UC Davis MMPC-Live Protocol

Ex vivo assessment of barrier function /gut permeability

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Replaces version: none
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Summary:

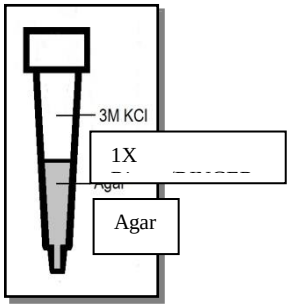
Gut tissue (by region; *e.g.* ileum & colon) will be opened along the mesenteric border and mounted in Ussing chambers (Physiologic Instruments, San Diego, CA, USA), exposing 0.1 cm2 of tissue surface area to 2.5ml of oxygenated Krebs-glucose (10mM) and Krebs-mannitol (10mM) at 37°C on the serosal and luminal sides, respectively. The paracellular pathway and transcellular pathway will be measured as the flux of FITC conjugated to 4 kDa Dextran (FD-4, Sigma) and horseradish peroxidase (HRP Type VI, Sigma; 44 kDa), respectively. FD-4 (400µg/ml) and HRP (200µg/ml) will be added to the mucosal chamber and samples will be collected from the serosal chamber every 30 min for 2 hours. Concentration of FD-4 is measured via fluorescence at excitation 485 nm, emission 538 nm. O-dianisidine substrate is used to detect HRP at absorbance 450 nm.

Reagents and Materials:

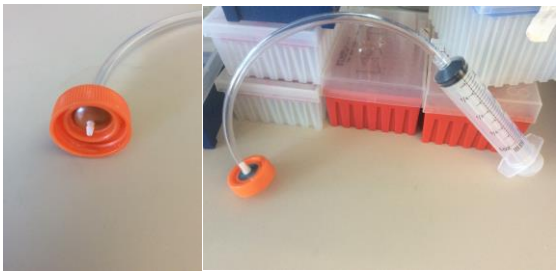
Reagent/Material	*Vendor*	*Stock Number*	
Ultrapure Agarose	Invitrogen	16-500	
Glucose	Sigma Aldrich	G5767-500G	
Ringer's or Krebs-Ringer-Bicarbonate solutions			
95% Oxygen/5% CO2 tank			
Mannitol	Sigma Aldrich	M9647-500G	
DMEM low glucose	Invitrogen	11885-084	
FITC-dextran 4000 (FD4)	Sigma Aldrich	FD4-1G	
Horseradish Peroxidase (HRP), type IV	Sigma Aldrich	P8375-25KU	
O-Dianisidine dihydrochloride	Sigma Aldrich	D9154-50TAB	
Corning™ 96-Well Solid Black Polystyrene Microplates (Costar 3915)	Fisher Scientific	07-200-590	
MaxiSorp (442404) ELISA assay plates	Fisher Scientific	12-565-136	
Ussing chamber system		Physiological Instruments	

Protocol:

Tip Preparation



1. Make 3% solutionose of agar in Ringers or RINGERS solution*. Place 0.75g agarose and 25 ml Ringers Solution into 50 ml conical. Use orange conical lid attached to 30ml syringe for degassing agar. Fill a 500ml beaker halfway with dH2O and place conical inside. Heat on hot plate.
2. When the solution thickens, use syringe to suck out air bubbles. You should see bubbles coming up on the sides of the conical but the center should look clear
3. Stick 5ml syringe (without needle) straight down into the agar and suck it up.
4. Back fill tip by inserting tip into syringe and pushing agar into the tip until the agar fills the lower quarter
5. Immediately use syringe with filling needle to backfill tip with Ringers.
6. Store in tip box filled with Ringers Solution in the refrigerator for up to one month.



Electrode Assembly

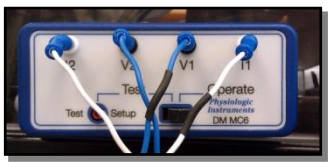
1. (Optional) Apply thin film of silicone vacuum grease (stopcock grease) to the black rubber seal on the threaded shaft of the electrode.
2. Remove backfilled tip from the container and screw onto the electrode. To avoid drying, electrodes + tips should always be kept in Ringers solution.

Collecting Tissue

1. Collect intestinal tissue and remove the intestinal content. Do not scrape too much.
2. Immediately place it in DMEM with low glucose Medium.
3. Open up tissue, wash in Ringers.

Setting up and Calibrating the chambers

1. Prewarm the RINGERS solution in the 37°C waterbath.
2. Turn on the 47°C circulating water bath and allow it to circulate for 20-30 minutes before starting the calibration. Power button on the back, press the blue power button in the front, and the arrow button to start water circulation. Water bath is filled with dH_2O



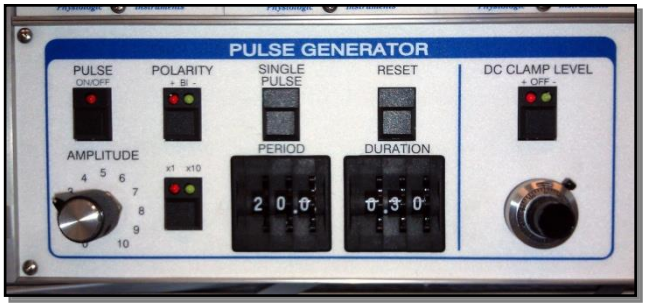
3. Turn on clamp apparatus. Turn on the computer. Make sure each electrode set is on “operate” if there is a sample for that set and on “test” if there are no samples.

4. Check **Clamp** Module. For each set, check:

   METER: Green light on "**V**"
   FUNCTION: All lights **OFF**
   MODE: Green light on "**V**"
   OFFSET: All lights **OFF**
   KNOBS: Both knobs turned to **zero** (all the way to the left).

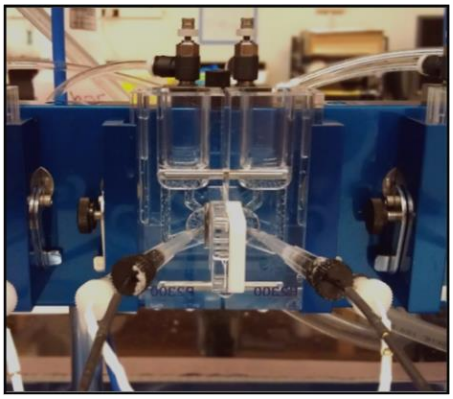
5. Check **Pulse Generator** Module.

   PULSE: All lights are **OFF**
   POLARITY: All lights **ON**
   AMPLITUDE: Set on "**3**" and red light on **x1**
   PERIOD: Set to **20.0** seconds
   DURATION: Set to **0.30** seconds
   DC CLAMP LEVEL: All lights **OFF**
   KNOBS: Turned to **zero**



6. Check **Master Control** Module. All lights should be **OFF**.

7. Mount the chamber, sliders, and electrodes.



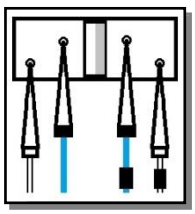
   CHAMBER: Connect two pieces together.

   SLIDERS: White side on the left, transparent side on the right. Pull silver clamp located on both sides down and turn black knob to tighten chambers and sliders into place.
   - Sliders used for rat intestines are 0.5cm^2
   - Sliders used for mice intestines are 0.3 or 0.1cm^2

   ELECTRODES:
   Test each set of 4 electrodes in RINGERS's solution.

   a) To test black electrodes: Press **OPEN** function. Values should be between -3 and +3. If not, change tip. If that doesn't work, change electrode.

   b) To test white electrodes: **METER** light on red for "**I**". Press black button (push to Adj) and values should be around 51-54 for tips made with Ringers. If not, change tip. If that doesn't work, change electrode.



      **** Do not press the black button too long, it can damage the electrode. ****

   c) Set up electrodes in the chamber.

   d) Add Ringers solution to the chamber. Pull each electrode slightly back out to eliminate bubbles.

   e) Change METER light back to green for "**V**"

   f) To calibrate black electrodes: Press offset and adjust knob A to reflect "0"
      If original value was negative, leave offset on "**+**" before adjusting knob.

If original value was positive, leave offset on "-" before adjusting knob.

g) To test white electrodes: Press black button and turn knob B to reflect "0"

h) **Calibration should be done with warmed medium as temperature will affect the voltage sensing electrodes. Touch them minimally once calibrated.**

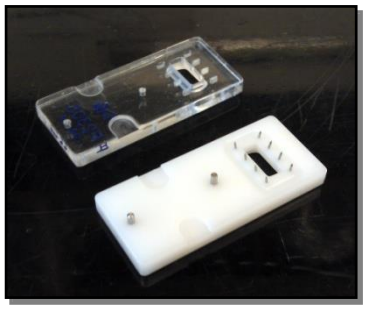
8. Prepare Glucose and Mannitol solutions the day of the experiment or prepare ahead of time and store at 4°C for no more than a week. Warm in the 37°C water bath and keep warm during experiment set up.

9. Recalibrate electrodes and adjust offset.
10. Remove RINGERS's solution from the chamber with a vacuum syringe.

11. Be certain the entire back of the chamber is completely dry. If a leak occurs at any point, dry the chamber.

12. Open silver clamp and turn black knob to release sliders from the chamber. Mount tissue on the slider and set up slider in the chamber. To mount tissue:

    a. Loosen the pressure in the chambers by turning the black knob on each side of the chamber. Then remove the slider.

    b. Dry the inside of the chamber with a Kimwipe and tweezers

    c. Cut the intestine from the mesenteric border to open it up. The intestinal samples are usually between 1-2cm. (Be careful not to use intestinal parts with Peyer patches, as these areas have greater permeability.)

    d. Pinch the tissue carefully into the white part of the slider with the mucosal side facing up. Use the 8 pins as anchorage. Close it with the transparent side (as in a sandwich).

    e. Mount tissue with mucosal/luminal side facing the left and serosa on the right

mucosa	serosa

    f. Once the tissue is in the slider, mount it in the Ussing chamber. Tighten it by turning the knob on each side of the chamber.

13. Turn on 95% O_2 / 5% CO_2 gas tank. On the tank, top knob on the tank to open up the tank. Silver knob on left to open up the flow to the chamber. **Do not touch the black knob.** Adjust bubbles per chamber (last chamber will not be easy to slow down, keep bubbles coming, put lid on chamber if needed)
    a. Gas should be passed through beaker filled with H2O.

14. At the same time, slowly add 2.5ml of Ringers+10mM glucose to the chamber that will be in contact with the serosal side (right) and 2.5ml of Ringers+10mM mannitol to the chamber that will be in contact with the mucosal side (left). Check for bubbles. As needed, use a syringe to aspirate out the bubbles.

mannitol	glucose

    a. When the tissue is mounted in the chamber, there should be a negative current reading

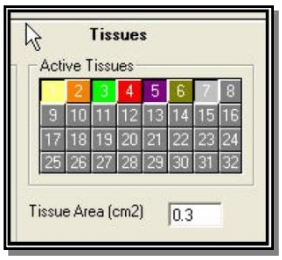
15. On the computer's desktop, click on the "Acquire and Analyze" software. If no error message pops up, then it's a good connection. Otherwise, do the following:
    a. Restart computer. As Windows tries to restart, press F2.
    b. Select "onboard devices"
    c. Select "LPT Port Mode"
    d. Scroll down to hardware and make sure the connection is on "EPP"
    e. Exit setup menu and continue with restarting Windows. Open up the software and the error message should disappear. If not, try restarting a few times. If that doesn't work, call technical services.

Data Acquisition

1. On the **File** menu, select **New Experiment**

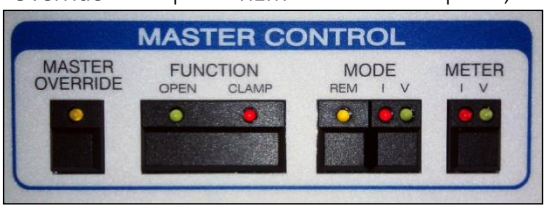
2. Enter a new filename in the **Save Experiment** dialog box and select the destination directory for your data. Then press **Save**.

3. Next the Experiment Properties dialog box will appear. Find the tab labeled **Tissues**, and under **Active Tissues**, select the tissues from which you will be collecting data. To select tissues, left-click on the colored square representing each tissue. If the square looks depressed that means the tissue is selected (i.e., will acquire data on this channel), and if it looks like it is in the up button position, it is not selected. In the example figure to the right, tissues 1, 3, 5, and 7 are selected to collect data.



    a. Plug in tissue area according to what you are using
4. Now we need to take a zero reference reading to calibrate the measurement. Select **Acquire > Reference** from the main menu.

5. Under Master Control in the clamp apparatus, press **Master Override** and press **REM**. On the computer, click on "**reference**". The table should be populated with data. All values should be less than 0.1. If any of the boxes show up with a red or yellow color, that is a warning that the reference is out of range, and that you should check your clamp setup and run the Reference again. Yellow is ok. Red is not good but you can recalibrate and add new tissue and take new reference.

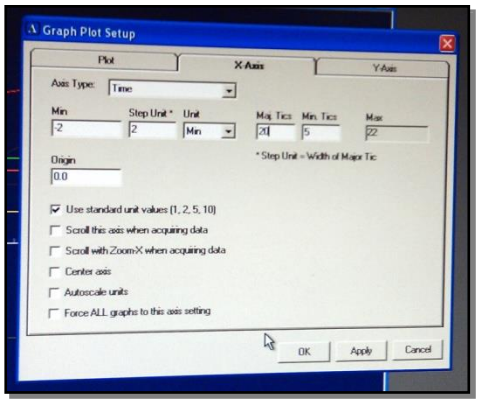


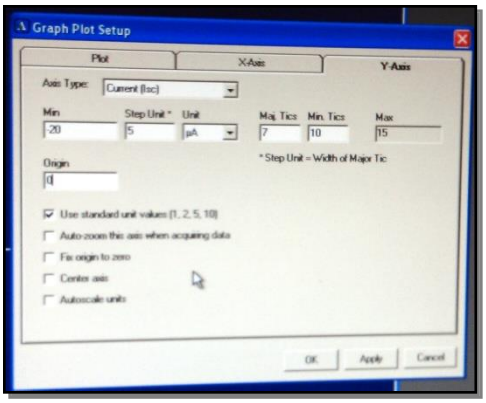
6. Press the  "running man" icon on the toolbar to start data acquisition. .

7. Individual chambers can be selected by selecting "remote" on desired chambers.

8. Pulse speed can be set by selecting the red-slow, yellow-medium, or green-fast stop sign

9. Double-click on the graph to adjust axis settings on the Current graph.





X axis: Maj ticks = 60

Y axis: Maj ticks = 20 Min=80

10. Create a new graph for Conductance
    a. Y axis: change axis type to conductance. Mag ticks= 20, orgin =0
    b. X axis: Same as other graph

11. Let the program acquire data for at least 15 minutes before proceeding. (Ideally 30 min)

Sampling

1. After 25min of acquiring data, record base line Isc and G.

2. At time 0, collect 2x 100 µl from the serosal side and place in black plate or a 0.5 ml tube. (Store at 4°C). Replace with 200 ul Ringers+glucose

3. Remove 150 µl from the mucosal side and add sample of choice.

mucosa	serosa

ie: **FITC-dextran 4000 (FD4)** → 100 µl to mucosal side [small: goes through tight junction, paracellular]
Stock solution: 10 mg/ml
TdB Consultancy AB 1g
Horseradish Peroxidase (HRP) (SIGMA) → 50 µl to mucosal side [big: goes through transcellular]
Stock solution: 10 mg/ml

4. Collect 2x 100 µl from serosal side at desired time point (15, 30 or 60 min) and place in black plate or 0.5 ml tube. (Store at 4°C)

5. After 2 hours, Click on  "stop" icon on the toolbar to stop data acquisition.

6. Turn off chamber, computer, and water bath.

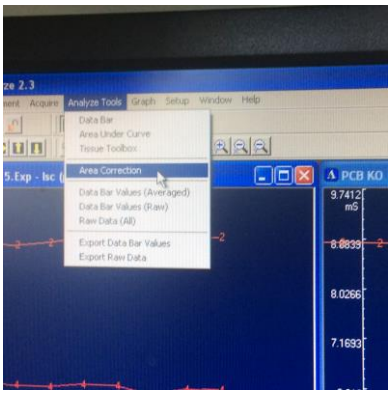
Taking tissues at different times

1. Selecting the tissues you are ready to use on the Experiment/Properties screen. Once you select the tissues on which to take data you will likely be required to take a reference. Do it. Start recording data.

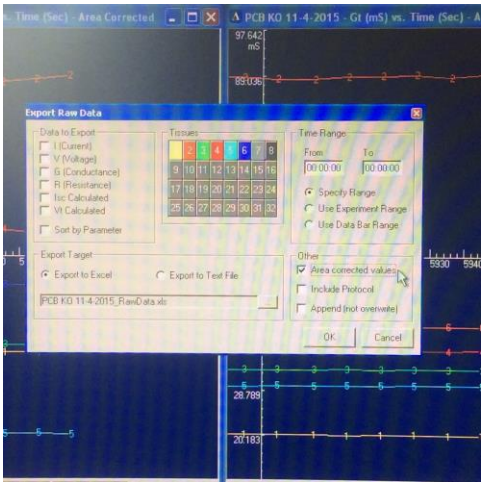
2. Mount your next set of tissues and when ready, Stop the data acquisition and click on Experiment/Properties. Add the new tissues to the selection and press Save. You will have to Reference the new tissues (actually it will reference all of them again) and then start taking data again. This can all be done in a matter of about 10 seconds if you're organized.
3. Continue this until all tissues are mounted.

Data collection

1. Values should be "area corrected" for real values.



2. To export data to excel: Under Analyze Tools, select "Export Raw Data." Select parameters under "data to export." "Use experimental range" or "specify a range" for the test range. Select "export to excel." Be sure that "area corrected values" is selected.



Testing Tissue Viability

Adding 10 uM forskolin to the chamber, which raises levels of cyclic AMP with resultant active net ion transport in viable tissue (as assessed by an increase in Isc). Add to both sides. Isc should increase and G should decrease
Discard nonresponsive tissues.

Shut Down and Clean up

1. Turn off O_2. Aspirate media from chambers with a vacuum syringe.

2. Pull out O_2 tubes then electrodes.

3. Loosen clamp and knobs for chambers. Disassemble and let sliders and chambers sit in soap (conrad) and water for a few minutes.

4. Clean chamber apparatus with soap and water to wash off buffer residue.

5. Wash chamber and sliders with soap and water. Final rinse with distilled water.

6. Rinse bottom of tips (part exposed to chamber) briefly in water.

7. IF setting up for the next day: shake out water in chambers, dry outsides being certain the backs are completely dry, add insert (outside also dry), and set up on apparatus. Attach electrodes and fill with ringers solution.

8. IF done for the week: let everything air dry. Detach tips from electrodes and return to box filled with ringers. Care for electrodes is as following:

TO CLEAN TIPS:

TIPS: Soak old tips in warm water. Use syringe filled with water to push out old agar and then put tips into clean H2O. Push water through tips. Push air through dips. Dry tips in incubator. Tips must be dry before adding new agar.

Electrode Maintance

1. Current driving (white) electrodes:
    a. Use 600 grit wet/dry sand paper to remove buildup. Electrode should become silver and shiny
    b. soak in bleach until it turns black. (centrifuge tube works well)
    c. rinse with water
2. Voltage measuring (black) electrodes:
    a. Rinse with water
    b. Gently rub sensing end on paper towel. Should see small black spot appear on towel.

FD4 & HRP Assays

Always do FD4 assay first. Complete both assays within 2-3 days of experiment.

FD4 Assay

Prepare standards for standard curve (Can make up these solutions and use them for up to 2 months):

Standard	*Diluent*	*Standard #*	*[FD4]*
50 µl of FD4 (10mg/ml)	450 µl of ringers	A	1 mg/ml
50 µl of A	450 µl of ringers	B	0.1 mg/ml
60 µl of B	540 µl of ringers	1	10000 ng/ml
150 µl of #1	450 µl of ringers	2	2500 ng/ml
150 µl of #2	450 µl of ringers	3	625 ng/ml
150 µl of #3	450 µl of ringers	4	156.3 ng/ml
150 µl of #4	450 µl of ringers	5	39.1 ng/ml
150 µl of #5	450 µl of ringers	6	9.8 ng/ml
150 µl of #6	450 µl of ringers	7	2.4 ng/ml
--	450 µl of ringers	Blank	0 ng/ml

** Use black 96 well plate **

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Blank										
B	7	7										
C	6	6										
D	5	5										
E	4	4										
F	3	3										
G	2	2										
H	1	1										

SAMPLES IN DUPLICATE

1. Add 100 µl of each standard to indicated wells.

2. Read fluorescence Excitation: 485 nm
   Emission: 538 nm

3. Plot the standards against the known concentration and fit a 5-parameter logistic curve (5PLC) and use this to interpolate the concentration of FD4 in your samples.
4.

HRP Assay

1. Mix up working buffer (25 ml for 1 plate/50 ml for 2 plates, *etc.*; 20 ml is actually needed/plate)

2. Dissolve 1 tablet of o-dianisidine (Sigma Aldrich #D9154-50TAB) in 1ml of ddH_2O

> ⚠ **TOXIC!** Work under the fume hood when using o-dianisidine

3. Prepare standards for standard curve:

HRP Dilution	*Diluent*	*Standard ID*	*[HRP]*
10 µl of HRP (10mg/ml)	90 µl of ringers	AA	1 mg/ml
20 µl of AA	180 µl of ringers	A	100 µg/ml
20 µl of A	180 µl of ringers	B	10 µg/ml
20 µl of B	180 µl of ringers	C	1 µg/ml
75 µl of C	175 µl of ringers	1	300 ng/ml
80 µl of #1	160 µl of ringers	2	100 ng/ml
80 µl of #2	160 µl of ringers	3	33 ng/ml
80 µl of #3	160 µl of ringers	4	11 ng/ml
80 µl of #4	160 µl of ringers	5	3.7 ng/ml
80 µl of #5	160 µl of ringers	6	1.2 ng/ml
80 µl of #6	160 µl of ringers	7	0.4 ng/ml
--	180 µl of ringers	Blank	0 ng/ml

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Blank										
B	7	7										
C	6	6										
D	5	5										
E	4	4										
F	3	3										
G	2	2										
H	1	1										

SAMPLES IN DUPLICATE

4. Add 40 µl of sample or standard/well.
5. Add 320 µl of the o-dianisidine solution to the 40 ml working buffer, (160µl to 20 ml for 1 plate)
6. Add 4 µl (2 µl for 20 ml/1 plate) of 30% hydrogen peroxide to the o-dianisidine working dilution. *Use extreme caution, 30% hydrogen peroxide will cause severe burns instantaneously.*
7. Set up the Microplate reader for the kinetic HRP assay protocol (450 nm kinetic reads every 35 seconds for 9 min). **Do this immediately before adding the substrate** (next step).

   Note: If the computer goes to sleep with the software running, a complete restart of the computer and microplate reader is required and will require the assay to be redone because of the time required.

8. Add 160 µl of this reaction substrate solution per well using a multichannel pipet.
9. Quickly go the microplate reader and read the plate for 9 minutes. Getting plate into the reader should take less than one minute from the time the last substrate is added.
10. The bottom of the file has a table that shows the slope of the line of each reading (for absorbances below 0.9). Use this as the value for each sample. Plot the standards against the known concentration and fit a 5-parameter logistic curve (5PLC) and use this to interpolate the concentration of HRP in your samples.

Notes:

HRP Dilution	*Diluent*	*Standard ID*	*[HRP]*
10 µl of HRP (10mg/ml)	990 µl of ringers	A	100 µg/ml
50 µl of A	450 µl of ringers	B	10 µg/ml
50 µl of B	450 µl of ringers	C	1 µg/ml
180 µl of #C	420 µl of ringers	1	250 ng/ml
200 µl of #1	400 µl of ringers	2	83 ng/ml
200 µl of #2	400 µl of ringers	3	33 ng/ml
200 µl of #3	400 µl of ringers	4	13 ng/ml
200 µl of #4	400 µl of ringers	5	5.3 ng/ml
200 µl of #5	400 µl of ringers	6	2.1 ng/ml
200 µl of #6	400 µl of ringers	7	0.9 ng/ml
--	450 µl of ringers	Blank	0 ng/ml

An alternative, higher volume standard curve appears above.

Read the FD4 assay first. Samples that have > 350 FIU, probably result from having a hole in them. Those that have > 120 FIU/well are probably also damaged (but will likely need to be diluted if you want an accurate value for HRP). In addition to analyzing 40 µl of undiluted sample, also analyze 40 µl of 1:10 dilution. To do this, add 4 µl of sample to 36 µl of ringers directly to the wells of the assay plate.

Reagent Preparation:

Ringers Component Stock Solutions:
Store at RT

1) 2.3 M NaCl:
134.41g NaCl
in 1L ddH_2O
2) 48mM $K_2HPO_4 \cdot 3H_2O$ +8mM KH_2PO_4
10.96g $KH_2PO_4 \cdot 3H_2O$ or 8.36g K_2HPO_4
1.09g KH_2PO_4
In 1L ddH_2O
3) 0.5M $NaHCO_3$
42.01g $NaHCO_3$
in 1L ddH_2O
4) 24mM $MgCl_2 \cdot 6H_2O$+ 24mM $CaCl_2 \cdot 2H_2O$
4.88g $MgCl_2 \cdot 6H_2O$
3.53g $CaCl_2 \cdot 2H_2O$
in 1L ddH_2O

Ringers Buffer (1L)
Store at 4°C
Combine 50ml each of Ringers componenet solution **1**,**2** and **3** into 700ml ddH_2O

Bring pH to 7.3 with 1M HEPES, free acid.

Add 50ml Ringers component solution **4**
Bring total volume to 1L with ddH_2O

Glucose 1M stock:
Store at -20°C

Glucose 9g
Ringers solution 50 ml

Mannitol 1M stock:
Store at -20°C
Mannitol 9.1g
Ringers solution 50 ml

Glucose 10mM:
Make fresh

1M Glucose 1ml
Ringers solution 99 ml

Mannitol 10mM:
Make fresh

1M Mannitol	1ml
Ringers solution	99 ml

10 mg/ml FD4
Prepare 900µl aliquots & store at -20°C

Dissolve 1g vial of FD4 in 100 ml of Ringers solution and aliquot (900µl).

10 mg/ml Horseradish Peroxidase (HRP) *this actually 2.5KU/ml, which is nominally 10mg/ml*
Prepare 450µl aliquots & store at -20°C

Dissolve 25KU vial of HRP in 10 ml of Ringers solution and aliquot (450µl).

0.2 M Na_2HPO_4:
Autoclave or store at 4°C

Na_2HPO_4, anhydrous (FW 141.96)	2.84 g
ddH_20	to 100 ml

0.2 M NaH_2PO_4,:
Autoclave or store at 4°C

NaH_2PO_4, monohydrate (FW137.99)	13.80 g
ddH_20	to 500 ml

HRP Working Buffer (0.1 M $NaP0_4$, pH 6.3)
Make fresh

Buffer	*20 ml*	*40 ml*	*80 ml*	*160 ml*
0.2 M Na_2HPO_4	2.3 ml	4.5 ml	9 ml	18 ml
0.2 M NaH_2PO_4	7.8 ml	15.5 ml	31 ml	62 ml
MilliQ H_20	10 ml	20 ml	40 ml	80 ml