PROTOCOLS

I. Bradford's method to quantify total protein concentration

(Bradford, D, M. M. A rapid and sensitive method for the quantitaton of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemestry, 722: p. 248-254, 1976)

Preparation of Bradford's reagent (1 liter):

Coomassie Brilliant Blue G (Sigma 98%) ..... 102,4 mg.

Ethanol 95% ……..... 50 mL

Phosphoric acid 85% .(w/v)..... 100 mL

Mili-Q water q.s.p..... 1000 mL

OBS: Protect from light all steps.

Steps for Bradford's reagent:

• **Step 1**: Weight out Coomassie.

• **Step 2:** Add alcohol to Coomassie Blue in a 100mL Becker, covered with aluminum paper.

• **Step 3:** Agitate with a stir bar for 1 hour.

• **Step 4:** Pour this solution in one 1000-mL Erlenmeyer (protected from light with aluminum paper).

• **Step 5:** Add 100 mL of phosphoric acid.

• **Step 6:** Complete volume to 1000,00mL using Mili-Q water (or DD water).

• **Step 7:** Stir manually.

• **Step 8:** Filter twice with paper filter (in the dark). Store solution in a bottle covered with aluminum paper.

Calibration of Bradford's curve

Reagents: **1.** BSA solution: 1mg/mL in Mili-Q water (standard BSA solution).

2. Bradford's reagent.

• Preparation of BSA concentration gradient:

? 5µg/100µL (100µL of standard BSA solution + 1900µL Mili-Q water) [BSA 1].

? 10µg/100µL (200µL of standard BSA solution + 1800µL Mili-Q water) [BSA 2].

? 15µg/100µL (300µL of standard BSA solution + 1700µL Mili-Q water) [BSA 3].

? 20µg/100µL (400µL of standard BSA solution + 1600µL Mili-Q water) [BSA 4].

? 25µg/100µL (500µL of standard BSA solution + 1500µL) Mili-Q water [BSA 5].

? 30µg/100µL (600µL of standard BSA solution + 1600µL Mili-Q water) [BSA 4].

? 35µg/100µL (700µL of standard BSA solution + 1500µL) Mili-Q water [BSA 5].

? 40µg/100µL (800µL of standard BSA solution + 1600µL Mili-Q water) [BSA 4].

? 45µg/100µL (900µL of standard BSA solution + 1500µL) Mili-Q water [BSA 5].

? 50µg/100µL (1000µL of standard BSA solution + 1000µL Mili-Q water) [BSA 10].

• Summary of BSA dilution

Tubes	Concentration (µg/100 µL)	Standard BSA solution	H2O mili-Q
Branco	0	0 µL	2.5 mL
1	5 µg	100 µL	2.5 mL
2	10 µg	100 µL	2.5 mL
3	15 µg	100 µL	2.5 mL
4	20 µg	100 µL	2.5 mL
5	25 µg	100 µL	2.5 mL
6	30 µg	100 µL	2.5 mL
7	35 µg	100 µL	2.5 mL
8	40 µg	100 µL	2.5 mL
9	45 µg	100 µL	2.5 mL
10	50 µg	100 µL	2.5 mL

• **Step 1:** After preparation of BSA solutions at different concentrations, Vortex them and read after 15 min. at the spectrophotometer (595nM) in triplicates.

- ✍ Coomassie dye binds to quartz cuvettes quite strongly; therefore, glass or plastic cuvettes should be used.
- ✍ Warm spec for ~ 15 minutes before the assay.
- ✍ Zero the spec with the blank (without BSA).

• **Step 2:** Write down absorbances and determine standard curve using Excel.

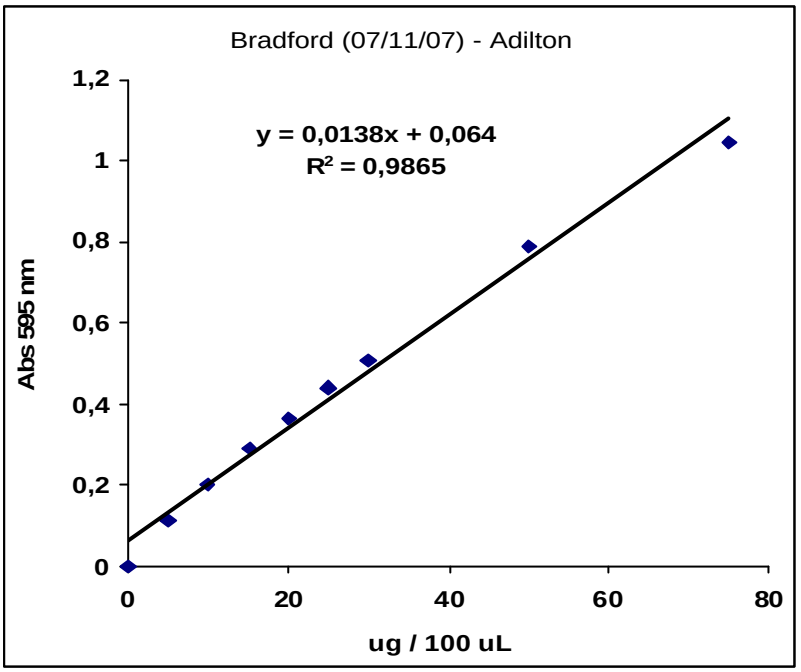
<u>Bradford's standard curve (Excel):</u>

• Add BSA concentrations in the first column;

• Add the second column with absorbances read in the spectrophotometer.

• Select two columns at the same time and ask Excel to make am X Y graph. Write µg/100µL for the "X" axle and Abs 595nM for the "Y" axle.

• Once the graph has been generated, add trend line by clicking on right bottom of mouse. Use linear regression, showing R^2 and equation (example below, for demo only).



• From the equation above, calculate the calibration factor using the following formula:

F = 1/a (from the equation y = aX + B).

Observations:

1. When Bradford's becomes one month old, please make a new curve. After tow months, prepare another reagent.

2. Bradford's reagent makes covalent associations with basic aminoacids of the proteins.

Bradford's assay:

Blank: 100 µL water + 2500 µL Bradford's reagent.

Samples: 100 µL of sample + 2500 µL Bradford's reagent.

Read blank with distilled water and then samples (595 nm)

Then calculate protein concentration by the formula: C = (F x A x D) / 100, where

F: fator de calibration factor; A: absorbance; D: dilution factor. The standard volume for Bradford's procedure is 100 µl. Thus, whatever dilution we use, the valor of D will have to express the dilution from that volume.

II. TWO-DIMENSIONAL ELECTROPHORESIS

II.1. Electrofocusing (1st dimension)

1. Reswelling of strips

? Preparing reswelling buffer

Urea 7 M	8,4g
Tiurea 2 M	3g
DTT 65 mM	5mg
IPG BUFFER 0,5%	5µl
CHAPS 0,5 %	5mg
Bromophenol blue (4 % in water – 4 g in 100 µlr)	(2 drops)

For 20 ml of urea/tiourea 9M (stock solution): add these quantities to Mili-Q water (qsq). Filter using paper filter and vacuum pump (the 9M urea/tiourea solution should be stored in 0.5 or 1-ml aliquots at - 20ºC).

Add these reagents in 0.5 mL of 9M urea/ tiourea

Obs: Prepare reswelling buffer right before use.

• **Step 1:** Weight out 5mg of DTT and 5mg of Chaps.

• **Step 2:** Remove urea/tiourea solution form freezer. Important: this solution tends to precipitate in the Eppendorf tube. Thus, bring urea/tiourea back to solution by inverting the tube a few times. Do not Vortex.

• **Step 3**: Add reagents to the tube containing 0.5 mL of urea/tiourea (thawed and solubilized). Get urea back into solution by mixing/inverting the tube several times. NO need to Vortex it.

- First add the IPG BUFFER 0.5% (5µL)
  - ✍ pH range of the buffer should match strip's pH. However, we have observed that it is ok if the IPG buffer had a pH that includes the limit of strip pH.
- Then add DTT and CHAPS.

• **Step 4**: Mix until precipitate is dissolved.

• **Step 5**: Add a couple of drops of bromophenol blue.

Observations

1. Always use new aliquot of 9M urea/tiourea for **reswelling** of strips

Reswelling of strips

• **Step 1**: Calculate the volume of sample corresponding to the total protein to be loaded in the gel. Reswelling solution volume is 250µl, from which sample volume should be deducted.

- Vol. of sample + reswelling solution = 250 µl (from 13 cm strips).

- Add sample volume to the reswelling solution in an Eppendorf tube.

• **Step 2:** Adjust level of *Immobile DryStrip Reswelling Tray* in a flat bench.

Also:

- ✍ Mark the size of the strip, by the side of the reswelling tray. This will help loading of the sample on the well and the strip;
- ✍ Load the sample (sample + reswelling solution = 250 µl) in the well of the reswelling tray.
- ✍ Distribute the samples as even as possible in the well. Get rid of bubbles as much as possible.

• **Step 4:** Remove strips from package and pill off the plastic attached to it.

- ✍ May want to write down the serial number of each strip and make sure location of samples is recorded properly.

• **Step 5:** Lay strip on the well, on the top of the sample solution, trying to avoid bubbles.

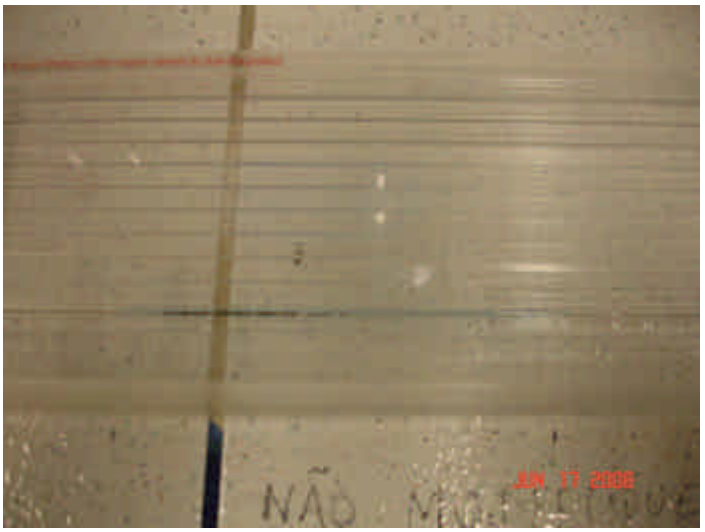
- ✍ Strip shuould get in contact it all te solution in the well.
- ✍ If bubbles are seen, try to press the strip a little to get rid of them. Don't press it too much to avoid damaging the gel.

• **Step 6**: Pipette mineral oil on top of strip (~ 2 ml). Load oil from the ends of strip to the middle of them, so that oil covers the entire strip. Make sure oil covers the entire strip.

• **Step 7:** Close the tray and let it reswell *over night* (15 - 20 h) at TR.

Resweling tray and IPG strip

Resweling tray with lid on: sample + reswelling buffer has been loaded, with strip and mineral oil.



Isoelectric focusing (IEF)

• **Step 1:** Remove strips from reswelling tray e lay them on tissue paper to get rid of excess of oil.

- ✍ **Remember:** Do not lay strip with gel touching the paper. Gel must face up.
- ✍ **Also:** Turn on the equipment (*Ettan IPGphor II*) and check the programa:

Phase 1: 100V; 1h
Phase 2: 500 V; 2 h
Phase 3: 5000V; 2:30 min
Phase 4: 10000 V - 18000VhT

• **Step 2:** Cut two pieces of electrode strips (paper; ~ 2 cm) for each strip. Wet these papers with Mili-Q water. These pieces of paper will go between the electrode and the end of the strips. May help remove salts and impurities.

• **Step 3:** Verify if there is enough mineral oil in the wells of IPGphor.

- ✍ Change oil of the equipment every month.

• **Step 4:** Lay strip in the well, with gel up.

* Watch the orientation of the electrical poles of strips and equipment: + and - .

• **Step 5:** After setting the strip in the well, lay the wet pieces of paper on the extremities of the strips, with some overlay.

- ✍ Paper should get in touch with the gel:

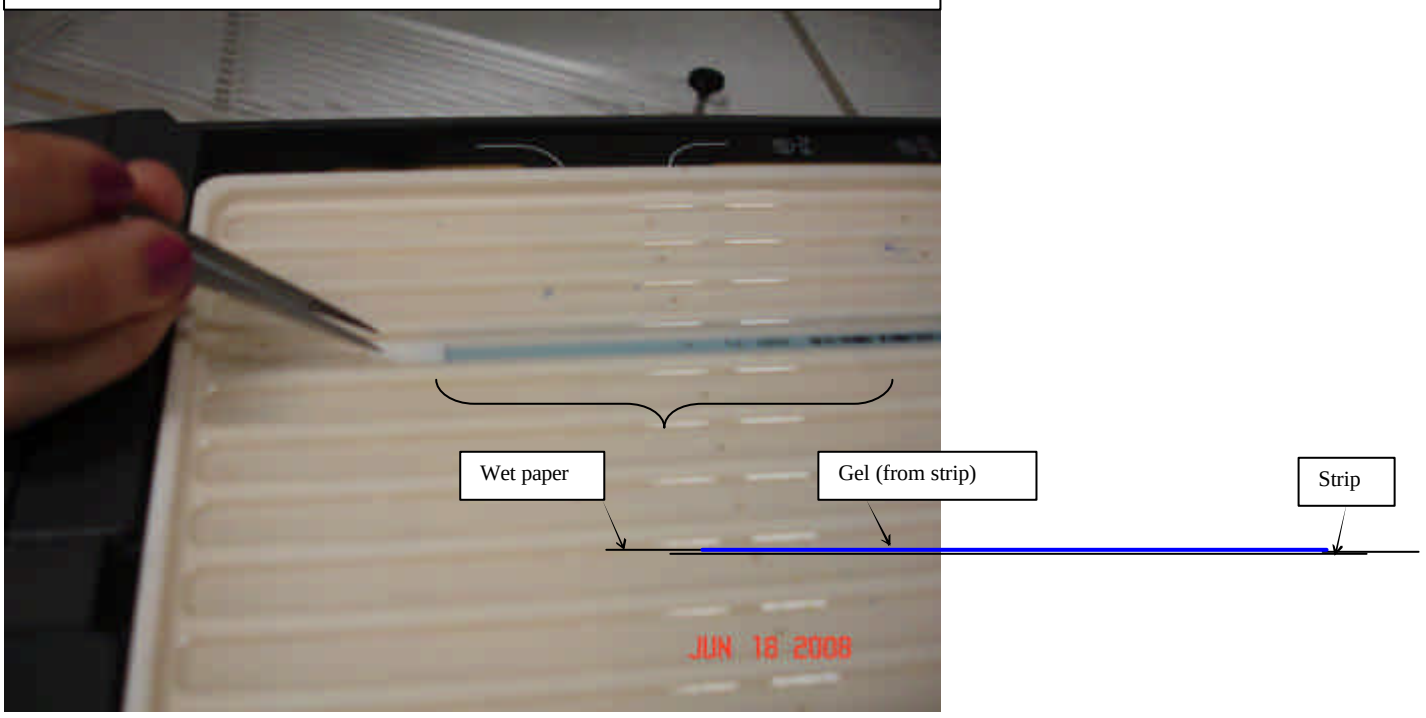
• **Step 6:** Put the electrodes on the edges of strips, precisely on the electrode strips. Then, close the equipment.

• **Step 7:** Press START.

- ✍ check number of strips and program again.

Loading strip on IPGphor

Adjusting paper strip on the edge of IPG strip



IPGphor ready, with strip

2nd Dimension - SDS-PAGE.

Preparation of 15% acrylamide gels

Cleaning and setting up glass plates

• **Step 1**: Wash plates with water and special detergent and then with Mili-Q water.

Clean with ethanol (70 %) and set plates on paper towel, on the bench.

Put silicone on edges of spacers and set them on plates.

Then mount plates and spacers, as many gels as we have to run.

Tie clamps and set plates on the apparatus, turning knobs at the bottom.

• **Step 2:** Balance the system that will receive the gel.

• **Step 3:** Add Mili-Q water to the system to check if there is leak.

- ✍ if there is any leak, remove water and rearrange the plates and may use more silicone gel too.
- ✍ Plates should NEVER leak.

• **Step 4:** If there is no leak, remove water and dry the plates internally.

- ✍ Balance the system at the end (before filling plates with gel).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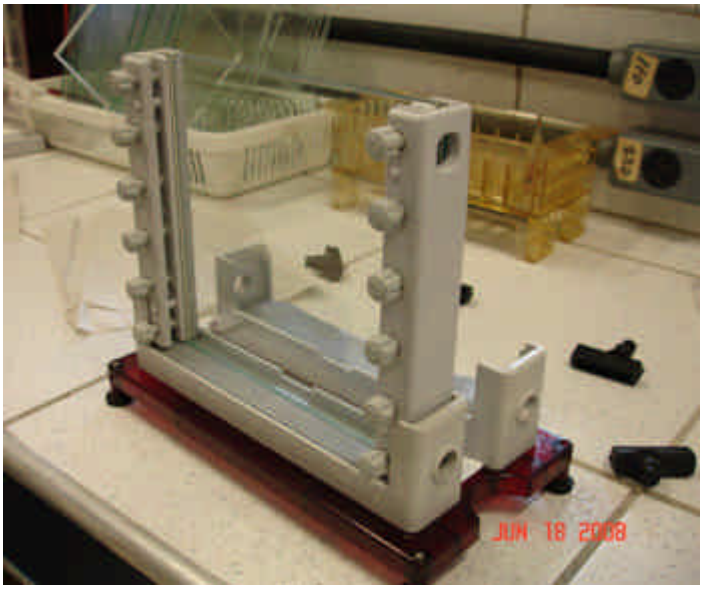
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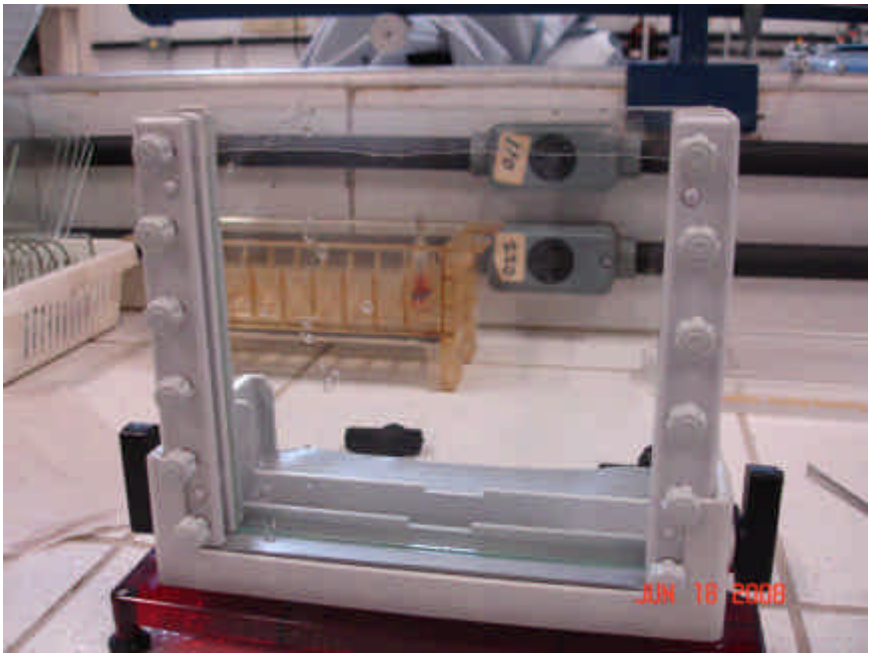
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Making polyacrylamide gels (15 %) - 4 gels

1. Mili-Q water: 28.17 ml
2. Solution A (filtered and degassed): 60.0 mL
   - Acrylamide 30%
   - Bis-Acrilamide 0,8%.
3. Solution B (filtered and degassed): 30.0 mL
   - Tris-HCL 1,5 M pH 8,8
4. SDS 10% ? 1,2 mL
5. Amonium persulfate (10%): 540µL
6. TEMED ? 90µL

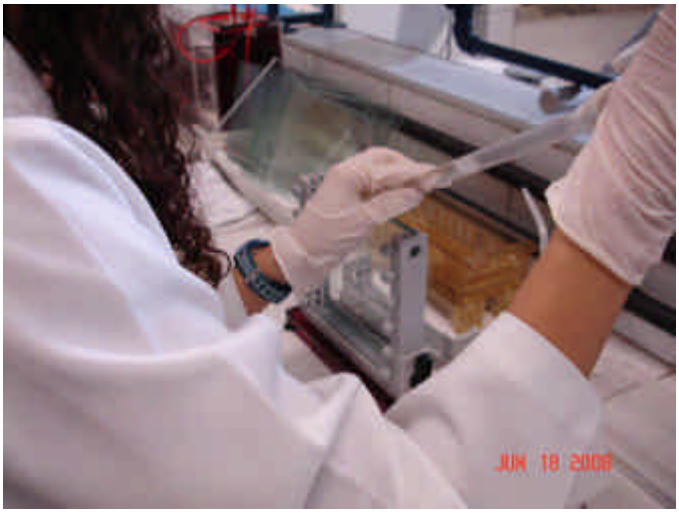
• **Step 1:** Pour water in a Becker and then add solutions 2, 3, 4 and 5. Mix and take it to degas for 1 minute.

• **Step 2:** Add TEMED to the solution, stir a few times e then load the solution to the chambers. Add all the way up to the edge, leaving only a space for the strips.

• **Step 3:** Load butanol on the top of gel to equalize the gel and drive bubbles out.

- ✍ Let gel polymerize for 1 hr.

OBS.: After polymerization, remove butanol and add Mili-Q water to the top of gel. If gel is not going to be used on that day, cover it with plastic film, add some water to the package and store it in the refrigerator.



Equilibration of strips: carried out right before the 2nd dimension.

Solution for equilibration of strips:

Tris (50mM – final concentration)	1.675 ml (pH 8.8 1.5 M)
Glicerol 87%	15.0 ml (for 100 %)
Urea	18.020 g
SDS 2%	1.00 g
Bromophenol blue	Trace
Mili Q water (q.s.p)	50 ml

Step 1: Weight out 57.8 mg of DTT and 69.3 mg of IAA per strip;

Step 2:

- ✍ Add the DTT (57.8 mg) to 3 mL of equilibrium solution and let the solution shake for 15 min. (gentle) – Tube 1
- ✍ Add the IAA (69.3 mg) to 3 ml of equilibrium solution and let it shake (gentle) for 15 min. or until the IAA is solubilized – Tube 2
    - o Equilibration of strips is always done immediately before the second dimension;
    - o Use 10 ml tubes to mix the DDT and IAA with the equilibration solution.

Step 3:

- ✍ Incubate strip in Tube 1 (DTT + equil. solution) for 15 min, at gentle shaking;
- ✍ Then remove strip from Tube 1 and incubate it in Tube 2 (IAA + equil. solution), for 15 min and gently shaking as before.

OBS: If we have, for instance, 4 strips, make 12 ml of DTT solution and 12 ml of IAA solution, then divide these in 3 ml aliquots for incubation of strips individually.

After strips are incubated with equilibrium solutions (DTT and IAA solutions), proceed to the 2nd dimension:

Running buffer should have been made up previously.

Running buffer (10x):

Tris-HCl 0,25 M pH 8,8	30,285 g
Glicine 1,92 M	144,13 g
SDS 1%	10 g
Mili-Q water	1000 ml

Step 2: Remove strip from Tube 2 and let it sit on the middle of the gel. Put strip on top of the gel and try not to move it too much to avoid damaging the gel (of the strip). Avoid, as much as possible, the gel strip getting in touch with the glasses.

- If strip is not entirely mounted on the gel, pipette a few drops of running buffer on the top of it and try to move down gently until the strip sits properly on the gel.
- Right down the orientation of the + and – ends of the strips.
- Get rid of bubbles.
- Important to have made the polyacrylamide gel as high as possible in the glass plates. This prevents problems when we transfer the strips to the top of that very gel.

Step 3. Add a paper filter with 3 µl of the molecular weight standards at the acidic end of the strip, already sit on the gel.

Step 4. Add agarose to the top of the strip to help seal it on the polyacrylamide gel. Get rid of bubbles.

Agarose solution (0.5%):

Running SDS buffer	100 mL
Agarose	0,5 %
Bromophenol blue.....	0,002%

- Make the agarose solution previously and store it at 4 oC;
- Agarose may take around 30-40 min to become liquid. So, let it heat well before the 2 dimension is started.
- Let the agarose cool down before using on the gel. Hint: when we can hold the Erlenmeyer with the agarose solution, it can be used to seal the gel.

Step 5. Add running buffer (1x) on the top of the 2D system and add 5.5 l at the bottom

Setp 6. Adjust the plates on the system and finish mounting the system.

- Before mounting the system: remove the plates with the gels, release the screws and raise the plates a little to improve contact with the lid. Also, add silicone on the top edges of the plates to improve sealing.

Step 7: After the system is mounted, connect the hoses for the cooling water;

Step 8: Turn on the power supply and select the program by pressing "view":

- Voltage: 120V - 150V
- 20 – 25 mA per gel for the first 15 min.
- Then 40 mA per gel until the end of run.

Step 9: Press RUN. Make sure current is going through.

- Watch bromophenol blue to decide when the run should be stopped. Sometimes, we need to let the gel run 30 or 40 min. after the bromophenol blue hits the end of the plate. Let gel run as much as possible to get a decent separation of proteins.