JAN 25, 2024

DOI:
dx.doi.org/10.17504/protocols.io.bp2l6xwmklqe/v1

Protocol Citation: Michael X. Henderon, Lindsay Meyerdirk 2024. α-Synuclein Protein Preparation (Large scale). **protocols.io** https://dx.doi.org/10.17504/protocols.io.bp2l6xwmklqe/v1

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

Created: Dec 21, 2023

α-Synuclein Protein Preparation (Large scale)

In 1 collection

Michael X. Henderon[1], Lindsay Meyerdirk[1]

[1]Van Andel Institute

michael.henderson

ABSTRACT

This protocol details α-synuclein protein preparation in a large scale.

ATTACHMENTS

932-2406.docx

GUIDELINES

Adapted from: Xiao Tu, Kelvin Luk, Jonathan Branch, Patrick O'Brien, Dustin Covell, Katelyn Becker, Volpicelli-Daley, Laura A et al. "Addition of exogenous α-synuclein preformed fibrils to primary neuronal cultures to seed recruitment of endogenous α-synuclein to Lewy body and Lewy neurite-like aggregates." Nature protocols vol. 9,9 (2014): 2135-46. doi:10.1038/nprot.2014.143

Last Modified: Jan 25, 2024

PROTOCOL integer ID: 93624

Funders Acknowledgement:
Aligning Science Across Parkinson's
Grant ID: ASAP-020616

MATERIALS

Materials

- LB + 100 mg/L ampicillin (LB/Amp) plates
- Competent BL21(DE3) RIL cells (Stratagene- A) (20 µL aliquots)
- α-synuclein gene of interest cloned into pRK172 NdeI-HindIII site
- 100mg/mL Ampicillin stock
- 40 mL Dounce homogenizer and pestle A
- Dialysis tubing (Spectrum Labs, Membrane tubing 12-14 kD)
- Fisher Scientific Sonic Dismembrator Model 500 (Sonicator)
- FPLC
- Collection tubes
- Superdex 200 Column
- 1000 mL 0.22 µm filter unit
- 15% SDS—PAGE gels
- Amicon Ultra Centrifugal Filter devices (Amicon Ultra 15 10K MWCO)
- MonoQ column (GE Health, HiTrap Q HP 645932)

Note

For truncated synucleins, another column may be necessary.

Media and solutions

Lysogeny Broth (LB)

	A	B	C
	Ingredient	Amount	Final Conc.
	Bacto-tryptone	10g	
	Yeast Extract	5g	
	NaCl	10g	
	MilliQ H2O	To 1 L	

Autoclave and cool before use.

Terrific Broth (TB)

	A	B	C
	Ingredient	Amount	Final Conc.
	Bacto-tryptone	48 g	
	Yeast extract	96 g	
	Glycerol	16 mL	
	MilliQ H2O	To 4 L	
	10x Phosphate Buffer	Add 50 mL to 450 mL TB	

Autoclave and cool before use.

10x Phosphate Buffer (PB, 1L)

	A	B	C
	Ingredient	Amount	Final Conc.
	KH2PO4	23.2 g	
	K2HPO4	125.4 g	
	MilliQ H2O	To 1 L	

0.5 M IPTG

	A	B	C
	Ingredient	Amount	Final Conc.
	IPTG	120 mg	0.5 M
	MilliQ H2O	1 mL	

High Salt Buffer (50 mL/L culture)

	A	B	C
	Ingredient	Amount	Final Conc.
	5 M NaCl	150 mL	750 mM
	0.5 M Tris, pH 7.6	20 mL	10 mM
	0.5 M EDTA	2 mL	1 mM
	0.5 M PMSF	2 mL	1 mM
	MilliQ H2O	To 1 L	

Add protease inhibitors 1:1000 and PMSF immediately prior to use.

5 M NaCl (1 L)

	A	B	C
	Ingredient	Amount	Final Conc.
	Sodium Chloride pH to 7.4	292.2 g	5 M
	MilliQ H2O	To 1 L	

0.5 M Tris, pH 7.6 (1 L)

	A	B	C
	Ingredient	Amount	Final Conc.
	Tris-base	60.57 g	0.5 M
	HCl, pH to 7.6	~30 mL	
	MilliQ H2O	To 1 L	

0.5 M EDTA, pH 8.0 (500 mL)

	A	B	C
	Ingredient	Amount	Final Conc.
	Sodium hydroxide pellets	10 g	0.5 M
	MilliQ H2O	400 mL	
	EDTA pH to 8.0	96.05 g	
	MilliQ H2O	To 500 mL	

Gel Filtration Dialysis Buffer (1.25 L/L culture)

	A	B	C
	Ingredient	Amount	Final Conc.
	5 M NaCl	50 mL	50 mM
	0.5 M Tris, pH 7.6	100 mL	10 mM
	0.5 M EDTA	10 mL	1 mM
	0.5 M PMSF	10 mL	1 mM

	A	B	C
	MilliQ H2O	To 5 L	

Add PMSF immediately prior to use.

Gel Filtration Buffer (1 L)

	A	B	C
	Ingredient	Amount	Final Conc.
	5 M NaCl	10 mL	50 mM
	0.5 M Tris, pH 7.6	20 mL	10 mM
	0.5 M EDTA	2 mL	1 mM
	MilliQ H2O	To 1 L	

Buffer A (1L/L culture)

	A	B	C
	Ingredient	Amount	Final Conc.
	5 M NaCl	20 mL	25 mM
	0.5 M Tris, pH 7.6	80 mL	10 mM
	0.5 M EDTA	8 mL	1 mM
	0.5 M PMSF	8 mL	1 mM
	MilliQ H2O	To 4 L	

Add PMSF and filter with 0.2 µm filter (for column) immediately prior to use.

Buffer B (400 mL/L culture)

	A	B	C
	Ingredient	Amount	Final Conc.
	5 M NaCl	200 mL	1 M
	0.5 M Tris, pH 7.6	20 mL	10 mM
	0.5 M EDTA	2 mL	1 mM
	0.5 M PMSF	2 mL	1 mM
	MilliQ H2O	To 1 L	

Add PMSF and filter with 0.2 µm filter (for column) immediately prior to use.

Day 1: Transformation

1 Thaw E. coli (BL21(DE3)RIL) and DNA On ice.

2 Mix 1 µL – 2 µL DNA (α-synuclein in pRK172 plasmid) with 20 µL competent cells and incubate 5-10 minutes On ice.

3 Heat-shock cells for 00:00:45 in 42 °C water and place back On ice for 00:02:00. 2m 45s

> **Note**
>
> The microwave can be used to heat a beaker of water to 42 °C.

4 Add 200 µL LB (no antibiotics) and shake at 37 °C for 01:00:00. Warm Amp^{100} plate @ 37 °C. 1h

5 Plate 200 µL on agar bacteria plate with ampicillin and spread with glass cell spreader.

6 Incubate the plate inverted at 37 °C Overnight . 1h

Day 1: Transformation- Buffer Preparation

7 Make 4 L of TB and 400 mL of 10x phosphate buffer.

8 Add 450 mL of TB + 50 mL 10x phosphate buffer into 8x 2 L flasks. Add 200 mL to a 1L flask. Cover the tops with foil, and autoclave on the liquid setting.

9 Make sure to let flasks cool for at least 01:00:00 after they have been autoclaved, or if you're in a hurry, cool in the cold room for at least 00:30:00 . 1h 30m

Day 2: Making Starter Culture

10 Add 5 µL of 100 mg/mL Ampicillin to 5 mL SOC/TB/LB in a round bottom tube.

11 Pick a colony with a 20 µL pipet tip and drop the tip into the starter culture. Swirl around to ensure the colony detaches from the pipet tip.

12 Shake at 200 rpm at 37 °C Overnight . 30m

13 Add 0.5 mL of the starter culture to each500 mL flask of TB/phosphate buffer and allow to shake in 30m incubator @ 200 rpm for 00:30:00 without antibiotic.

14 After 00:30:00, add 0.5 mL ampicillin (100 mg/mL) and grow Overnight at 37°C. 2d 1h Expression will occur without induction Overnight. With such a small starter culture, the goal is for at least 24:00:00 growth.

Day 3: Protein Isolation

15 Transfer bacteria cultures to large centrifuge bottles (provided by glassware), make sure the bottles are balanced and centrifuge for 00:20:00 at 5000 x g and 4 °C (rotor F10S). 20m

15.1 Pour out the supernatant after each spin and keep adding more of the culture to the bottles (balanced) until all culture is centrifuged.

16

Note

THIS IS THE POINT TO FREEZE THE PELLETS IF YOU ARE NOT PROCEEDING WITH THE PREP.

If so, scrape out the pellets and transfer to a labeled 50 mL conical and store at -20 °C. Otherwise:

17 Pour off the supernatant and carefully scrape out pellet with a spatula into a 500 ml beaker. Re-suspend pellets using cold High Salt Buffer. Use 15 mL of the buffer to re-suspend the pellet, pour off into the beaker, and then use 5 mL of the buffer to rinse out the bottle. About 50 mL of High Salt Buffer per liter of cell culture will be used (total after the next step).

18 Break down pellets in beaker with manual agitation. Pour roughly 40 mL at a time into a 40 mL dounce homogenizer.

19 Homogenize with pestle A (>15 times), making sure that you have broken up all clumps. Repeat with remaining bacteria until all is homogenized and poured into a small plastic beaker.

19.1 Rinse the homogenizer with the high salt buffer 2-3 times to get the remaining clumps and cell lysate out of the homogenizer

20 Heat electric deep fryer on high heat until water is rapidly boiling, being sure to fill the pot up so that a tube rack can be submerged.

21 Sonicate On ice (in a plastic or metal beaker) at 35% for 00:01:20 total ON time (5 sec ON, 2s 1m 20s OFF, 8 min total time).

21.1 You will have to attach the tip to the sonicator, make sure the tip is 1-2cm from the bottom of the cup taking care not to hit/break the tip.

22 Transfer the homogenate to 50 mL conical tubes (~3/4 full).

Note

MAKE SURE ALL TUBES ARE BALANCED AT THIS TIME.

22.1 Boil the homogenate for 00:20:00. 20m

23 Make 5 L gel filtration dialysis buffer and put in the cold room.

24 After boiling, bury homogenate tubes in ice and place them in the cold room for at least 00:30:00 or 30m until they feel cold.

25 Centrifuge cold lysate for 00:20:00 at 11800 x g and 4 °C (rotor F21). 20m

26 OPTIONAL: Take an aliquot of the supernatant. Add 5X sample buffer and run on a 15% gel.

27 Cut dialysis tubing (12-14 kD tubing) and wash in the dialysis buffer to soften the tubing.

28 Pipet supernatant into the tubing, close with clamps (**leave an air bubble to promote floatation of the tubing), put the tubing in dialysis buffer and leave in cold room Overnight on the stirrer (stir slowly!).

29 If you are doing gel filtration tomorrow, prep the ÄKTA **(S.1)**, and start the program for the gel filtration column for loading tomorrow. **(S.2)** Lines should be set up as:

- A1-Size Exclusion Buffer
- A2-Sterile Distilled Water
- B1-20% Ethanol
- B2-20% Ethanol

30 Clean-up: wash all beakers and tubes with bleach and water. Wash dounce homogenizer with DI water and methanol.

Day 5: Gel filtration

31

Note

Concentration and gel filtration steps can be done on separate days if the concentration takes too long. Gel filtration is done with 1-2 runs/L of cell culture and up to 3 mL of sample/run. Make sure the fraction collector has 24/run 15 mL tubes and is centered correctly. After column prep, the fraction tube should be transferred to the fraction collector arm.

Filter the protein through a 0.22 µm filter (1000 mL filter unit due to large surface area).

32 Concentrate down to 2-4 mL/L of cell culture in Amicon Ultra Centrifugal Filter devices at 4000 x g , 15m 4 °C , 00:15:00 /cycle or Sartorius Vivaspin 15R at 5000 x g .

Note

This step may take you all day if you prepare a large scale of synuclein.

33 Once all the protein is concentrated down to 10 mL total, filter through a 0.45 µm syringe filter into a 50mL conical tube.

34 Load the protein sample onto the column using the A1 inlet, **MAX of** 13 mL**.**

34.1 When the sample is getting close to the bottom of the red stopper, pause the ÄKTA and add 1-2mL of Gel Filtration Buffer into the 50mL conical so you can pull up more protein, continue the program and stop it when it is close to the bottom again.

> Note
>
> **MAKE SURE YOU PULL UP NO AIR.**

35 Once the sample is all loaded, continue to the “next breakpoint” to start the elution

a. **Manual->Execute Manual Instructions**
   i. **Other-> Next Breakpoint->Insert->Execute**
      1. Select **continue**
         a. The program should start up again

36 Make 4 L of Buffer A, and place in the cold room.

37 Take 10 µL of the even fractions, mix with 2.5 µL 5x sample buffer run on 15% acrylamide gels (will need 2 to see all the fractions of interest).

38 Coomassie stain (or Instant Blue) the gels and collect the clean fractions from the gel filtration, primarily avoiding the high molecular weight protein that has a similar charge to α-synuclein **(E.1).**

39 Cut dialysis tubing (12-14 kD tubing) and wash in Buffer A to soften the tubing.

40 Pipette the fractions into the tubing and dialyze the fractions against Buffer A Overnight . 15m

41 After the elution, the program will continue automatically to wash the column, and store it in 20% Ethanol.

Day 6: MonoQ Column (1)

42

Note

MonoQ Column separates by ion affinity and may need to be calibrated based on overall charge, and the ramp may need to be determined empirically. 25%B has worked for tagged proteins before, α-synuclein elute at a higher %B than tagged α-synuclein. 1-2 runs/L of cell culture and up to 20 mL of sample/run. Make sure the fraction collector has 33/run 15 mL tubes and is centered correctly. After column prep, the fraction tube should be transferred to the fraction collector arm.

Connect the MonoQ column between ports 2A (top) and 2B (bottom) **(S.1)**

43 Switch inputs A1, A2 and B1 and start the MonoQ program **(S.3).** Lines should be set up as:

- A1-Buffer A

- A2-Sterile Distilled Water
- B1-Buffer B
- B2-20% Ethanol

44 Load the sample using A1. While the sample is loading collect the flow through to test so you know you are not losing protein that is not sticking to the column.

44.1 When the sample is getting close to the bottom of the red stopper, pause the ÄKTA and add 3 mL of buffer A into the 50 mL conical so you can pull up more protein, continue the program and stop it when it is close to the bottom again.

> Note
>
> **MAKE SURE YOU PULL UP NO AIR**

45 Once the sample is loaded, continue to the “next breakpoint” to start the wash and anion exchange.

46 Take 10 µL of the even fractions as they come off, mix with 2.5 µL 5x sample buffer run on two 15% acrylamide gels. If you will run a second day of MonoQ, you can pick the fractions to keep based on the UV trace alone.

47 Coomassie stain the gels and collect the clean fractions from the gel filtration, primarily avoiding proteins that are not α-synuclein **(E.2).** This will primarily manifest right before and right after the main peak.

48 Dialyze the fractions against Buffer A (or DPBS if this is pure enough) Overnight. 15m

Note

To increase purity >90% a second day of MonoQ is recommended. Dialyze the selected fractions back into buffer A Overnight.

Day 6: MonoQ Column (2)

49 Repeat steps 44-48 above, and keep fractions based on coomassie and UV trace and dialyze into DPBS, 20m

pH 7.0 Overnight.

50 The primary goal is to get rid of truncated α-synuclein. This shows up as a hump on the later end of the a-synuclein peak and can be visualized as truncated forms on the Coomassie-stained gel **(E.3).**

Day 7: Concentrate and Aliquot

51 Concentrate down to approximately 2 mL of α-synuclein or molar equivalent in Amicon Ultra Centrifugal Filter devices at 4000 x g, 4 °C, 00:10:00/cycle. 10m

Note

The concentration needs to be over the 5 mg/mL or 15 mg/mL concentration at which the protein will be aggregated.

51.1 Measure the protein concentration by a BCA assay with sample dilutions of 1:10, 1:20, 1:40, 1:60, 1:80, 1:160, 1:320, 1:640.

51.2 Load only 10 µL of each sample into the BSA assay.

51.3 Take the average of the concentration estimations for which values are in the linear range of the BSA curve.

> **Note**
>
> You can expect 10-50 mg protein/L culture, depending on the construct.

52 Filter the protein through a 0.22 µm filter. Since this is a small volume, the small blue syringe filters are ideal to minimize sample loss.

53 Aliquot the protein into tubes with the protein type, concentration, date and initials.

54 Freeze at -80 °C (monomer) or proceed with aggregation (fibrils).

55 For aggregation, aliquot the protein out into low binding tubes.

> **Note**
>
> You want at least 750 µL per tube, 1 mL is ideal.

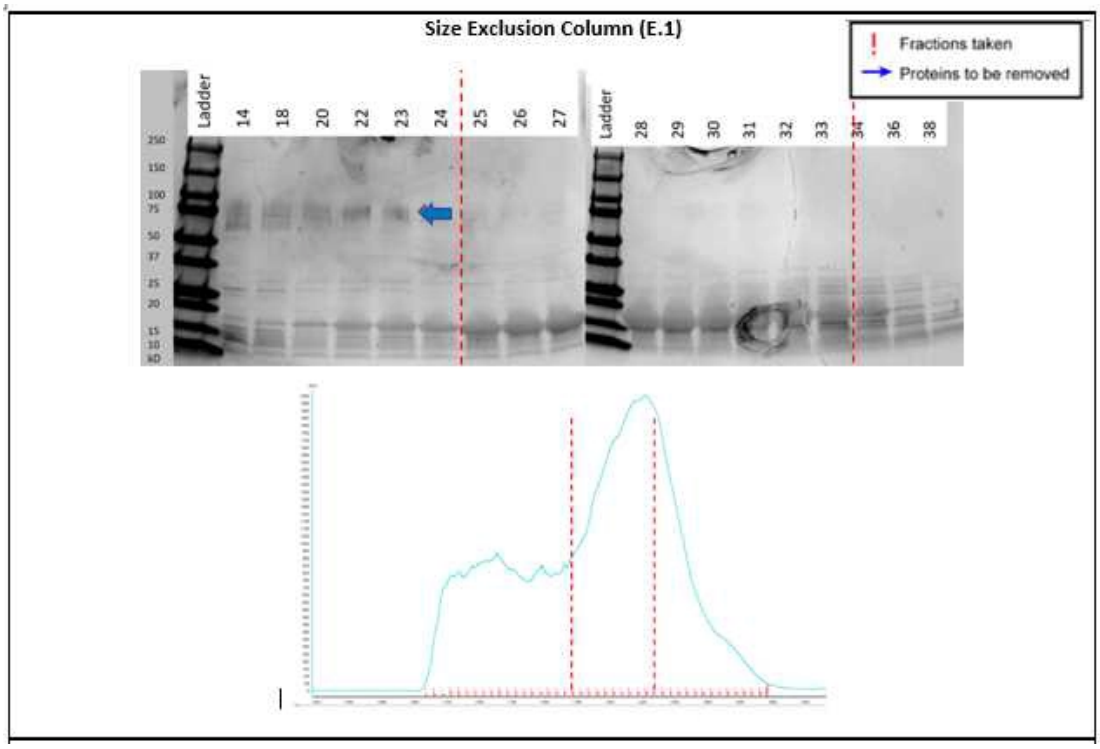
56 Place tubes in a heated shaker at 1000 rpm at 37 °C for 168:00:00. 1w

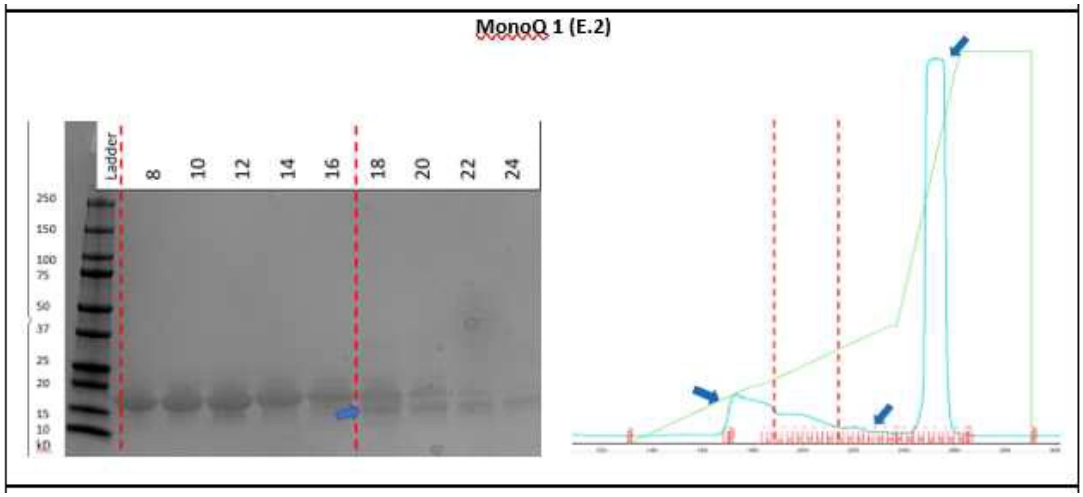
57 After 7 days, aliquot the fibrils into small aliquots to avoid freeze thaw cycles, and store in the mid/back of the -80 °C freezer.

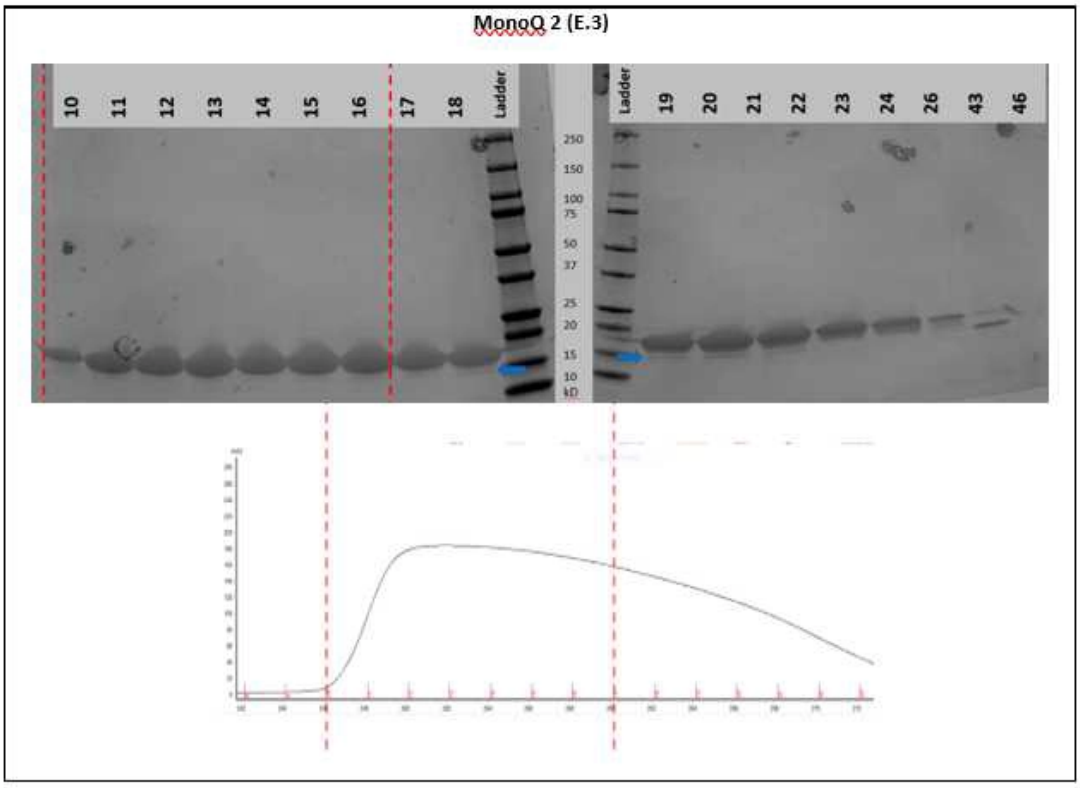
58 For quality control the sedimentation assay and thioflavin T assay should be done after they fibrillization (see additional protocols).

Examples of Mouse Alpha Synuclein Prep done on 12.2020

59







Supplemental Information: S.1 Preparing the AKTA Chromatography Syste…

60 Place all the lines in their corresponding bottles.

61 Using the 25 mL Syringe pull 20 mL through the lines at the correct pump location, do this slowly, if you go too quick you can introduce air into the system die to back pressure. To do this:

61.1
- Go to **Manual->Execute Manual Instructions->Flow path**
  a. Then you can choose **Inlet A** or **Inlet B**
    i. Then choose your flow path (**A1** or **A2** for A, **B1** or **B2** for B)
      a. Select **Insert**
    ii. Hit the **Execute** button

61.2 You can now pull the liquid out of the corresponding pumps you just turned on. Do this by:

- Turning the knob on the outside of the pump and inserting the syringe tip into the opening and slowly pulling back
- Make sure to tighten the pump knobs once you have finished pulling the lines

Note

Repeat this procedure until you have pulled out 20 mL from each line.

Supplemental Information: S.1 Preparing the AKTA Chromatography Syste…

62

Note

ALWAYS DETACH BOTTOM TO TOP, AND ATTACH TOP TO BOTTOM!

Manual->Execute Manual Instructions-Insert the following instructions

62.1 **i. Pumps**
1. System Flow: **2 ml/min**
2. Pressure control: **System Pressure**
   a. **INSERT**

62.2 **ii. Flow Path**

1. **Inlet B-> B2-> INSERT**
2. **Column Position->** select **1-> INSERT**

62.3 **iii. Alarms**

1. Alarm system pressure-> **Enabled**
2. High alarm = **0.5 mPA**
    a. **INSERT**

63 Confirm on the screen that the B2 path is highlighted and flowing over the column to waste at 2 mL/min or slower.

64 Unscrew the bottom connector from the Q column (20% Ethanol should be dripping out) and cap the bottom.

> **Note**
>
> The pressure alarm is going to go off, this is normal.

65 Loosen the connector at the top of the column, and hit continue on the program.

66 Fill the top of the Q column with 20% Ethanol and screw the top cap on.

> **Note**
>
> If connecting the Superdex 200 immediately, you can let the Ethanol drip for a minute while you change them. Otherwise, hit pause.

Supplemental Information: S.1 Preparing the AKTA Chromatography Syste…

67 If you just detached the Q column you can continue on, if the system was off perform the steps above (1.i->1.iii), their should be 20% Ethanol dripping from the top connecting line.

68 Insert the column into the holders on the ÄKTA carefully (bottom should be in line with the bottom of the pumps).

69 Remove the cap from the top of the column, 20% Ethanol should start dripping out of the tubing from the column. Remove the tubing from 1A location on the ÄKTA (this is the location the 20% Ethanol should be dripping from), connect the column to the port on the machine in a drip to drip fashion.

Note

IF YOU SEE ANY AIR BUBBLES DETACH IMMEDIATELY and let the solution flow back out of the tubing.

70 Detach the buffer reservoir from the bottom of the superdex column, you should see It dripping out now.

71 Attach the tubing to the line coming from the 1B position on the ÄKTA.

72 Buffer should be flowing and the column should be ready to go.

Supplemental Information: S.1 Preparing the AKTA Chromatography Syste…

73 **Manual-> Execute Manual Instructions**-Insert the following instructions

73.1 i. **Pumps**
1. System Flow: **2 ml/min**
2. Pressure control: **System Pressure**
    a. **INSERT**

73.2 **ii. Flow Path**
1. Inlet B-> B2-> INSERT
2. Column Position->select **1 -> INSERT**

73.3 **iii. Alarms**
1. Alarm system pressure-> **Enabled**
2. High alarm = **0.5 mPA**
    a. **INSERT**

74 When you see 20% Ethanol dripping from system, you can detach the bottom connector and attach the buffer reservoir, allow the ÄKTA to flow over the column until the reservoir is filled so that the stopper is pass the line.

75 Detach the top of the column, 20% Ethanol should be dripping out, quickly cap the top of the tubing off.

76 Carefully remove the column and store it in the bottom of the fridge.

Supplemental Information: S.1 Preparing the AKTA Chromatography Syste…

77 If you just detached the Superdex column you can continue on, if the system was off perform the steps above (1.i->1.iii), there should be 20% Ethanol dripping from the top connecting line.

78 Attach the tubing to the port 1A on the machine, when 20% Ethanol starts dripping from the tubing, remove the cap from the Q trap.

79 Fill the top of the Qtrap with Ethanol so that it is bubbled up at the top and attach the connector to the column.

> **Note**
>
> The alarm will go off, that is normal.

80 Remove the cap from the bottom of the column and continue to run the program on the computer.

81 Connect the line from port 1B to the column.

82 The column should now have the 20% Ethanol running over it.

S.2 Size Exclusion Chromatography Program- No Wash Size Exclusion As…

83 **Method settings:**

- column selected (HiLoad 26/600 Superdex 200pg)
- pressure limit should be 0.5 MPa,
- column volume (CV): 318.557ml
- column position: 1
- flow rate 2.6 ml/min
- control the flow to avoid overpressure should be selected.

84 **System prep:**

- A2 is selected
- Injection valve with capillary loop is selected to be washed
- Fraction collector is selected to be washed

85 **Column Wash:**

- 2.6 ml/min (select use the same flow rate as in methods setting)
- A2 is shown 0% B1
- Wash is 1 CV sent to waste (~ 320 mL)

86 **Equilibration:**

- 2.6 ml/min (select use the same flow rate as in methods setting)
- A1 is shown, 0% B
- Equilibrate until 2 CV (~ 640 mL)

87 **Miscellaneous**

87.1 Message is selected “load sample”.

87.2 Pause after message is selected.

88 **Load sample**

88.1 Select “reset UV monitor”.

88.2 Select “use the same flow rate as in method setting”.

88.3 A1 should be shown with 0% B.

88.4 Equilibrate until “the total volume is 0.2 CV” is selected.

89 Elution

89.1 Select “use the same flow rate as in method settings”.

89.2 A1 should be shown.

89.3 Isocratic elution should be selected Volume: 1.5 CV 0% B.

89.4 Fractionate: select using fraction collector

1. Fraction type: Peak Fractionation
2. Peak Frac settings: 50.00 mAu for both start and end level
3. Peak fractionation volume 5 mL

89.5 Select start fractionation after 0.2 CV.

90 **Water wash**

90.1 Select use the same flow rate as in method settings.

90.2 A2 should be shown as the inlet A.

90.3 Wash until: select “the total volume is 2 CV.

90.4 Fractionate: select “in waste”.

91 Ethanol equilibration B2.

S.3 Henderson Lab MonoQ Qtrap HP Protocol

92 This method will include the following steps

System prep

- Wash the system with 20 mL volume per position at 10 mL per min from inlet A1. Make sure the column position “by-pass” is selected.

93 **Equilibration**

- 5 ml/min wash of column with A1 inlet for 5CV

94 **Miscellaneous**

- pauses the system with the message “load sample”

95 **Load sample**

- 2 ml/min through A1 for 20 CV

96 **Column wash**

- 2 ml/min wash of column with A1 inlet for 5 CV

97 **Elution-15%**

- 2 ml/min linear gradient elution from 100% A1 inlet to 15% B1 inlet over 10 CV.
- Collecting 2 mL fractions after UV goes to 25.00mAu

98 **Elution-30%**

- 2 ml/min linear gradient elution from 15% B1 to 30% B1 over 10 CV.
- Collecting fixed 2 mL fractions starting at 15%

99 **Elution-100%**

- 2 ml/min linear gradient from 30% B1 to 100% B1 over 5 CV.
- Collecting 2 mL fixed fractions

100 **Column wash**

- 5 ml/min 100% B1 inlet over 5 CV

101 **Equilibration of Column**

- 5 ml/min A1 inlet for 5 CV

102 **Ethanol wash**

- 5 ml/min 100% B2 inlet line for 5CV