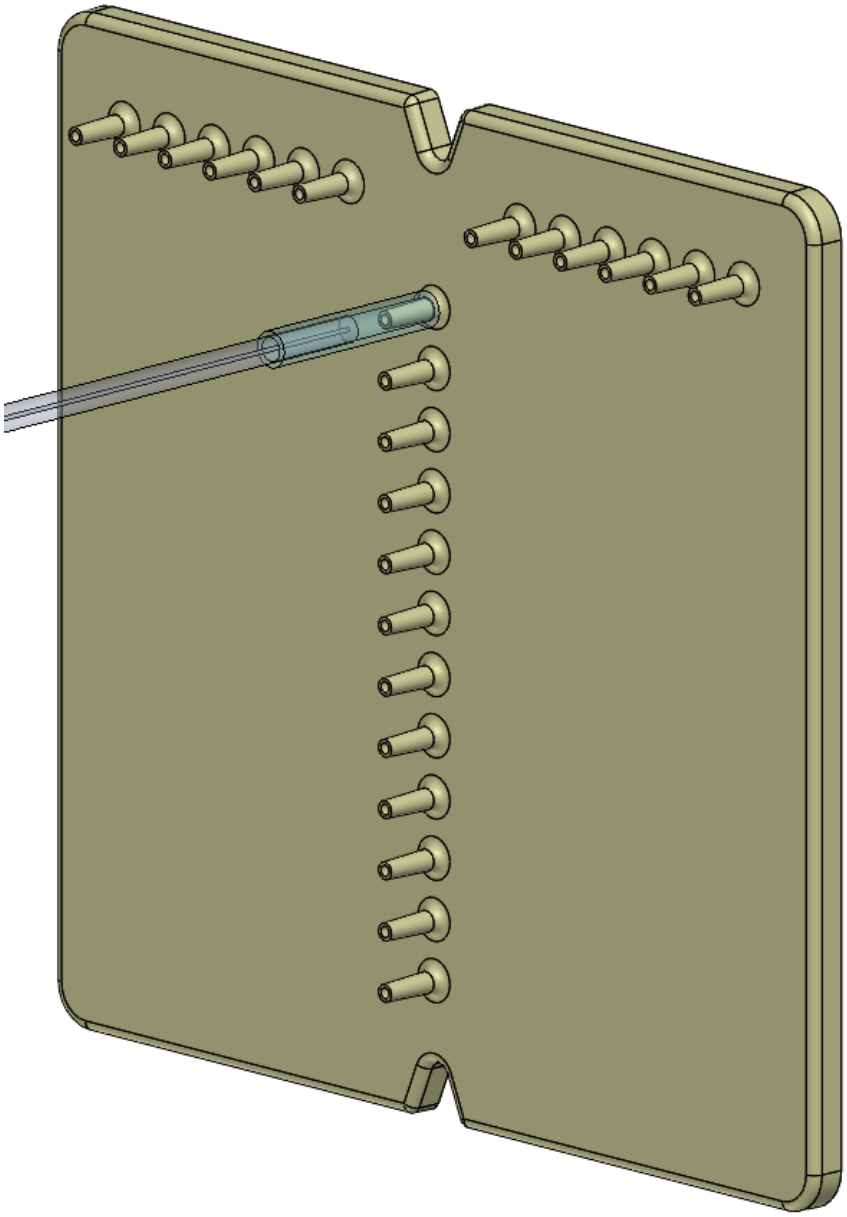


Figure 7. Pump tubing connection

5. Mount the tubing as indicated in Figure 8 to a pump cassette and clamp it to pump position # 1 (Pump position # 1 is the position closest to the body of the instrument).

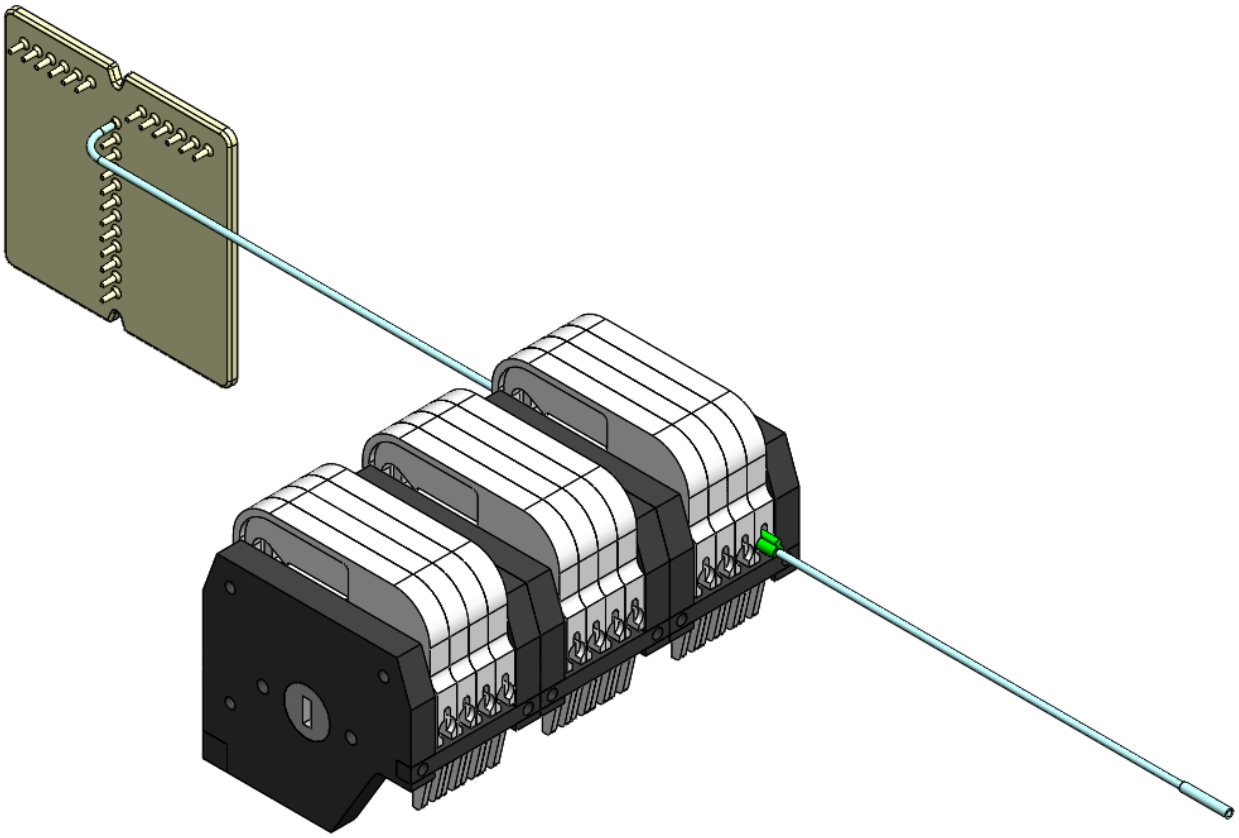


Figure 8. Microbore tubing mounted on pump position # 1 (Clamped Cassette)

6. Repeat steps 4. and 5. ONLY for the tubing (channels) intended to be used. Let the unused tubing sit inside the enclosure.
7. REMEMEBR, only install pump cassette right before turning pump ON. Do not leave pump cassettes with tubing in place for prolonged periods of time as tubing will deform permanently and affect flow rate. Leave pump cassettes inside enclosure to prevent misplacing them.

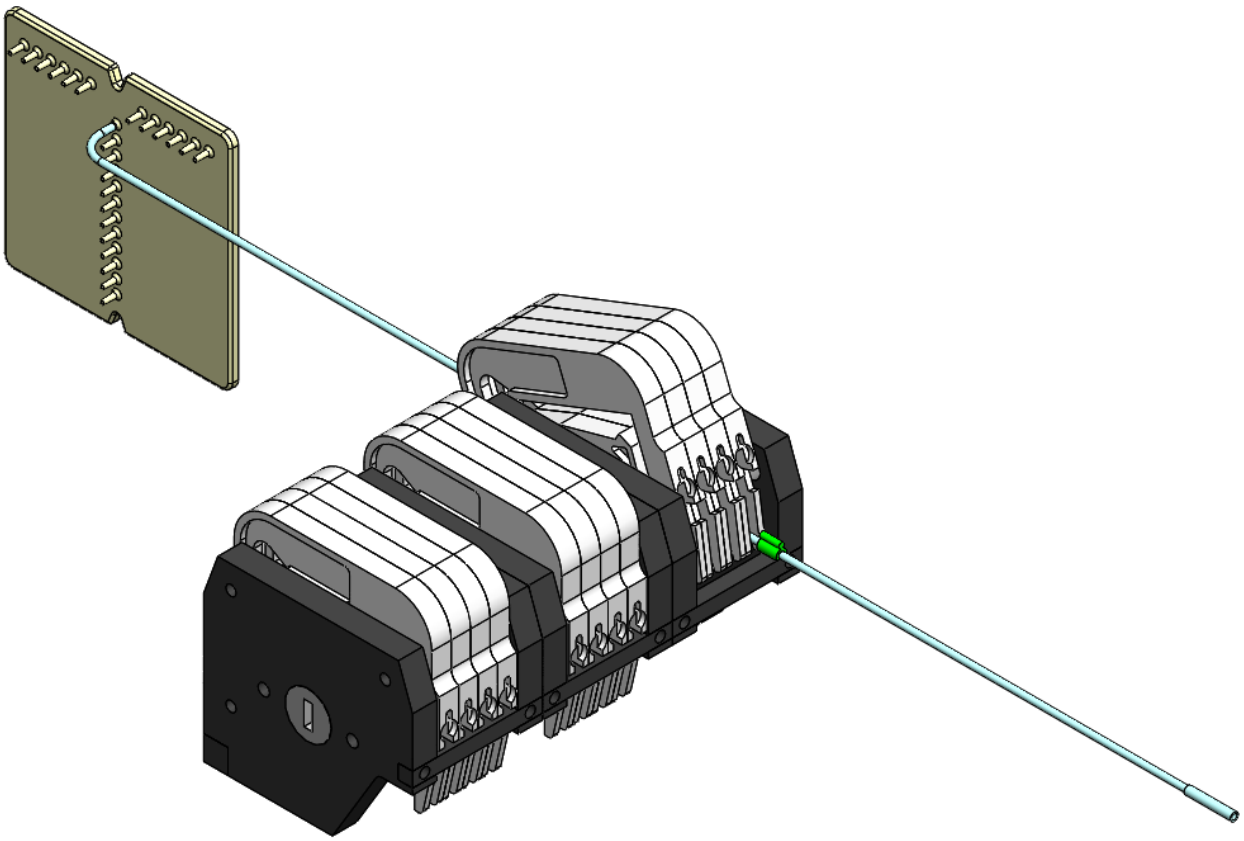


Figure 9. Microbore tubing mounted on pump position # 1 (Unclamped Cassette)

Entering a new protocol

As is the case for the other steps described above, it is time efficient to perform this step in advance of an experiment.

1. Click on PLAN PROTOCOL
    a. Under the SETUP section:
        i. Type in the number of chambers you will be using.
        ii. Enter the solution names
        iii. Select the 96-well plate style (2mL vs 0.5mL well volume)
        iv. Enter the number of rows to skip.
        v. Click on the floppy drive icon to save the protocol

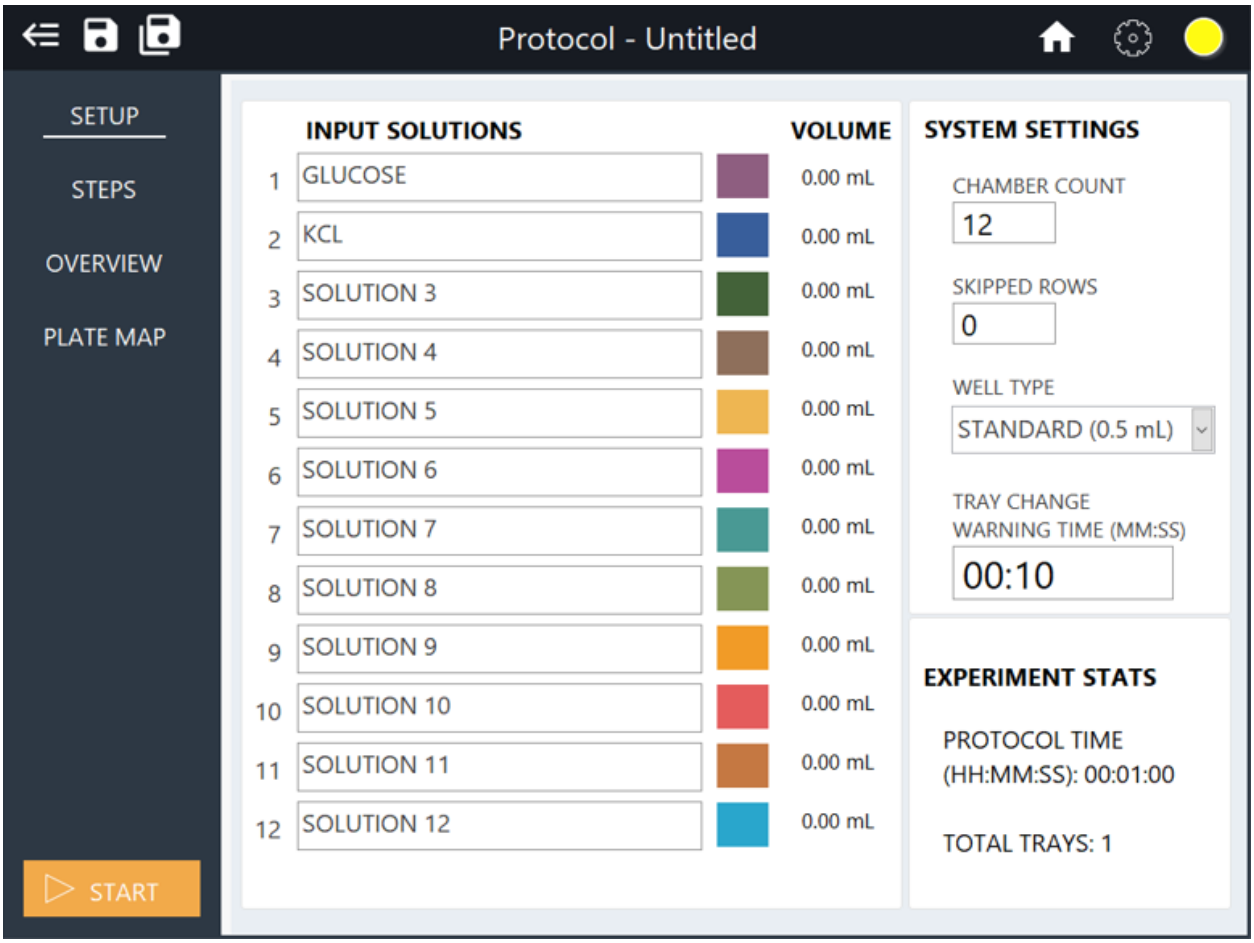


Figure 10. PROTOCOL – SETUP SCREEN

2. Click on STEPS
    a. Enter the following information for STEP 1
        i. # of Rows
        ii. Time per row (MM:SS)
        iii. Flow rate (uL/min
        iv. Stimulating solution from drop down menu
        v. Determine if the solutions will be mixed during step or not by clickin on the checkbox.
        vi. Select which chambers shall receive the selected solution (make sure the chamber selection matches the actual tubing setup).

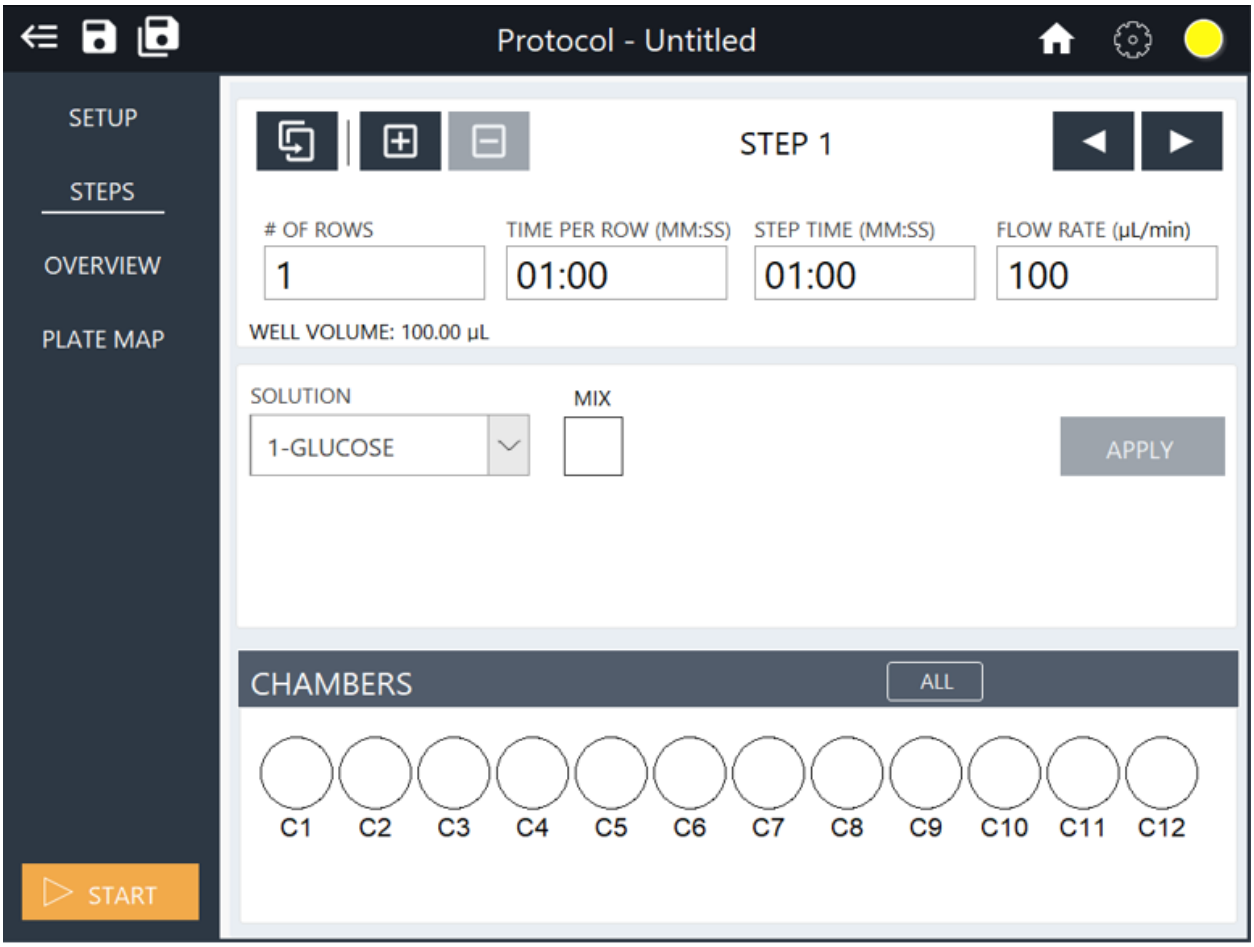


Figure 11. PROTOCOL – STEPS SCREEN

3. Click on OVERVIEW
    a. Check protocol in table format to ensure it matches experiment intent

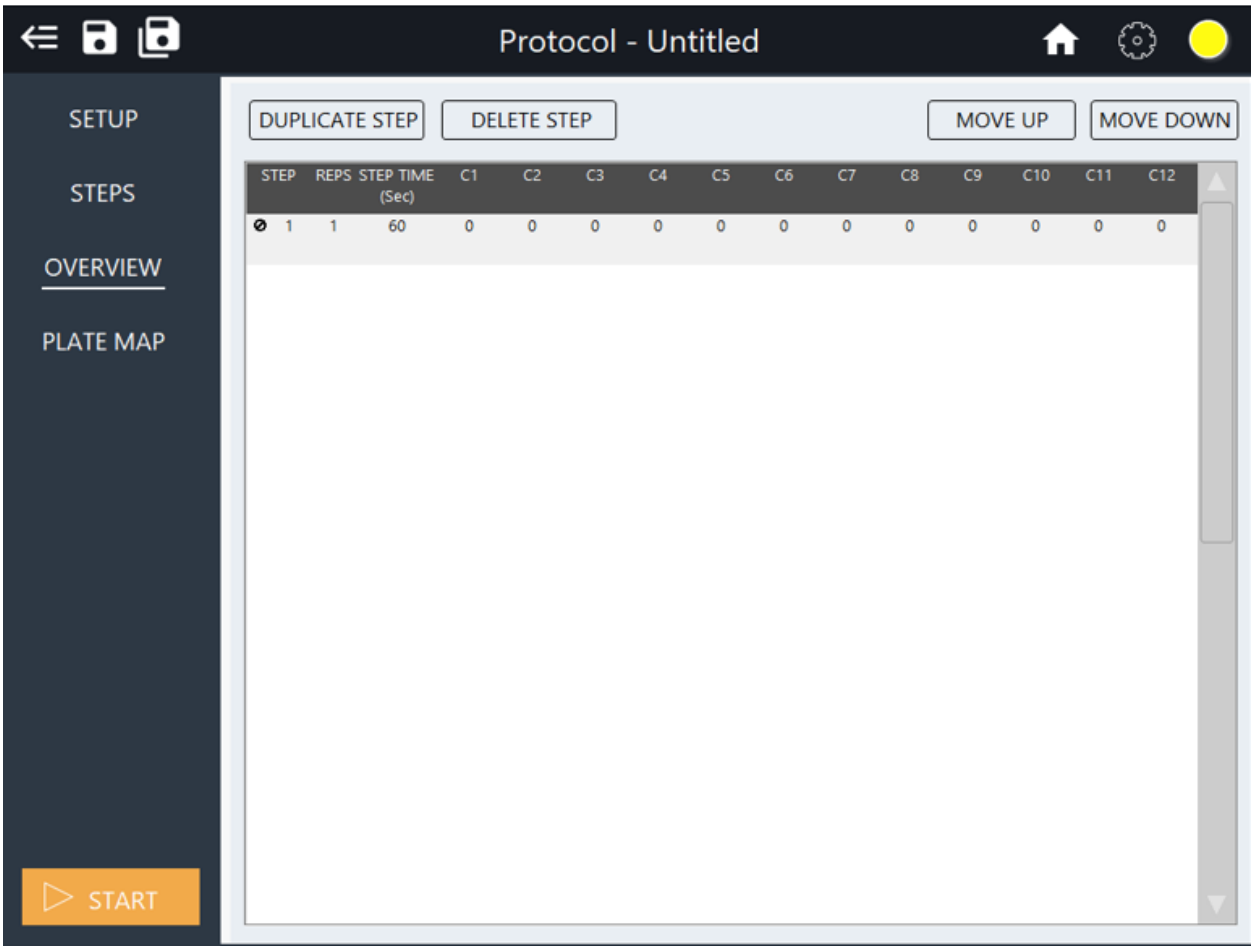


Figure 12. PROTOCOL – OVERVIEW SCREEN

4. Click on PLATE MAP
    a. Confirm the solutions are being dispensed in the correct wells.
    b. Scroll through all the 96-well plates by clicking the forward and back arrows

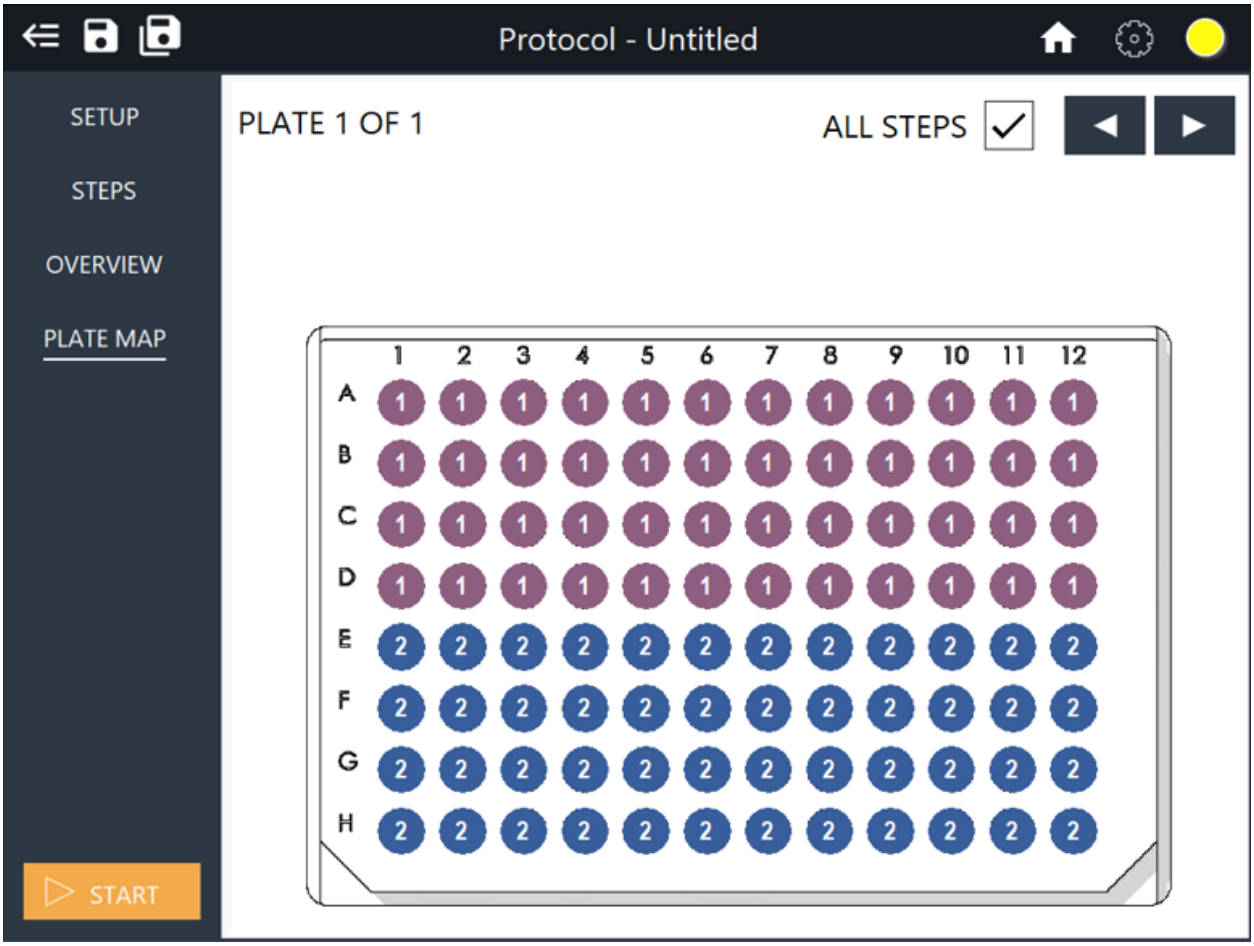


Figure 13. PROTOCOL – PLATE MAP SCREEN

5. Click on START to enter PROTOCOL EXECTUTION stage

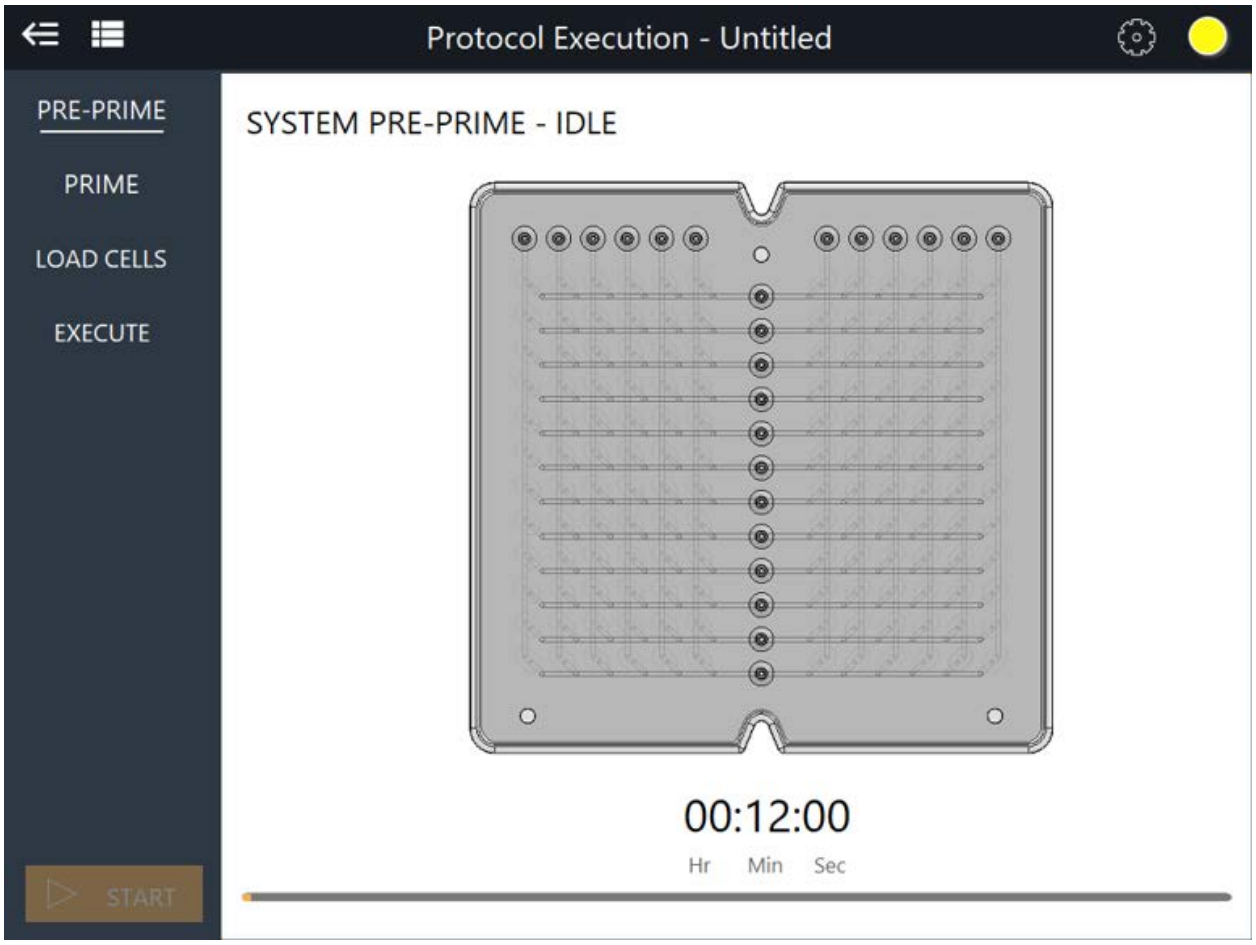


Figure 14. PROTOCOL EXECUTION – PRE-PRIME SCREEN

6. Click START to iniate PRE-PRIME sequence.
    a. Place all input tubing into buffer solution to prime all the channels.
7. Click on PRIME
    a. Place the tubing into corresponsing input solutions
    b. Click on START to initiate PRIMING sequence.

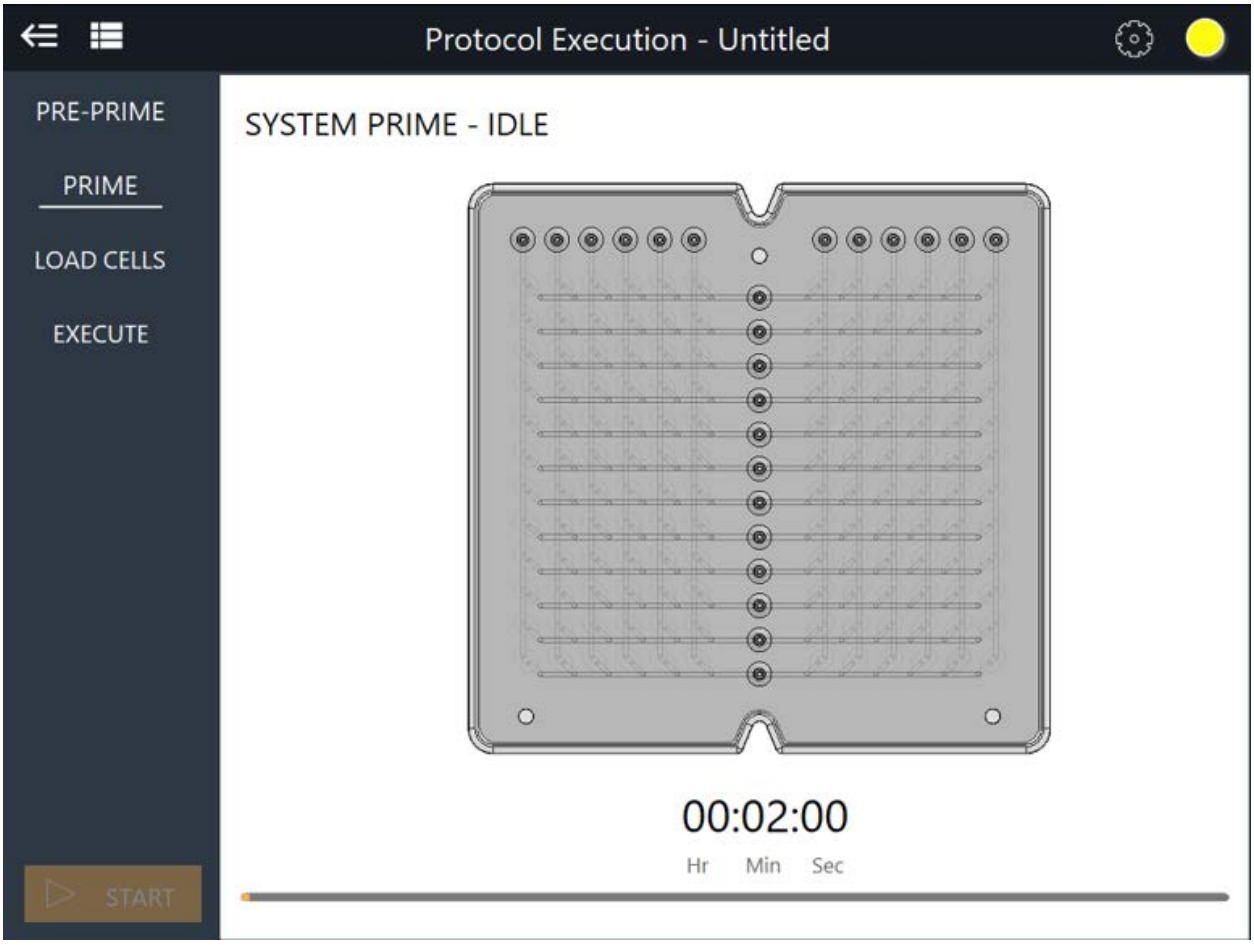


Figure 15. PROTOCOL EXECUTION –PRIME SCREEN

8. Click on LOAD CELLS

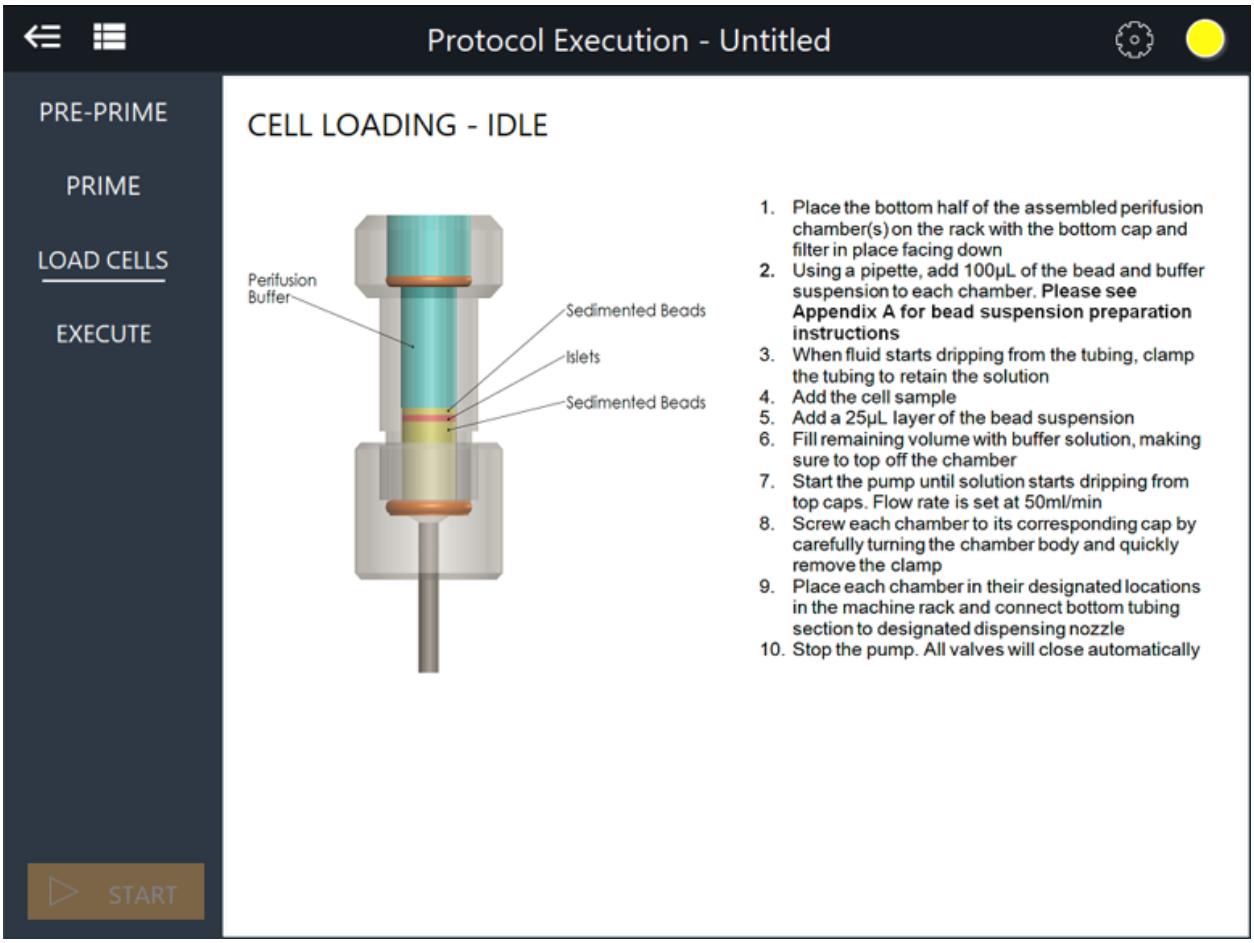


Figure 16. PROTOCOL EXECUTION – LOAD CELLS SCREEN

9. Click on EXECUTE
    a. Entire plate will be greyed out excep the actual row being dispensed into
    b. The solution # being dispensed will be shown inside each well.

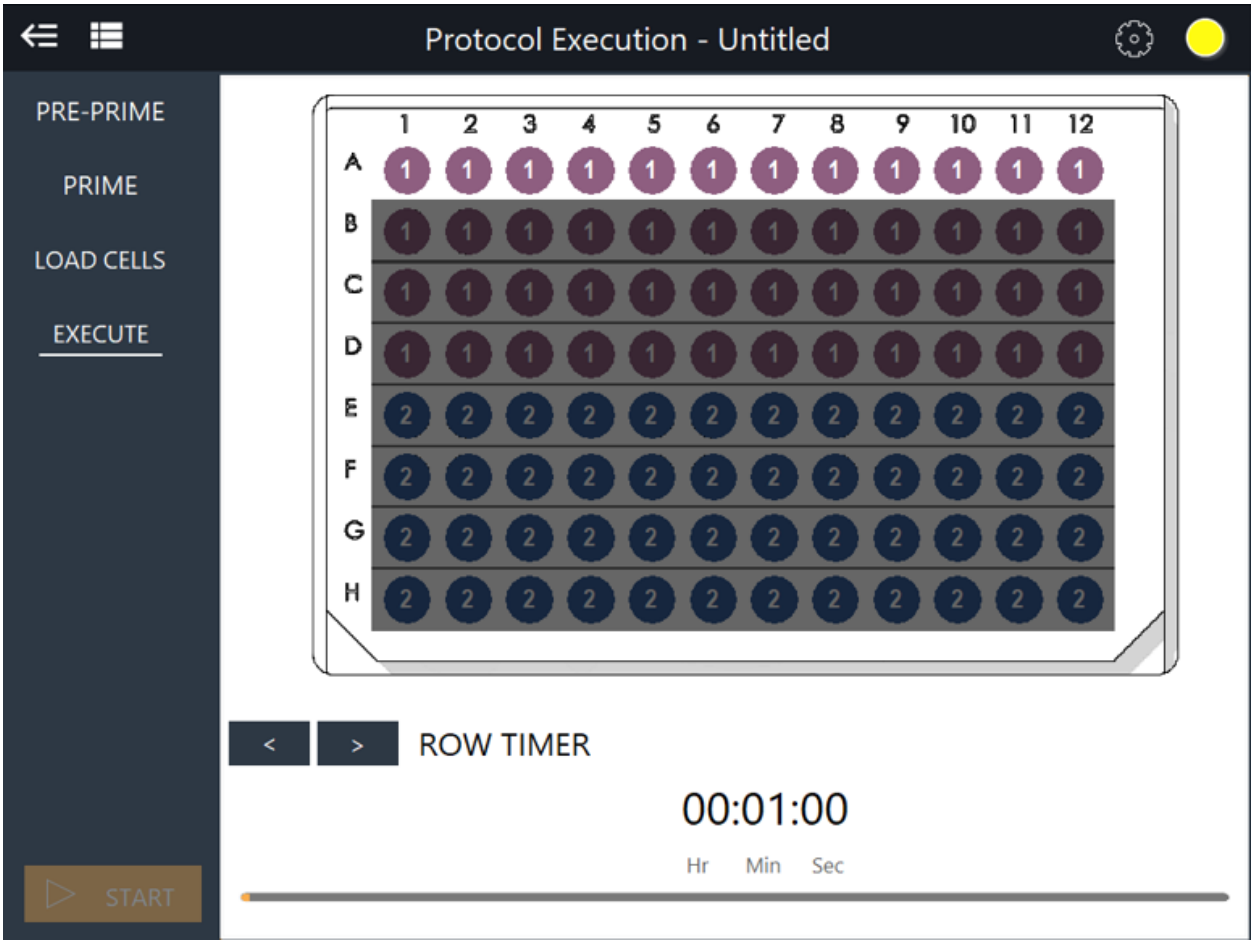


Figure 17. PROTOCOL EXECUTION – EXECUTE SCREEN

In the case where a gradient has been pre-programmed as part of the protocol, the simulated 96-well plate will show the wells split in half where the two solution #s and mixing ratio is displayed.

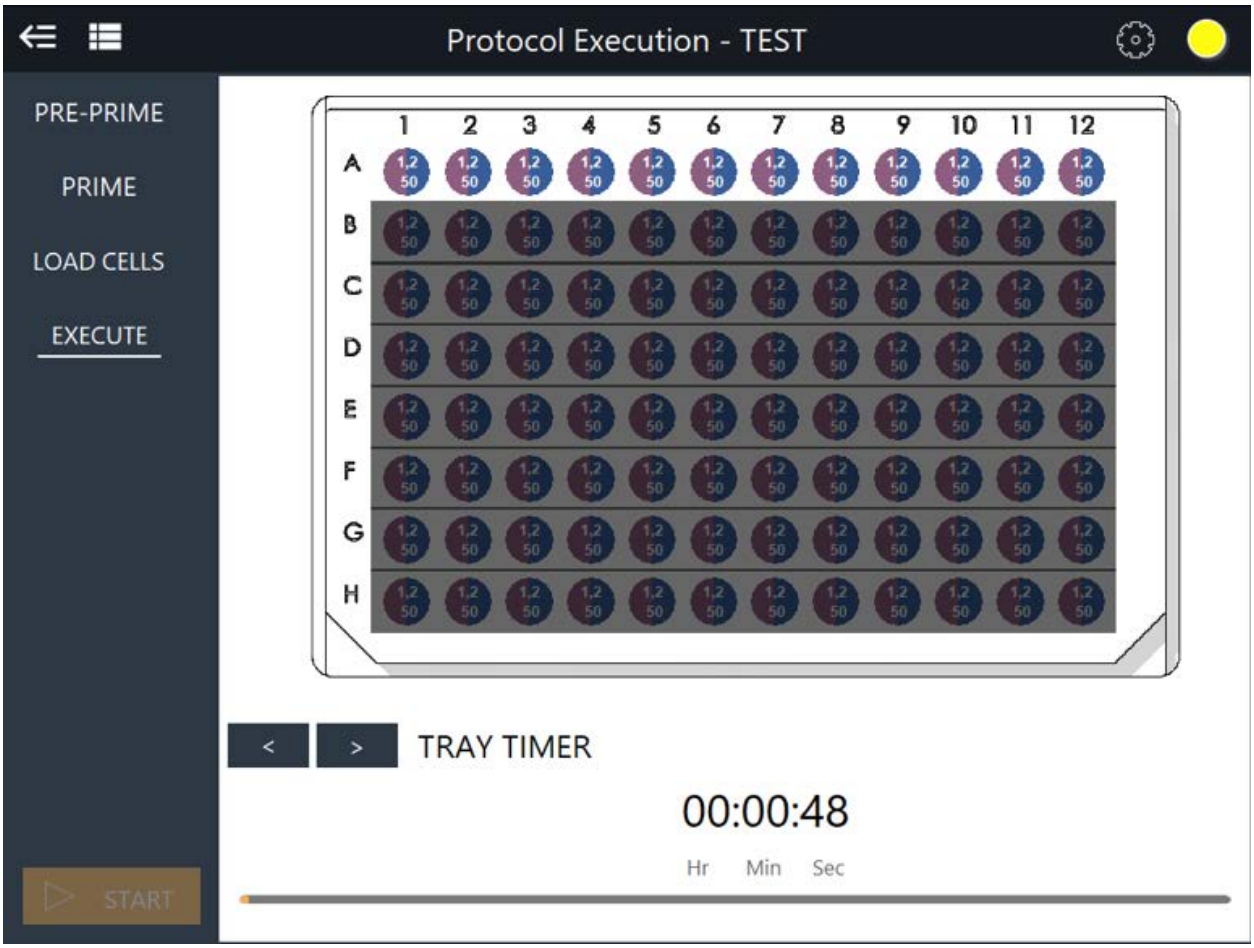


Figure 18. PROTOCOL EXECUTION – EXECUTE SCREEN - GRADIENTS

Sample protocols

Priming the system

1. Tubing must be properly setup with all input tubings in the buffer solution and all outputs dispensing through the top caps of the chambers into a waste tray beside the system.
2. Machine will automatically go into the priming mode once user hits the START button. You will be able to monitor the liquid flow through the microfluidic cartridge as the machine performs the priming.

Protocol to connect the chamber with beads and islets to the pump tubing

1. Start the pump until solution starts dripping from top caps. Flow rate is set at 50 ml/min
2. Screw each chamber to its corresponding cap by carefully turning the chamber body and quickly remove the clamp

3. Place each chamber in their designated locations in the machine rack and connect bottom tubing section to designated dispensing nozzle
4. Stop the pump. All valves will close automatically.

Running a protocol to collect the hormones released by the islets into the perfusate

Execute the protocol named “First Phase Insulin Release”

This a sample protocol that will perform well for rodent islets. The user needs to experiment for speficic conditions.

Solutions, prepared in PB with BSA and the adequate amount of glucose:

1. 2.8 mM of glucose (control)
2. 2.8 mM of glucose + drug concentration 1 (Test conditions 1)
3. 2.8 mM of glucose + drug concentration 2 (Test conditions 2)
4. 2.8 mM of glucose + drug concentration 3 (Test conditions 3)
5. 16.6 mM of glucose
6. 16.6 mM of glucose + drug concentration 1 (Test conditions 1)
7. 16.6 mM of glucose + drug concentration 2 (Test conditions 2)
8. 16.6 mM of glucose + drug concentration 3 (Test conditions 3)

Protocol:

Step	Reps	Time (sec)	Flow (µL/min)	Channel (A)	Channel (B)	Channel (C)	Channel (D)
1	21	165	100	1	1	1	1
2	7	120	100	1	1	1	1
3	7	90	100	1	2	3	4
4	14	30	100	5	6	7	8
5	14	120	100	5	6	7	8
6	7	150	100	1	1	1	1

Figure 19. PROTOCOL EXAMPLE

Association of channels (input tubing) with experimental test conditions

	Basal: 2.8 mM of glucose	Stimulation 2.8 mM of glucose	Stimulation 16.7 mM of glucose
Channel 1	Control	Control	Control
Channel 2			
Channel 3			
Channel 4	Control	Test conditions 1	Test conditions 1
Channel 5			
Channel 6			
Channel 7	Control	Test conditions 2	Test conditions 2
Channel 8			
Channel 9			

Channel 10	Control	Test conditions 3	Test conditions 3
Channel 11			
Channel 12			

Figure 20. ASSOCIATION OF INPUT WITH TEST CONDITIONS

Other sample protocols:

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

Figure 21. FIRST PHASE INSULIN RELEASE PROTOCOL EXAMPLE

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

Figure 22. FIRST AND SECOND PHASE INSULIN RELEASE PROTOCOL EXAMPLE

Loading the islets into the chambers

5. Place the assembled perifusion chamber(s) on the rack (see Figure 3).
6. Add 100µL of PB to each chamber to wet the fiberglass filter.
7. Add 100-150µL of premixed bead suspension to each chamber. The exact amount depend on how much PB was added to the dry beads. Do not add more than ¼ chamber of sedimented beads (Figure 23). It is important to have a column of PB in the chamber to trap any bubble floating (Figure 23). If necessary, remove any excess of beads with a pipette. If there is not PB on

top of the sedimented beads, adding more PB to the chamber will facilitate the removal of beads in excess.

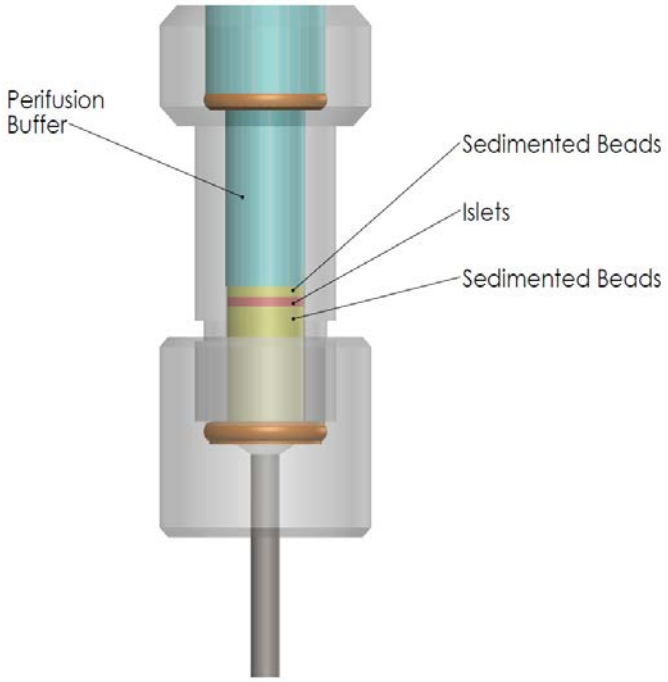


Figure 23. CELL LOADING

8. When the beads sediment, clamp the silicone tubing with a paper clip to avoid the beads drying.
9. 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.
10. Add a couple drops (with a micropipette and 100 µL 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.
11. Fill the chamber with perifusion buffer; make sure to top off the chamber.
12. Start the pump until the perifusion buffer starts dripping from the top caps; the idea is to screw the cap to the body of the chamber trapping as little air as possible. REMEMEBER, AIR BUBBLES ARE THE ENEMY; HOWEVER, NO NEED TO PANIC. AS LONG AS THE BUBBLES DO NOT REACH THE ISLETS INSIDE THE CHAMBER EVERYTHING WILL BE FINE. You can set the flow rate to 50µL/min to prevent wasting to much perifusion buffer.
13. Remove the paper clip clamp and screw the chamber to its corresponding cap by carefully turning the chamber body.
14. Connect the bottom outlet of the chamber to the tubing already attached to the proper nozzle (see Figure 1) and place the chamber in the designated locations in the rack inside the enclosure.
15. Repeat the process for every chamber.
16. Ensure that perifusion buffer is dripping from every nozzle. If 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 THEREFORE A CAUSE OF SPURIOUS RESULTS.

Recovering the islets from the chambers

1. Disconnect the tubing attached to the bottom cap of the chamber.
2. Remove the top cap (see Figure 24).
3. Close the top of the chamber with the tip of your index finger.
4. Remove the bottom cap and introduce the body of the chamber into a micro-centrifuge tube (see Figure 24). If the fiberglass filter remains attached to the body of the chamber, remove it with a pair of forceps.
5. Add 1 ml of Hank's balanced salt solution (HBSS with Ca^{2+} and Mg^{2+}) from the top to flush down beads and islets.
6. Remove the body of the chamber from the micro-centrifuge tube.
7. Close the lid of the micro-centrifuge tube.
8. Centrifuge at 300g for 5 minutes.
9. 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.

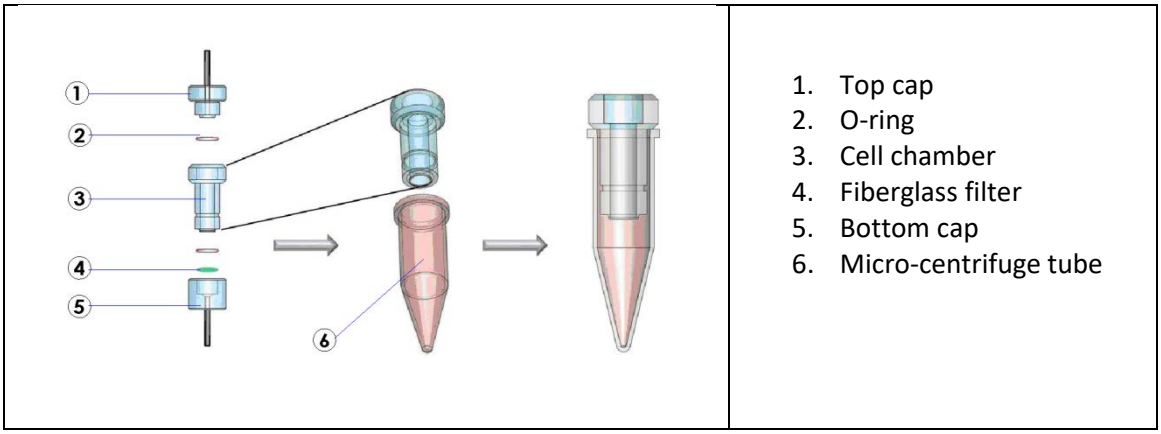


Figure 24. Recovering Islets from Perifusion Chamber

Normalizing the insulin output by the 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.

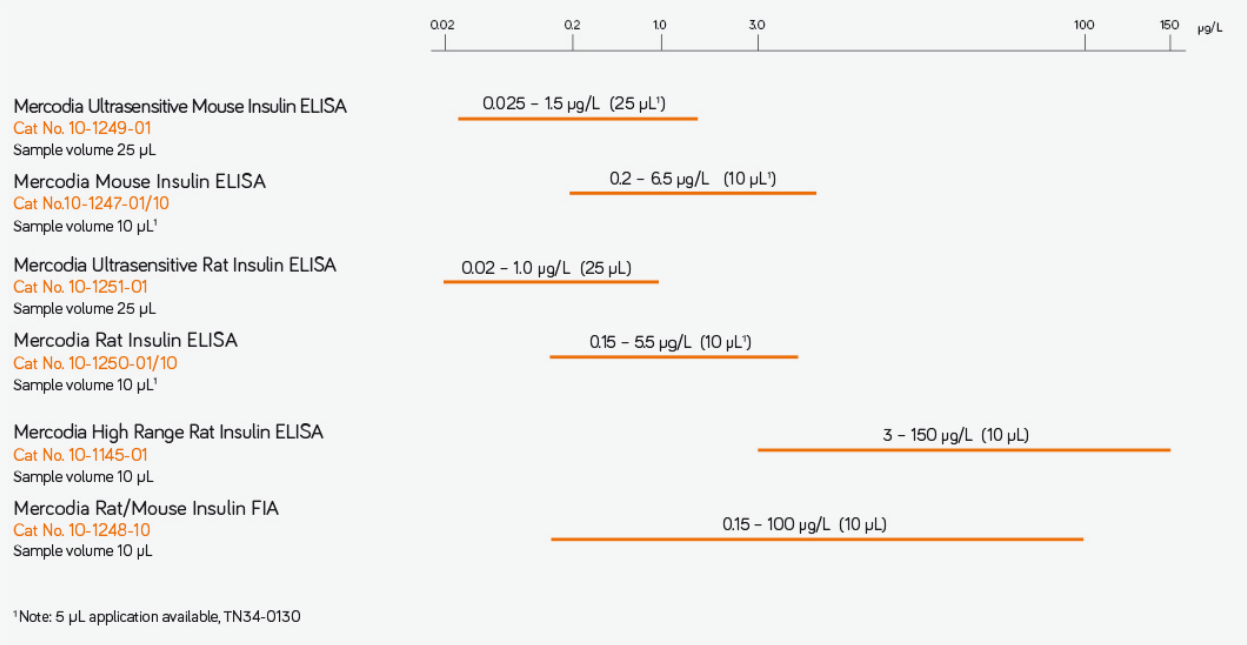
3. Quantify the DNA using the NanoDrop (Thermo Scientific) or your method of choice.

Measuring the hormones released by the islets into the perfusate

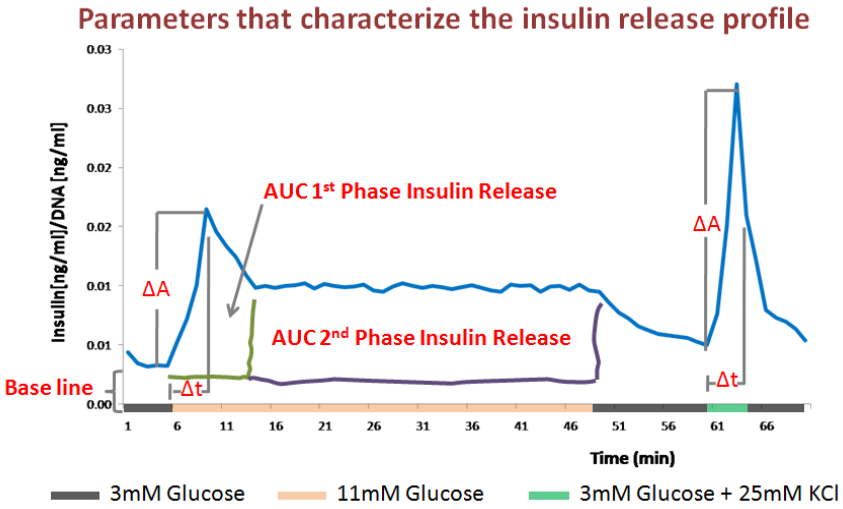
There are many suitable assays in the market, but the following two assays have been tested by Perifusion users with good results.

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 standard, 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/)



What can be calculated from the insulin release profile



AUC= Area Under Curve