May 14, 2019

UC Davis - Ex vivo assessment of barrier function-gut permeability

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Protocol status: Working
We use this protocol and it's working

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Abstract

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 0.3 cm^2 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-Dextran 4000 (FD-4, Sigma –Aldrich) and horseradish peroxidase (HRP Type VI, Sigma Aldrich), 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.

Materials

MATERIALS

- Ultrapure Agar **Invitrogen - Thermo Fisher Catalog #**16-500
- Glucose **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**G5767-500G
- Ringer's or Krebs-Ringer-Bicarbonate solutions
- Oxygen tank
- Mannitol **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**M9647-500G
- DMEM low glucose **Invitrogen - Thermo Fisher Catalog #**11885-084
- FITC-dextran 4000 (FD4) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**FD4-100MG
- Horseradish Peroxidase (HRP) type IV **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**P8375-25KU
- O-Dianisidine dihydrochloride **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**D9154-50TAB
- Corning™ 96-Well Solid Black Polystyrene Microplates (Costar 3915) **Fisher Scientific Catalog #**07-200-590
- MaxiSorp (442404) ELISA assay plates **Fisher Scientific Catalog #**12-565-136
- Ussing chamber system **Physiologic Instruments**

Note:
Sigma-Aldrich, RRID:SCR_008988
Fisher Scientific, RRID:SCR_008452

Reagent Preparation:

Ringer's Stock Solutions::

1) 2.3 M NaCl:
   134.41g NaCl
   in 1L ddH_2O

2) 48mM $KH_2PO_4.3H_2O$ +8mM KH_2PO_4
   10.96g $KH_2PO_4.3H_2O$
   1.09g KH_2PO_4
   In 1L ddH_2O

3) 0.5M $NaHCO_3$
   42.01g $NaHCO_3$
   in 1L ddH_2O

4) 24mM $MgCl_2.6H_2O$+ 24mM $CaCl_2.2H_2O$
   4.88g $MgCl_2.6H_2O$
   3.53g $CaCl_2.2H_2O$ in 1L ddH_2O

Ringers Buffer (1L):
Combine 50ml each of solution 1,2 and 3 into 700ml ddH_2O
Bring pH to 7.3 with 1M Hepes
Add 50ml solution 4
Bring total volume to 1L with ddH_2O

Glucose 1M stock:
Glucose 9g
Ringers solution 50 ml

Mannitol 1M stock:
mannitol 9.1g
Ringers solution 50 ml

Glucose 10mM:
1M Glucose 1ml
Ringers solution 99 ml

Mannitol 10mM:
1M Mannitol 1ml
Ringers solution 99 ml

OR ALTERNATIVE BUFFERS

3M KCl:
KCl 111.8 g
Fill ddH20 up to 500 ml

10X Krebs Solution pH 7.2 (1L) (store at 4C):
NaCl (145nM) 69 g
KCl 3.5 g
KH_2PO_4 1.6 g
$MgSO_4$ anhydrous 2.9 g
$NaHCO_3$ 20.8 g
Fill ddH_2O up to 1 liter
pH solution to **pH 7.2**

10X CaCl2 Solution (500ml) (Store at 4°C):
$CaCl_2$, $2H_2O$ 0.55 g
Fill ddH_2O up to 500 ml

KRB Solution (1L) (Store at 4°C):

10X Krebs-Ringer Soln. 90 ml
10X $CaCl_2$ Soln. 10 ml
ddH_2O 900 ml

1X KRB + Glucose 10mM:

Glucose 180 mg
KRB solution 100 ml

1X KRB + Mannitol 10mM:

Mannitol 180 mg
KRB solution 100 ml

1

Tip Preparation

1. Make 3% solution of agar in Ringers or KRB solution*. Place 0.75g agar and 25 ml Ringers/KRB into 50 ml conical. Use orange conical lid attached to 30ml syringe for degassing agar. Fill a 500ml beaker halfway with dH_2O and place conical inside. Heat on hot plate.

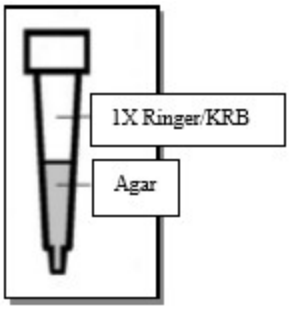
2. When the solution thickens, use syringe to suck out air bubbles. You should see bubbles coming up on the sides of the conical but the center should look clear

3. Stick 1ml syringe (without needle) straight down into the agar and suck it up.

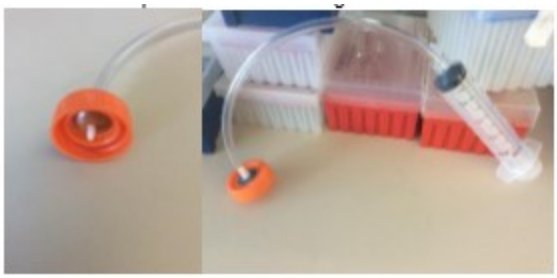
4. Back fill tip by inserting tip into syringe and pushing agar into the tip until the agar fills the lower quarter

5. Immediately use syringe with filling needle to backfill tip with Ringers/KRB

6. Store in tip box filled with Ringers/KRB in 4deg



*3M HCl can also be used in place of KRB or Ringers



2 **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 KRB's solution.

3 **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 KRB.

4 **Setting up and Calibrating the chambers**

1. Prewarm the KRB 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

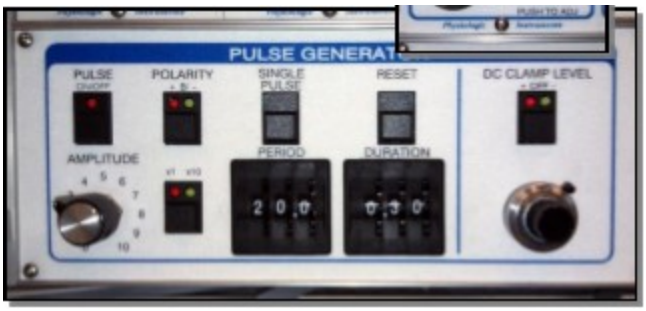
3. Turn on clamp apparatus. Turn on the computer. Make sure each electrode set is on "operate" if there is a sample for that set and on "test" if there are no samples.

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**

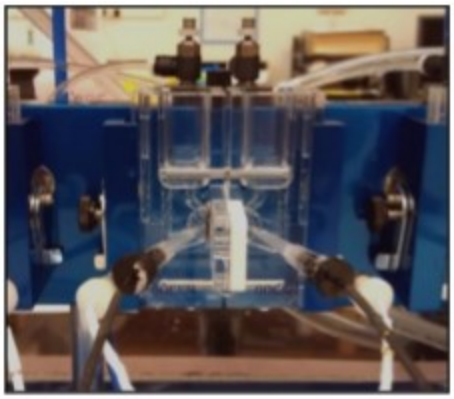


6. Check **Master Control** Module. All lights should be **OFF.**

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 0.5cm²
- Sliders used for mice intestines are 0.3 or 0.1cm²

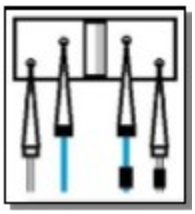


ELECTRODES: Test each set of 4 electrodes in KRB's solution.

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 or Krebs, and 60-67 for tips made with 3M KCl. 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 KRB's 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 waterbath and keep warm during experiment set up.

9. Recalibrate electrodes and adjust offset.

10. Remove KRB'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.

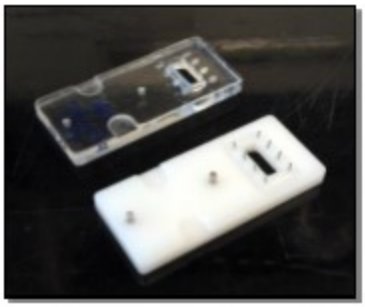
b. Dry the inside of the chamber with a kimwipe and tweezers

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/lumenal side facing the left and serosa on the right

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.



mucosa ---- serosa

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 H_2O.

mannitol	glucose

14. At the same time, slowly add 2.5ml of Krebs+glucose to the chamber that will be in contact with the serosal side (right) and 2.5ml of Krebs+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.

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.

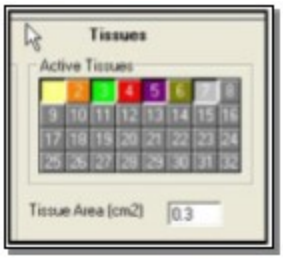
5 **Data Acquisition**

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.

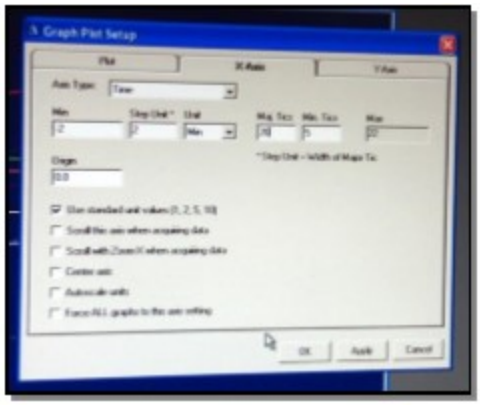
6. Press the

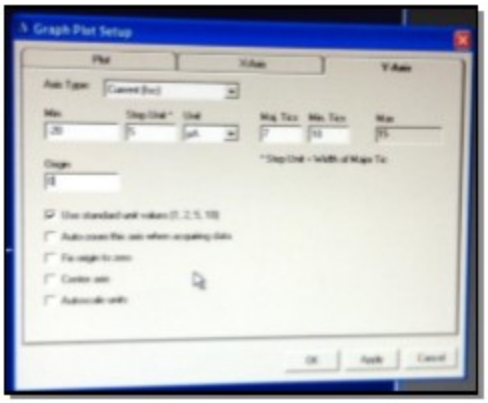
"running man" icon on the toolbar to start data acquisition

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)

6 **Sampling**

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

2. At time 0, collect 2× 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 trancellular]

Stock solution: 10 mg/ml

4. Collect 2× 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.

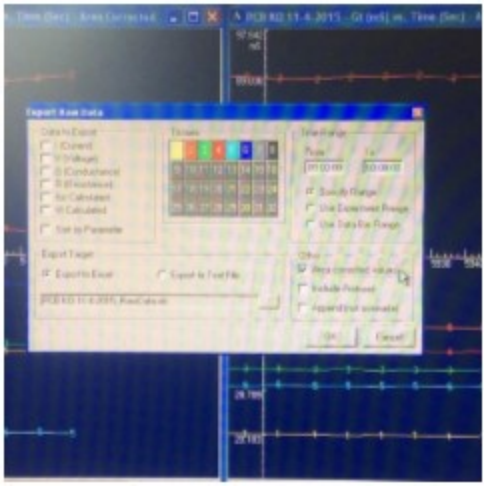
2. Mount your next set of tissues and when ready, Stop the data acquisition and click on Experiment/Properties. Add the new tissues to the selection and press Save. You will have to Reference the new tissues (actually it will reference all of them again) and then start taking data again. This can all 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.

7 **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.

8 **Electrode Maintance**

1. Current driving (white) electrodes:
   a. use nail file or 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.

9 **Assays**

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

10 **FD4 Assay**

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

100 µl of FD4 (10mg/ml)	+	900 µl of ringers →	**A**	1 mg/ml
100 µl of A	+	900 µl of ringers →	**B**	0.1 mg/ml
100 µl of B	+	900 µl of ringers →	**C**	0.01 mg/ml
200 µl of C	+	1800 µl of ringers	→	**1** 1000 ng/ml
750 µl of 1	+	250 µl of ringers →	**2**	750 ng/ml
667 µl of 2	+	333 µl of ringers →	**3**	500 ng/ml
500 µl of 3	+	500 µl of ringers →	**4**	250 ng/ml
500 µl of 4	+	500 µl of ringers →	**5**	125 ng/ml
500 µl of 5	+	500 µl of ringers →	**6**	62.5 ng/ml
	+	500 µl of ringers →	**7**	0 ng/ml

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

SAMPLE
IN DUPLICATE

** Use black 96 well plate **100 µl/well. Read fluorescence (Excitation 485 nm; Emission 538 nm)

11

HRP Assay

Sodium Azide 0.1 % is made from 1 % stock (5ml into 45 ml of H20)
To make 1%, use 1g Sodium Azide in 100ml H20

1. Mix 6 ml of Solution A and 44ml of Solution B. (3 into 22 for 1 plate)

Solution A:
Store at 4°C

Na_2HPO_4	7.5 g
ddH_20	500 ml

Solution B:
Store at 4°C

NaH_2PO_4, hydrated	7.8 g
ddH_20	500 ml

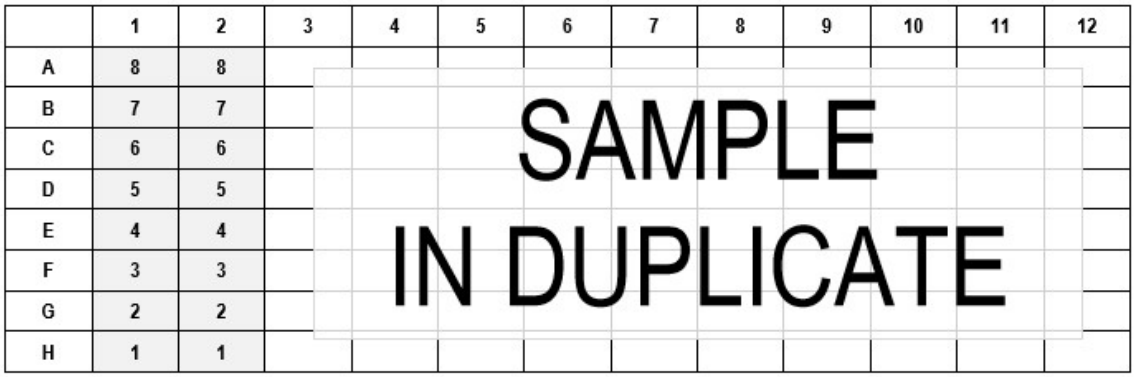
pH 6.0 (with NaOH)

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:

10 µl of HRP (10mg/ml)	+	990 µl of ringers →	**A**	100 µg/ml
50 µl of A	+	950 µl of ringers →	**B**	5 µg/ml
50 µl of B	+	950 µl of ringers →	**C**	250 ng/ml
400 µl of C	+	600 µl of ringers →	**1**	100 ng/ml
800 µl of 1	+	200 µl of ringers →	**2**	80 ng/ml
800 µl of 2	+	200 µl of ringers →	**3**	64 ng/ml
800 µl of 3	+	200 µl of ringers →	**4**	51.2 ng/ml
800 µl of 4	+	200 µl of ringers →	**5**	40.960 ng/ml
800 µl of 5	+	200 µl of ringers →	**6**	32.768 ng/ml
500 µl of 6	+	500 µl of ringers →	**7**	16.384 ng/ml
	+	500 µl of ringers →	**8**	0 ng/ml



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

Plates: LS1143 Elisa 96 Well from Central Services

4. Add 400 µl of the o-dianisidine solution to the Solution A + Solution B mix, (200 ul for 1 plate)

5. Add 5 µl (2.5ul) of 30% hydrogen peroxide to the Solution A + B mix.

6. Add 160 µl of this reaction solution per well.

7. Start the timer 10 minutes.

8. Stop the reaction with 100 µl of 0.1% Sodium Azide (NaN3) /well

9. Read absorbance at 450nm.