EJERCICIOS, PROBLEMAS, CASOS PRÁCTICOS

Universidad de Jaén

Facultad de Ciencias Experimentales

Protein purification protocol with AKTA

Protocolo de purificación de proteínas mediante cromatografía de afinidad AKTA

María del Carmen Marín Pérez

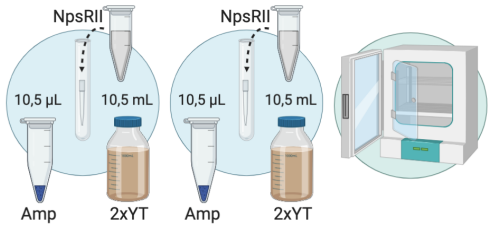
04/05/2025

E.coli PROTEIN PURIFICATION WITH AKTA

Feb. 1, 2025 Revised by María del Carmen Marín Pérez, Ph.D.

1. PRE-CULTURE

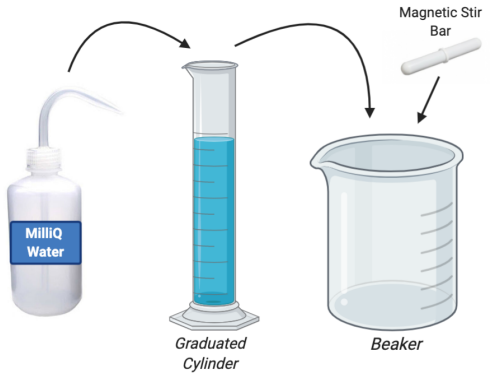
Step 1



Attention! *You have to work all the time around the Bunsen lighter to avoid further contamination of the sample and employed material.* ***Attention!*** *Use sterilized tips.* Prepare two glass tubes (since we have to prepare 2 L culture of cells, one tube for each L) and pour **10.5 mL** of sterilized 2xYT medium into each glass tube and add **10.5 μL** of the appropriate antibiotic (Amp) (50 mg/mL) just before use (**relation 1000 (medium) : 1 (Amp)**). Transfer *E.coli* strain of your protein (stock with Glycerol at -80°C, or LB argar plates colonies) to each test tube with a sterilized tip. Incubate **overnight at 37°C with continuous shaking (200 rpm)**.

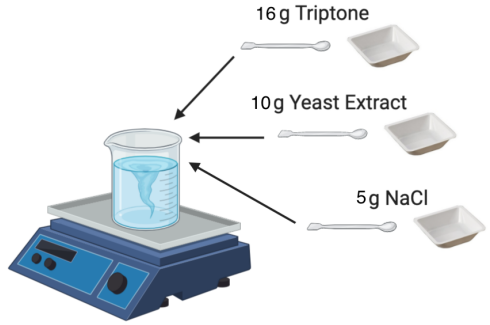
2. PREPARATION OF E.coli MEDIUM (2xYT)

Step 1



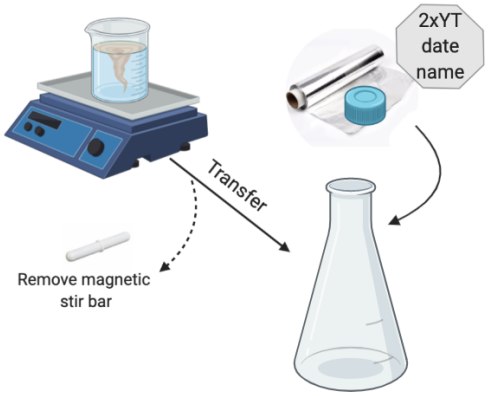
Pour 1000 mL milliQ water into each 1 L beaker (prepare two 1 L breakers, because we have 2 L culture). After that, introduce a stir bar magnet.

Step 2



Using three different spoons and weight baskets, add 16 g of Triptone, 10 g of Yeast Extract and 5 g of NaCl on each breaker. Mix it until it is mostly dissolved. Prepare 2 x 2 L conical flasks for your experiment. Reason: too much medium results in less oxygen in the medium and a poor growth of bacteria.

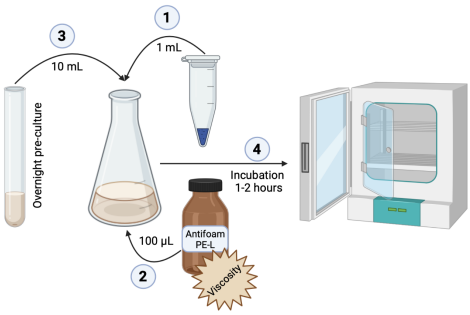
Step 3



Transfer each 1 L solution from each 1 L beaker to each 2 L conical flask. ***Attention!*** *Use a magnet to remove your magnetic stir bar inside the beaker.* Once the solution is inside the conical flask, cover the mouth of the conical flask with a silicone stop (for bacterial culture is BIO-SILICO) and aluminium foil. Please, write the medium's name, date and your name in the top and **Autoclave it**.

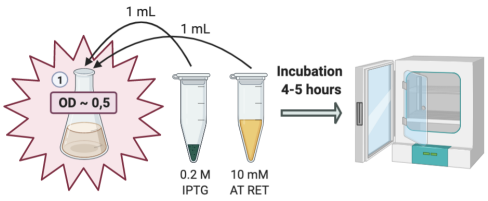
3. LARGE SCALE CULTURE

Step 1



Attention! *Use sterilized tips.* The next day, transfer 1 mL of antibiotic (Amp) (50 mg/mL) (relation 1000:1) and 100 μL of Antifoam PE-L, to avoid bubbles during the incubation with shaking, on each conical flask. Then, transfer 10 mL of each overnight pre-culture tube (relation 100:1) into each conical flask. Incubate at **37°C with continuous shaking (200 rpm)** for **2-3 hours**.

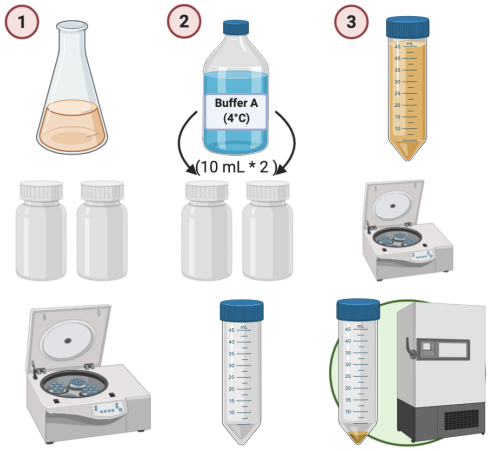
Step 2



Check the Optical Density (OD) of each culture after 1h and 30 min. Continue incubation **until OD reaches 0.5 - 0.7**. ***Attention!*** *Use sterilized tips.* To do that, transfer 4 mL from each conical flask into a new glass tube. Also, prepare 0,1 M IPTG solution before use (this concentration can be different, it depends of your protein expression optimization). Add 1 mL all-trans RET (10 mM stock in -80°C, final concentration 10 μM) and 1 mL IPTG (final concentration 0,01 mM) en each conical flask. Incubate at 37°**C with continuous shaking (200 rpm)** for **4 hours**.

4. CELL COLLECTION

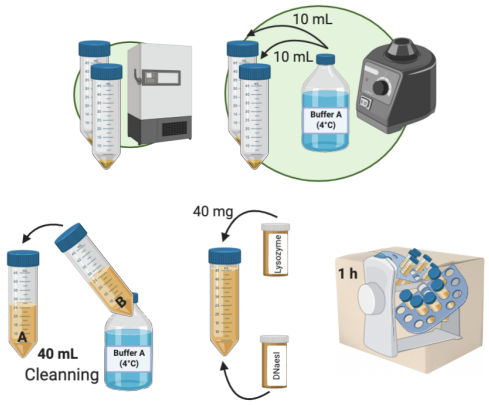
Step 1



Transfer the content on each conical flask inside two plastic bottles. Both bottles have to be the same content. Then, centrifuge them at **8000 rpm, 10 min and** 4°**C**. Discard the supernatant. In each bottle add 10 mL of Buffer A (store in the fridge at 4°C) (see Buffer preparation table on page 4). Re-suspend the cells and transfer the content of 2 bottles into one 50 mL Falcon tube. Again, add 10 mL of Buffer A in your bottles and re-suspend the remaining cells. Transfer the content into the previous 50 mL Falcon tube. Centrifuge both 50 mL Falcon tubes at **8000 rpm, 10 min and 4°C**. Discard the supernatant. Again centrifuge both tubes up to 8000 rpm and then stop the centrifuge machine. Discard the supernatant with the help of pipette. Covert them with aluminium foil and store them at -80°C, or continue with the cell disruption process.

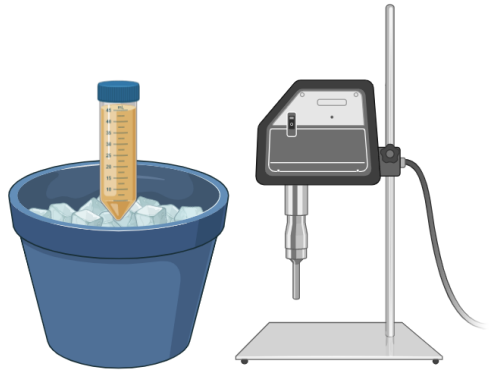
5. CELL DISRUPTION AND CENTRIFUGATION

Step 1



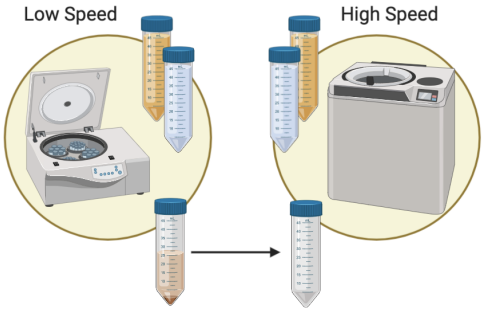
Thaw the pellets (in case you frozen them) and re-suspend it (each tube) in 10 mL Buffer A (stored in the fridge at 4°C) with the help of vortex. Then, transfer the content of 50 mL Falcon tube B on 50 mL Falcon tube A, and use 5 mL Buffer A to clean Falcon tube B. Again transfer it into Falcon tube A. Add Buffer A up to 40 mL. Add 40 mg of Lyzozyme (1 mg/mL) and a small amount of DNaseI to the cell suspension. Then, cover the tube with aluminium foil and mix rotating at room temperature for **1 hour (speed 15)** on the AS One mini rotator ACR-100.

Step 2



For cell disruption:
1) Attach the suitable probe
2) Prepare 200 mL MilliQ water in a beaker and put it bellow the standard probe (up to 2 cm).
3) Turn ON the Device and push TUNE button until it automatically finishes.
4) Prepare your Falcon tube holder in a mixture of ice and water and remember to run all sonication on ice.
5) Function tabs set as follow: 8/12 seconds of ON/OFF cycles (3 cycles) and 20 minutes/cycle with a power set of 50% - 40% - 35%, respectively.

Step 3



Do low speed centrifugation to get rid of cell debris. Centrifuge the 50 mL Falcon tube at **80000 rpm for 10 min at 4°C**). Please, remember introduce a balance (in case you only have one Falcon tube).

After that, transfer the supernatant from the Falcon tube into a specific tube for High speed Ultracentrifugation and fill it with buffer A. Please, remember introduce a balance with the exact same weight.

Set the adjustments for centrifugation: **1 h, 35,000 rpm and 4°C**. While the door is close, press "Vacuum" to start the vacuum and cooling process. Release the vacuum by pressing "Vacuum" again. Place the tubes within the rotor P45AT-1012 and tighten the rotor well. Then, put the rotor in the Ultracentrifuge. Close the door and press "Vacuum". Wait until the "Vacuum"process is finished and, then press "Start". Have a look at the vacuum display while acceleration. After centrifuge finished, you can go ahead with next step or you can hereafter get rid of the supernatant and freeze the pellets at -80°C.

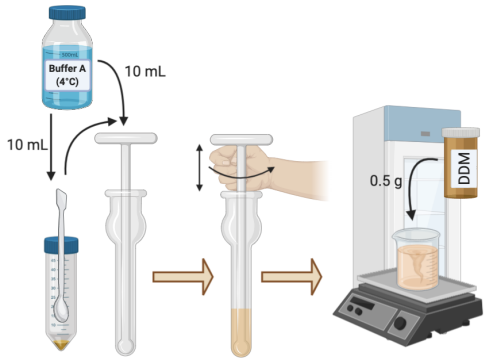
6. SOLUBILIZATION

Step 1

Buffers preparation: Keep them on the fridge (4°C). **Filter (vacuum filter unit (Advanctec VH050P)) all the buffers before add DDM, even those which do not contain DDM. Then include the DDM to avoid gas and bubbles in lines and tubes. You can wash out the disposable filters with DW and re-use them again later.**

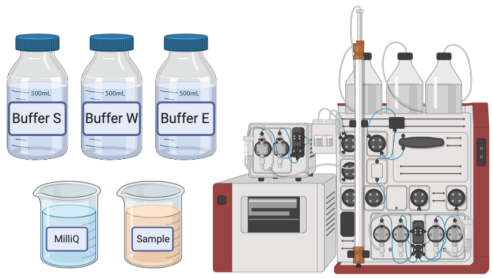
Buffer	Component	Final Concentration	Amount/L
Buffer A	Tris/HCl pH 8.0	50 mM	6.06 g
	$MgCl_2$ $6H_2O$	5 mM	1.01 g
Buffer S	MES/NaOH pH 6.5	50 mM	9.76 g
	NaCl	300 mM	17.53 g
	Imidazole	5 mM	0.34 g
	$MgCl_2$ $6H_2O$	5 mM	1.01 g
Buffer W	MES/NaOH pH 6.5	50 mM	9.76 g
	NaCl	300 mM	17.53 g
	Imidazole	50 mM	3.40 g
	DDM	0.1%	1.00 g
	$MgCl_2$ $6H_2O$	5 mM	1.01 g
Buffer E	Tris/HCl pH 7.0	50 mM	6.06 g
	NaCl	300 mM	17.53 g
	Imidazole	300 mM	20.42 g
	DDM	0.1%	1.00 g
	$MgCl_2$ $6H_2O$	5 mM	1.01 g
Dialysis Buffer	Tris/HCl pH 8.0	50 mM	6.06 g
	NaCl	100 mM	5.84 g
	DDM	0.05% or 0.1%	0.5 g or 1.00 g

Step 2



Hereafter get rid of the supernatant (or thaw the freezer pellet at room temperature) re-suspend the pellets in **10 mL Buffer S** and transfer it to a Teflon in glass homogenizer for homogenization (use a spatula to get the pellet). Add **10 mL Buffer S** in the ultracentrifuge tube to clean the remaining pellets and transfer it again to the glass homogenizer. Do the manual pestle homogenization using slowly up and down and swirling push until you cannot see any clear big particles. Transfer the sample to a 100 mL beaker. Clean the glass homogenizer with another 10 mL of Buffer S, and transfer the content again into the 100 mL beaker. **Adjust the volume to 50 mL using Bruffer S**. Add a magnetic stirrer and 0.5 g of n-dodecyl-β-Dmaltopyranoside (DDM, 1% final concentration) and place it on a AS One Slim Stirrer at the 4°C fridge rotating overnight (speed 2). **The day after, do another run of the high-speed Ultracentrifuge like described above and this time just collect the Supernatant for AKTA purification.**

7. AKTA PURIFICATION



Note: Please proceed with the AKTA setting while high-speed Ultracentrifuge is running. Turn ON the AKTA purification device and following these steps for **PRE-CLEANING AND PREPARATION**:

1) **Home menu → Manual Run → change the Sample valve tab from Buffer to Sample**. Run the program with 200 mL MilliQ water. This step should be **manually** stop after 3-5 min.

2) **Home menu → Method run → Prepare system → Pump Wash B**. Run it while both Line B and A are already placed inside the MilliQ water bottles. This step will stop **automatically**.

3) **Home menu → Method run → Prepare system → Pump Wash A**. Run it while both Line B and A are already placed inside the MilliQ water bottles. This step will stop **automatically**.

4) Bring out the optimal HiTrap TALON crude columns (1 mL or 5 mL column) **out of fridge**. **Home menu → Method run → Prepare system → Column preparation**. Set the following parts: **Column Volume: 1/5 for 1/5 mL Column**, **Flow rate: 5** and then **run it**. While it is running, connect the Hitrap affinity column to the Column connection tube from the top first and the bottom to the system. This step will finish **automatically**.

6) **Home menu → Method run → Prepare system → Washout fractionation tubing**. Waste liquid is collected into 15 mL plastic tube which set on the fraction collector. **Please, check that it is empty.** To collect elution, set the appropriate number of 1.5 - 2 mL - 5 mL (depending of the elution volume that you select) plastic tube on the fraction collector. **Please, check the orientation fractionation tube.**

After the preparation steps finished, start the **PRE-OPTIMIZATION** following steps for purifications:

1) **Home menu → Manual Run → change the Sample valve tab from Buffer to Sample**. Run the program with 200 mL Buffer S. This step should be **manually** stop after 3-5 min.

2) Put the tube of Line B in Buffer E. **Method run → Prepare system →** Pump Wash B. Run it.

3) Put back the sample input line (the one that you have in the beaker with Buffer S) to the MilliQ water beaker. Put the tube of Line A in Buffer S. **Method run → Prepare system → Pump Wash A**. Run it.

4) **Home menu → Method run → Prepare system → Column preparation**. Set the following parts: **Column Volume: 1/5 for 1/5 mL Column**, **Flow rate: 5** and then **run it**. Run the Column preparation while tube line A is inside the Buffer S. This step will stop **automatically**.

Then, for starting the **PURIFICATION**, bring your 100 mL beaker with your sample after ultr-centrifuge and set for the input sample line and follow these steps: **Method run → Templates → Affinity(AC)**. Then set the required options **BEFORE** running: **Column Volume: 1/5 for 1/5 mL Column**, **Flow rate: 1/5**, **Sample volume: set 5 mL less of your final sample volume (example: if you have 65 mL, set 60 mL**, **Fractionation Volume: 1, 2, or 5 mL** (**set the tubes in advance**). Select the name of the Save in the USB tab (AC01-AC99) and **run it**.

If you used 1/5 mL column wait for 1/5 min. Then change the Line A (**take care of the machine noise before perform the change**) from Buffer S to Buffer W. Push to the "Edit" button on the screen, change the Flow rate speed settings to 1 for sample upload step (push the execute) and continue the method until the whole 50 mL (*60 mL*) sample loading finish. Once the sample upload finished again change the Flow rate speed again back to the settings to the Flow rate 5 (in the Edit tab) for sample upload and continue the method (**add Buffer S to avoid bubbles**) until finished automatically.

Start **CLEANING** steps of AKTA as following steps:
1) Put back the Line A and B tubes back into the MilliQ water bottle.

2)Manual Run and after changing the Sample valve tab from Buffer to Sample run the program with 150 mL MilliQ water (prepared in advance in the beaker as for the sample input line). This step should be manually stop after 3-5 min.

3) **Method run → Prepare system → Pump Wash B** and run it.

4) **Method run → Prepare system → Pump Wash A** and run it.

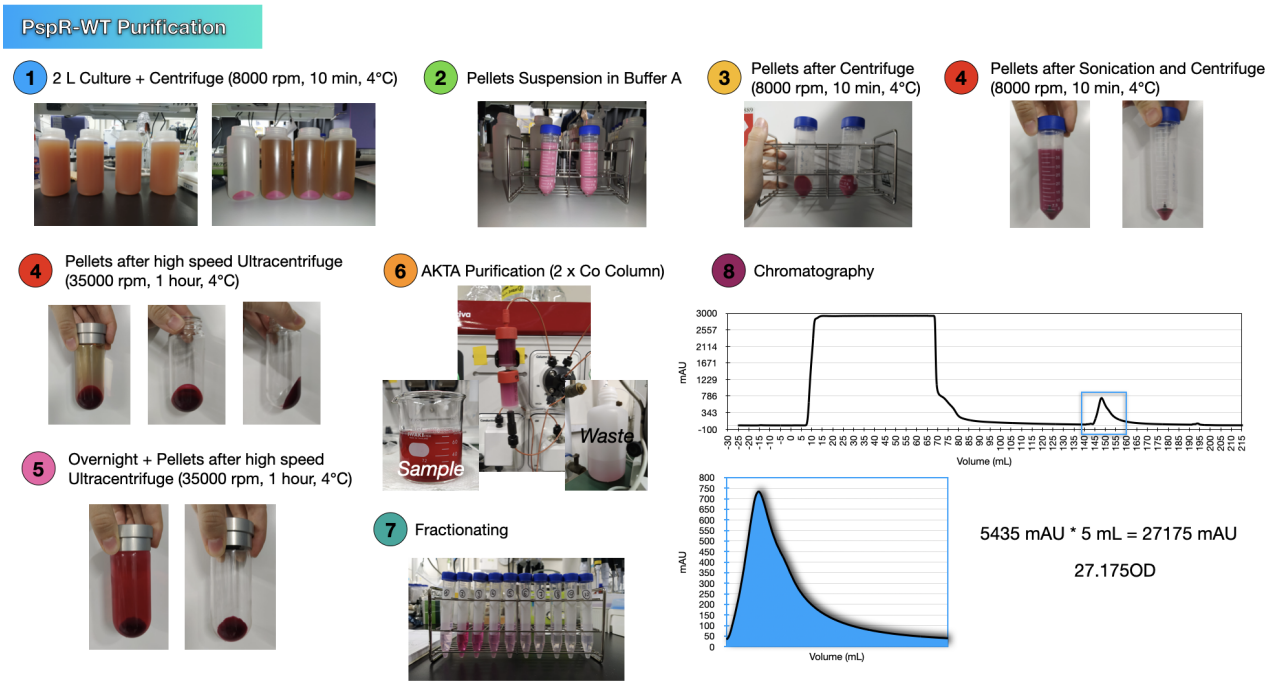
5) **Method run → Prepare system → Pump Wash A** and run it.

6) **Method run → Prepare system → Column preparation**: Set the following parts: **Column Volume: 1/5 for 1/5 mL Column**, **Flow rate: 5** (check bubbles inside the column). Start again the column preparation and after running start disconnecting the Hitrap affinity column from the column line tube but this time in reverse manner (from bottom of column and then top part of that).

7) Stop the procedure manually. **Method run → Prepare system →Washout fractionation tubing**. **Change the position of the waste tube from the eppendorf to the Falcon tubes**. All steps done and you can Turn OFF the AKTA system! You should wash out the waste container with water and DW.

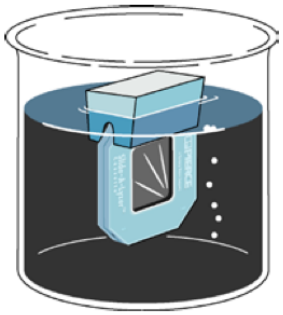
After the purification steps finished, choose the darker color fractionated tubes (the tubes which are include enriched interested purified protein sample). Record an absorption spectrum in the UV spectrometer, and calculate the OD.mL amount. You can keep them in the 4°C (covert them with aluminium foil) or go ahead with the dialysis procedure.

Schematic representation of protein purification



8. DIALYSIS

Step 1



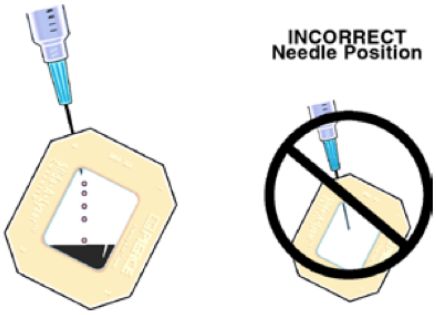
Hydrate the Membrane. Perform the following steps for cassettes requiring hydration and for all cassettes used with low sample volumes (***Attention!*** *Wear gloves.*):

1) Remove Slide-A-Lyzer Cassette (Slide-A-Lyze Dialysis (3.0-12 mL Capacity)) from its pouch and slip into the groove of an appropriate size buoy.

2) Immerse the cassette in 1L dialysis buffer. Hydrate the cassette(s) for at least 2 minutes in a 1000 mL beaker at room temperature.

3) Remove cassette from buffer and remove excess liquid by tapping the edge of the cassette gently on paper towels. ***Attention!*** *DO NOT BLOT THE MEMBRANE.*

Step 2



Add Sample. ***Attention!*** *Touch only the hard-plastic parts with gloves.*

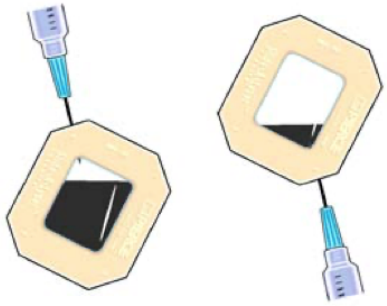
1) Centrifuge the sample(s). Fill the syringe with the sample, leaving a small amount of air in the syringe.

2) With the bevel sideways, insert the tip of the needle through one of the syringe ports located at a top corner of the cassette.

3) Inject sample slowly. Withdraw air by pulling up on the syringe piston. Remove the syringe needle from the cassette while retaining air in the syringe.

4) Insert the membrane(s) inside the same dialysis buffer beaker with magnet on AS One slim Stirrer at 4°C. Fridge for **2 hours**. After 2 hours, exchange the dialysis buffer once and keep it AS One slim Stirrer at 4°C fridge **overnight**. Next day, exchange for the last time the dialysis buffer one again and keep it on the magnetic Stirrer for 2 hours at 4°C.

Step 3



Remove Sample ***Attention!*** *Use caution to avoid contacting the needle to the membrane.*

1) Fill the syringe with a volume of air equal to the sample size. For low-volume samples, fill the syringe with a volume of air approximately equal to two times the sample volume.

2) With the bevel sideways, insert the tip of the needle through another syringe port located at a corner of the cassette. Inject air slowly into the cassette to separate the membranes.

3) Turn the unit so that needle is on the bottom and allow the sample to collect near the port.

Withdraw the sample into the syringe.
4)Transfer out the dialyzed purified protein sample(s) out of one corner into a new 1.5-2 or 15 mL tubes.
5) Record an absorption spectrum, write the information into a spreadsheet on the laboratory website and you are done. You can keep them afterward at 4°C (covert it with aluminium foil).