EDDU Protocols

Production of Recombinant α-Synuclein Monomers and Preformed Fibrils (PFFs)

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Name	Function:	Role	Date	Signature
Dr. Thomas Durcan	R&D	Director, MNI Early Drug Discovery Unit (EDDU)		

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1 Introduction

1.1 Objectives

This protocol describes how to:

- Produce and purify recombinant human and mouse α-synuclein in monomeric form from transformed bacteria
- Generate α-synuclein preformed fibrils (PFFs) from recombinant monomeric α-synuclein
- Fluorescently label α-synuclein PFFs

1.2 Protocol Overview

Using conventional methods for bacterial transformation and large-scale protein expression, plasmids containing glutathione S-transferase (GST)–tagged full-length recombinant human (NM_000345) or mouse (NM_001042451) α-synuclein are expressed in BL21 (DE3) *Escherichia coli* (**Figure 1**). In parallel, a plasmid containing GST-tagged recombinant 3C enzyme, which is capable of cleaving the GST tag from GST-tagged proteins, is also expressed. The GST-tagged proteins are purified from the bacterial cell lysates by affinity column chromatography.

The purified GST-α-synuclein protein is treated with GST-3C enzyme to remove the GST tag. Untagged α-synuclein protein is purified from the reaction using affinity column chromatography and then further purified using size exclusion chromatography.

The recombinant monomeric α-synuclein protein can be used for experiments directly, or for generating PFFs through aggregation. PFFs can then be fluorescently labelled if desired, depending on the experiment.

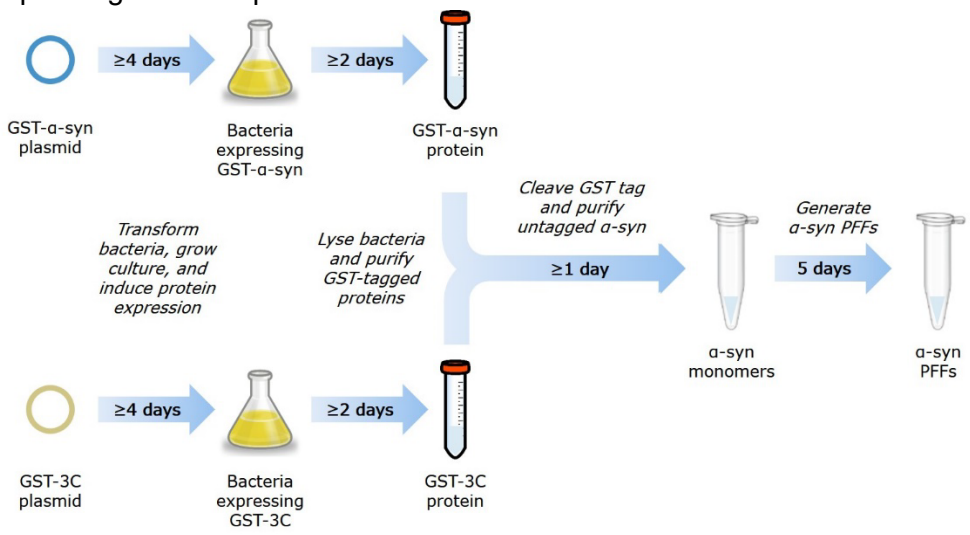


Figure 1. Protocol overview for the expression and purification of GST-tagged α-synuclein (GST-α-syn) and 3C enzyme (GST-3C), cleavage of GST tags and purification of untagged α-synuclein, and generation of α-synuclein PFFs.

Untagged α-synuclein protein (monomeric form) should be tested for bacterial endotoxin to ensure that levels are below the threshold for the endotoxic response in mice and human cells. Note that some animal species have a lower threshold for the endotoxic response and can only tolerate proteins produced in bacterial strains with a disabled endotoxin signal (e.g. ClearColi bacteria must be used to produce α-synuclein for marmosets).

1.3 List of updates from previous versions of the protocol

- Centrifuging speed was adjusted (page 14)
- Volume of cold wash buffer to be used was changed (page 14)
- The volume of sample to be loaded in the SDS mini gel was corrected (pages 14 and 16)
- PBS to replace cold wash buffer to wash the column (page 16)
- Updated sequence of inserts (pages 21 and 22)

1.4 Technical and safety considerations

The following information should be read before starting:

- All material and reagents should be sterile or autoclaved:
  - Glass flasks should be capped with aluminum foil and autoclaved to sterilize.
  - Lysogeny broth (LB) medium should be autoclaved on the liquid cycle for 30 minutes exposure time at 121°C to sterilize. After autoclaving, cool LB medium to room temperature before using.
- Use sterile technique and work over a flame during procedures involving bacterial cells and cultures.
- During the first procedure of expressing and purifying the GST-tagged proteins (section 3.1), it is possible to pause prior to sonication of the bacterial culture.
- Following purification of the GST-tagged proteins (section 3.1), it is necessary to proceed directly to the cleavage reaction (section 3.2). The purified GST-tagged proteins are sensitive to degradation should not be stored to perform cleavage reaction at a later time.
- All users of the ÄKTA pure protein purification system must be trained. The Superdex 200 16/600 columns should be used with care following proper training.
- All users of the Bioruptor Plus sonication device and water cooler must be trained.
- While working with α-synuclein PFFs:
  - Wear appropriate personal protective equipment at all times, including disposable gloves, a laboratory coat, a face mask, and protective goggles.
  - Perform all procedures in a sterile Class II biosafety (BSLII) cabinet.
  - Avoid inhaling PFFs or getting aerosolized PFFs in eyes.

- After working with α-synuclein PFFs:
  - Clean area with 1% SDS to destroy fibrils, then rinse area with sterile distilled water.
  - Dispose of all tips and tubes into a large empty bottle or a 50-mL tube containing 1% SDS. Store items in 1% SDS for up to 2 hours to disassemble the PFFs.
  - Dispose of gloves and face mask in an autoclavable biohazard bag to be sterilized.
- For further information on the use of SDS to break down α-synuclein PFFs, refer to Bousset et al. 2016. *J. Parkinson Dis*. (6) 143–151.
- Store α-synuclein PFFs at –80°C and thaw at room temperature. Do not store PFFs on ice or at 4 °C, as this causes dissociation and reduces activity. Avoid repeated freeze-thaw cycles.
- Do not vortex α-synuclein PFFs.

2 Materials

The material, reagents, and equipment listed in this document can be substituted for those supplied by other manufacturers. However, the performance of the assay may not be the same and may need to be optimized or redeveloped upon significant modifications to the materials and/or methods.

Refer to the product data sheet from the supplier for further details on storage and preparation instructions.

2.1 Labware

Item	Supplier	Catalogue #
Sterile needle, 22G 1½"	Becton Dickinson	305156
Aluminum foil	Sigma Aldrich	Z185140
Amicon Ultra-15 Centrifugal filter unit	Millipore	UFC900324
Autoclave tape	Fisher Scientific	15903
Chromatography columns, 10-mL, disposable (with stopcocks)*	Thermo Scientific	29924
Chromatography columns, 20-mL, disposable (with stopcocks)*	BioRad	732-1000
Column holder	Fisher Scientific	22-036466
Cryovial	Fisher Scientific	50-873-794
Erlenmeyer flask, glass, 125-mL	Sigma Aldrich	CLS4980125
Erlenmeyer flask, glass, 2-L	Fisher Scientific	FB5002000
Glass bottle with cap, 1-L	Fisher Scientific	C13951L
GSTrap 4B	GE Healthcare Life Sciences	28401745
Mini-PROTEAN® Tetra cell	BioRad	1658004
Mini-PROTEAN® Tetra cell casting module	BioRad	1658021
Parafilm	Sigma Aldrich	P7793
Petri dish, 100 mm x 15 mm	Fisher Scientific	FB0875712
Plate, 96 deepwell	Eppendorf	0030504305
Plate, 96-well, clear	BD Falcon	351172
Polypropylene bottles, 500-mL	Beckman Coulter	361691
Polypropylene bottles, 50-mL	Beckman Coulter	361694
Slide-A-Lyzer™ MINI Dialysis Device, 3.5K MWCO, 0.5 mL	ThermoFisher Scientific	88400
Spectrophotometer cuvette	Sigma Aldrich	C5416
Sterile disposable filter unit, 0.2-µm	ThermoFisher Scientific	566-0020
Sterile syringe filter, 0.2-µm	VWR	28145-501
Sterile syringe, 10-mL	Becton Dickinson	309604
Sterile syringe, 1-mL	Becton Dickinson	309628

Item	Supplier	Catalogue #
Superdex 200 16/600 column	GE Healthcare Life Sciences	28-9893-35
Support stand	Fisher Scientific	22-260469
Tubes, 50-mL	Fisher Scientific	14-959-49A

*Reusable chromatography columns may be used as an alternative to disposable columns (e.g. 50-mL reusable chromatography columns [Bio-Rad Laboratories #7374251]).

2.2 Reagents

Item	Supplier	Catalogue #	Stock conc.	Working conc.	Storage temp.
Acetic Acid, Glacial (Certified ACS)	Fisher Scientific	A38-450LB	100%	Various	RT
Acrylamide/ bisacrylamide (29:1), 30%	BioRad	1610156	30%	Various	4°C
Agar	BioShop	AGA001	NA	15 g/L	RT
Alexa Fluor™ 488*	Molecular Probes	A20000	5 mg/mL	~161 µg/mL	Stock: –20°C
Alexa Fluor™ 568*	Molecular Probes	A20003	5 mg/mL	~161 µg/mL	Stock: –20°C
Alexa Fluor™ 633*	Molecular Probes	A20005	5 mg/mL	~161 µg/mL	Stock: –20°C
Ammonium persulphate (APS)	Sigma Aldrich	A3678	10%	0.005%	RT
Ampicillin	Sigma Aldrich	A9393	100 mg/mL	100 µg/mL	Stock: –20°C Working: 4°C
Aprotinin	Sigma Aldrich	A1153	0.5 mg/mL	0.5 µg/mL	Storage: –20°C Working: 4°C
Benzamidine	Sigma Aldrich	12072	0.5 mg/mL	0.5 µg/mL	Storage: –20°C Working: 4°C
BL21(DE3) Competent *E. coli* cells	New England Biolabs	C2527H	NA	NA	–80°C
Bradford Assay Kit	ThermoFisher Scientific	23236	NA	NA	4°C
Bromophenol blue	Sigma Aldrich	B0126	100%	0.1%	RT
Coomassie stain, Bio-Safe™	BioRad	1610786	100%	100%	RT
Dimethyl sulfoxide (DMSO)	Sigma Aldrich	D8418	NA	NA	RT

Item	Supplier	Catalogue #	Stock conc.	Working conc.	Storage temp.
Dithiothreitol (DTT)	Biobasic	DB0058	1 M	Various	Stock: –20°C Working: RT
Ethanol (EtOH)	Fisher Scientific	P06EAAN	100%	20% (v/v)	RT
Glutathione	Sigma Aldrich	G4251	NA	20 mM	4°C
Glutathione Sepharose bead slurry	GE Healthcare Life Sciences	17075605	NA	NA	4°C
Glycerol	Fisher Scientific	G33-1	100%	Various	RT
Glycine	Wisent Biosciences	800-045-IK	NA	192 mM	RT
GST-α-synuclein plasmid, human	NA	NA	100 ng/µL	2 ng/µL	–20°C
GST-3C plasmid	NA	NA	100 ng/µL	2 ng/µL	–20°C
GST-α-synuclein plasmid, mouse	NA	NA	100 ng/µL	2 ng/µL	–20°C
Isopropyl-β-D-thiogalactoside (IPTG)	Biobasic	IB0168	0.5 M	300 µM	Stock: –20°C
LAL chromogenic endotoxin quantification kit†	Thermo Scientific	88282	NA	NA	4°C
LB broth (Miller)	BioShop	LBL407	NA	25 g/L	RT
Leupeptin	Sigma Aldrich	L2884	0.5 mg/mL	0.5 µg/mL	Stock: –20°C Working: 4°C
Methanol (Certified ACS)	Fisher Scientific	A412-1	100%	40%	RT
Paraform-aldehyde (PFA)	Thermo Fisher	28908	100%	4%	RT
Phenylmethane-sulfonyl fluoride (PMSF)	Sigma Aldrich	P7626	0.5 M	5 mM	Storage: –20°C Working: 4°C
Phosphate-buffered saline (PBS)	Wisent Bioproducts	311-012	10x	1x	RT
Protein molecular weight standards	BioRad	1610373	NA	NA	–20°C
SOC medium	New England Biolabs	B9020S	NA	NA	RT or 4°C

Item	Supplier	Catalogue #	Stock conc.	Working conc.	Storage temp.
Sodium chloride (NaCl)	Wisent Bioproducts	600-08	NA	400 mM	Working: 4°C
Sodium dodecyl sulfate (SDS)	Wisent Bioproducts	800-100	10%	Various	RT
Tetramethyl-ethylene-diamine (TEMED)	BioShop	TEM001.50	100%	Various	4°C
Tris-HCl	Wisent Bioproducts	600-126	Various	Various	RT
Triton X	Sigma Aldrich	X100	20%	0.5%	Stock: RT Working: 4°C

*Light-sensitive reagent. Stock and working aliquots should be covered in aluminum foil to minimize exposure to light.
†The LAL chromogenic endotoxin quantification kit contains components that are light-sensitive in lyophilized form (LAL and chromogenic substrate). Light-sensitive components should be covered to minimize exposure to light.

2.3 Equipment

Item	Supplier	Catalogue #
ÄKTA pure L protein purification system	GE Healthcare Life Sciences	29018224
Beckman Coulter Avanti J-25I centrifuge (with JLA-10.500 and JA-25.50 rotors)	Beckman Coulter	BE-J25
BioDrop DUO Spectrophotometer	BioDrop	80-3006-61
Bioruptor® Plus sonication device with metallic soundproof box (B01200001) and water cooler (B02010003)	Diagenode	B01020001
CPXH Series Ultrasonic Cleaning Bath	Fisher Scientific	15-336-126
Digital heating shaking drybath or thermomixer	ThermoFisher Scientific	88880027
	Eppendorf	2231000574
Ecotron Incubation Shaker (with cooling)	INFORS HT	Ecotron
Fisherbrand Model 120 Sonic Dismembrator with sound enclosure box	Fisher Scientific	NA
Getinge Castle Vacuum Gravity Steam Sterilizer (autoclave)	Getinge	700HC-E SERIES
Heating block	Eppendorf	5383000027

Item	Supplier	Catalogue #
Infinite® 200 PRO series plate reader	Tecan	INF-MPLEX
Low-speed orbital shaker	Corning LSE	6780-FP
New Brunswick Innova 42 incubator shaker	Eppendorf	M1335-0010
Nutating Mixer	VWR	8207-202
PowerPac Universal Power Supply	BioRad	1645070
Reciprocal shaking bath	Precision™	NA (discontinued)
Stirrer plate	VWR	97042-706
Temperature-controlled orbital shaker	Barnstead	MaxQ
Thermo Scientific Heraeus Megafuge 40R Refrigerated Centrifuge	Fisher Scientific	75004518
UNICORN™ 7.0.2 software	GE Healthcare Life Sciences	29115456
Vortex mixer	Corning LSE	6776

3 Protocol

3.1 Expression and purification of GST-α-synuclein and GST-3C enzyme

Materials:

- BL21 (DE3) competent *E. coli* cells
- Plasmid(s):
  - Human GST-α-synuclein expression plasmid (100 ng/µL)
  - Mouse GST-α-synuclein expression plasmid (100 ng/µL)
  - GST-3C enzyme expression plasmid (100 ng/µL)
- SOC medium
- Ampicillin (100 mg/mL)
- 125-mL Erlenmeyer flasks
- 2-L Erlenmeyer flasks
- 500-mL capped polypropylene bottles
- 50-mL capped polypropylene bottles
- 50-mL tubes
- 0.2-µm sterile syringe filter and 22G 1½" sterile needle
- 10- or 20-mL disposable chromatography columns
- Support stand and column holder
- Amicon Ultra-15 Centrifugal Filter Unit
- Parafilm
- IPTG (0.5 M)
- 1x PBS
- 20% EtOH in ddH_2O
- Glutathione sepharose bead slurry

- Solutions:

Solution	Components
LB medium (autoclaved)	• 1 L ddH_2O • 25 g LB broth
Resuspension buffer (80 mL; prepare fresh and chill on ice before use)	• ddH_2O • 25 mM Tris, pH 8 • 400 mM NaCl • 5% glycerol • 0.5% Triton X • 5 mM PMSF • 0.5 mg/mL benzamidine • 0.5 µg/mL leupeptin • 0.5 µg/mL aprotinin • 1 mM DTT
Wash buffer (500 mL; chill on ice)	• ddH_2O • 50 mM Tris, pH 8 • 400 mM NaCl • 5% glycerol • 1 mM DTT (add fresh before use)
Elution buffer (20 mL; prepare fresh and chill on ice before use)	• Wash buffer • 20 mM glutathione (once dissolved adjust pH to 8)

- LB agar plates containing 100 µg/mL ampicillin (see Appendix 4.4 for procedure)
- SDS-PAGE and gel staining materials (see Appendix 4.8 for procedure)
- Bradford Assay kit and materials (see Appendix 4.6 for procedure)
- 42°C water bath
- Temperature-controlled orbital shaker
- 37°C bacterial incubator
- Beckman Coulter Avanti J-25I centrifuge with JLA-10.500 and JA-25.50 rotors
- Spectrophotometer and cuvettes
- Fisherbrand Model 120 sonic dismembrator
- Bioruptor Plus sonicator
- Thermo Scientific Heraeus Megafuge 40R Refrigerated Centrifuge
- Nutating mixer

Procedure (for each plasmid):

1. Thaw vial of BL21 (DE3) competent *E. coli.* cells on ice for 10 minutes.
2. Add 1 µL (100 ng) of plasmid to the cells and gently flick the tube 4 to 5 times to mix. Incubate cells on ice for 30 minutes.
    - Do not vortex cells to mix.
3. Place cells in a 42°C water bath for 10 seconds. Transfer cells to ice and incubate for 5 minutes.
4. Add 950 µL of SOC medium to the cells. Shake cells at 200 rpm at 37°C for 1 hour.
    - During the incubation, warm LB-ampicillin agar plates to 37°C.
5. Spread 20 µL of cells onto warm LB-ampicillin agar plates and incubate plates at 37°C overnight.
6. Add 20 mL of LB medium to a 125-mL Erlenmeyer flask. Add ampicillin to the medium to a final concentration of 100 µg/mL.
7. Use a sterile 20 µL pipette tip to pick a single, isolated colony from the plate and eject it into the flask containing the LB medium. Shake culture at 200 rpm at 37°C overnight.
    - Plates with colonies can be stored at 4°C for up to 1 month. For long-term storage of transformed bacteria, prepare a glycerol stock (see Appendix 4.5 for procedure).
8. Add 500 mL of LB medium to each of four 2-L Erlenmeyer flasks. Add ampicillin to the medium in each flask to a final concentration of 100 µg/mL.
    - Larger flasks may be used if available as long as the volume of medium is appropriate for the flask size (e.g. 2 L of LB medium in one 8-L flask).
9. Transfer 5 mL of overnight culture to each of the 2-L flasks containing the LB medium. Shake at 200 rpm at 37°C and 200 rpm. Monitor the optical density at 600 nm (OD_{600}) until it reaches 0.6 (see Appendix 4.7 for procedure).
    - It usually takes 3 to 5 hours or longer for the OD_{600} to reach 0.6.
10. Add IPTG to the culture to a final concentration of 300 µM to induce protein expression. Reduce shaker temperature to 16°C and shake culture at 200 rpm at 16°C for 18 hours (overnight).

11. Divide the culture among six 500-mL polypropylene bottles, ensuring that all filled bottles are of equal weight. Close bottles with caps and centrifuge at 5000 G at 4°C for 30 minutes using a JLA-10.500 rotor.

    - Place each bottle into a canister, and then place each canister into a cavity in the JLA-10.500 rotor.
    - Place the rotor lid on top of the rotor and fasten the round knob securely into the centrifuge.

12. Remove the supernatant and resuspend each pellet with 20 mL of cold resuspension buffer. Combine the culture and divide into four 50-mL capped polypropylene bottles, ensuring that both filled bottles are of equal weight (approximately 30 mL per bottle).

    - If necessary, the supernatant may be stored at –80°C until ready to sonicate. Once the frozen supernatant has been thawed, proceed immediately to the next step.

13. Sonicate the cells on an ice bath using the sonic dismembrator on the following settings: 5 cycles; 30 seconds ON/30 seconds OFF, 60% power.
14. Close bottles with caps and centrifuge at 18,000 rpm at 4°C for 30 minutes using the JA-25.50 rotor.
15. After the lysates have been cleared by centrifugation, transfer the supernatant from each bottle into 50-mL tubes.
16. To check the expression of GST-α-synuclein, reserve 100 µL of the supernatant (cleared lysate) for analysis on a 14% polyacrylamide SDS-PAGE gel (see Appendix 4.8 for procedure).

    - Recombinant GST-α-synuclein will run at approximately 40 kDa on a 14% polyacrylamide SDS-PAGE gel (**Figure 2**).

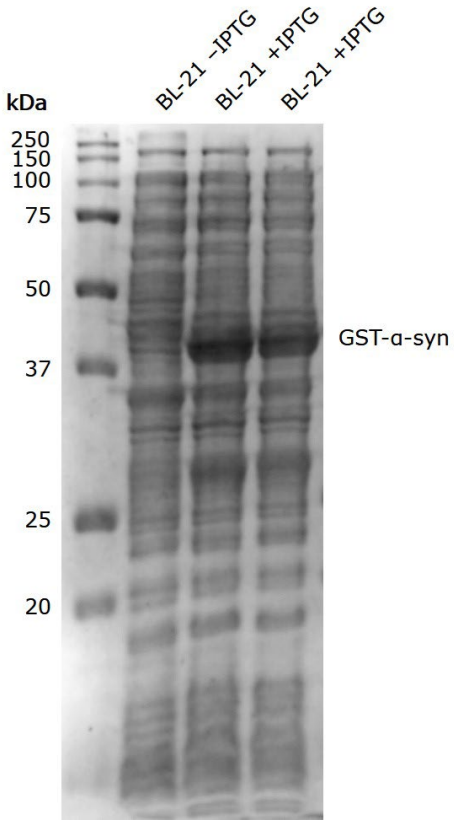


Figure 2. 14% polyacrylamide SDS-PAGE gel of recombinant GST-α-synuclein (GST-α-syn) in BL-21 cell lysate control (BL-21 –IPTG) and in BL-21 cell lysates induced with IPTG (BL-21 +IPTG).

17. Add 5 mL of glutathione Sepharose 4B bead slurry to two clean 50-mL tubes. Wash the beads by adding 30 mL of ddH_2O to each tube, centrifuge at 3000 rpm at 4°C for 3 minutes and remove liquid.
18. Wash the beads by adding 30 mL of cold wash buffer to each tube, centrifuge at 4°C at 3000 rpm for 3 minutes and remove liquid. Repeat this step two more times.
19. Pass the cleared lysates (from step 16) through a 0.2-µm sterile syringe filter into the 2 tubes containing the washed glutathione sepharose beads. Cap the tubes, seal with Parafilm, and incubate on a nutating mixer at 4°C for at least 24 hours.

20. Set up six 10-mL disposable chromatography columns in the column holders on the support stand. Place a vessel (e.g. beaker) under each column to collect flow-through. Transfer the lysate-bead mixtures to the columns. With the column stopcocks open, wash beads five times with 5 mL of cold wash buffer.

    - The beads will be retained in the column, while the liquid will flow through the columns.

21. Close the column stopcocks and place 50-mL tubes under the column to collect eluents.
22. Add 3 mL of cold elution buffer to each column and incubate for 10 minutes. Slightly open the column stopcocks and allow the eluents to drip into the 50-mL tubes. Repeat this step three more times.

    - The beads can be re-used to purify the same protein. Wash the columns containing the beads three times with 5 mL wash buffer then rinse three times with 5 mL ddH_2O. Add 20% EtOH to the column so that the beads are covered, and then transfer the bead solution to a clean 50-mL tube. Beads can be stored at 4°C until the expiry date on product label.

23. Combine the eluents and transfer to a single Amicon Ultra-15 Centrifugal Filter Unit. Centrifuge at 4000 xG at 4°C for 30 minutes.
24. Add 10 mL of PBS and centrifuge at 4000 xG at 4°C for 30 minutes. Repeat this step three times to concentrate the eluent to ≤4 mL.
25. Determine the protein concentration (see Appendix 4.6 for procedure).
26. Proceed immediately to Section 3.2 to cleave the GST tag off from the GST-α-synuclein. Reserve 20 µL of GST-α-synuclein to analyze by SDS-PAGE (see Section 3.2, step 9).

3.2 Cleavage of GST-α-synuclein and purification of untagged α-synuclein

Materials:

- Recombinant GST-α-synuclein protein
- Recombinant GST-3C enzyme
- 1.5-mL microcentrifuge tubes
- 96 deepwell plate
- Amicon Ultra-15 Centrifugal Filter Unit
- Superdex 200 16/600 column
- GSTrap 4B column
- Support stand and column holder
- SDS-PAGE and gel staining materials (see Appendix 4.8 for procedure)
- 1x PBS
- 20% EtOH in ddH_2O
- Solutions:

Solution	Components
Wash buffer (500 mL; chill on ice)	• ddH_2O • 50 mM Tris, pH 8 • 400 mM NaCl • 1 mM DTT (add fresh before use)
Elution buffer (20 mL; prepare fresh and chill on ice before use)	• Wash buffer • 20 mM glutathione (once dissolved adjust pH to 8)

- Bradford Assay kit and materials (see Appendix 4.6 for procedure)
- Thermo Scientific Heraeus Megafuge 40R Refrigerated Centrifuge
- Ultrasonic cleaning bath
- ÄKTA pure L protein purification system with UNICORN 7.0.2 software
- LAL chromogenic endotoxin quantification kit and materials (see Appendix 4.10 for procedure)

Procedure:

1. Add GST-3C protease to GST-α-synuclein at a 1:50 mass-to-mass ratio. Place the reaction mixture at 4°C overnight.
    - For example, add 0.3 mg of GST-3C protease to 15 mg of GST-α-synuclein.

2. Equilibrate a GSTrap 4B column connected to a 30-mL Luer Lok syringe. Place a vessel (e.g. beaker) under the column to collect flow-through. Wash column with 20 mL of ddH_2O to remove EtOH, and then wash with 20 mL of PBS.
3. Reserve 20 µL of the overnight reaction mixture to analyze by SDS-PAGE. Inject the remaining mixture into the GSTrap 4B column and collect the flow-through in a 50-mL tube. This is synuclein with GST-tag removed.

    - The column can be re-used to purify the same protein. Wash the column with 10 mL of cold elution buffer, then 10 mL of ddH_2O, and then 10 mL of 20% EtOH. Store at 4°C until the expiry date on the product label.

4. Transfer the synuclein to an Amicon Ultra-15 Centrifugal Filter Unit. Centrifuge at 4000 xG at 4°C for 30 minutes to concentrate to ≤4 mL. Reserve 20 µL of the sample to check synuclein purity on SDS-PAGE. If there are still some minor impure bands found, repeat step 2 to remove remaining GST or go further to steps 5 to 8 to remove other impurities.
5. Prepare filtered, sonicated PBS for the ÄKTA pure L system by passing 500 mL of PBS through a 0.2-µm sterile disposable filter unit and then sonicating for 3 minutes using an ultrasonic cleaning bath.
6. Start equilibration of the Superdex 200 16/600 column on the ÄKTA pure L system. The equilibration takes approximately 11 hours and should be run overnight so that the system is ready for the samples the next day. See Appendix 4.9 for instructions to set up and run the equilibration using the UNICORN software.
7. Purify the concentrated eluent with the Superdex 200 16/600 column on the ÄKTA pure L system. Run a sample method to fractionate the sample into a 96 deepwell plate, and then run a cleaning method to clean the column. See Appendix 4.9 for instructions to set up and run the sample and cleaning methods using the UNICORN software.

    - The sample method takes approximately 2 hours to run.
    - **IMPORTANT:** The column must be cleaned immediately after running the sample. The cleaning method takes approximately 15 hours to run.

8. Transfer the combined desired sample fractions to an Amicon Ultra-15 Centrifugal Filter Unit. Centrifuge at 3000 rpm at 4°C for 10 to 15 minutes.

9. To check the purity of the untagged α-synuclein, run a 14% SDS-PAGE gel alongside the 3C cleavage reaction (from step 3) and GST-α-synuclein (from Section 3.1; see Appendix 4.8 for procedure).

    - Recombinant untagged α-synuclein will run at approximately 15 kDa on a 14% polyacrylamide SDS-PAGE gel (**Figure 3**). The calculated molecular weight of α-synuclein is 14.87 kDa based on its amino acid sequence.

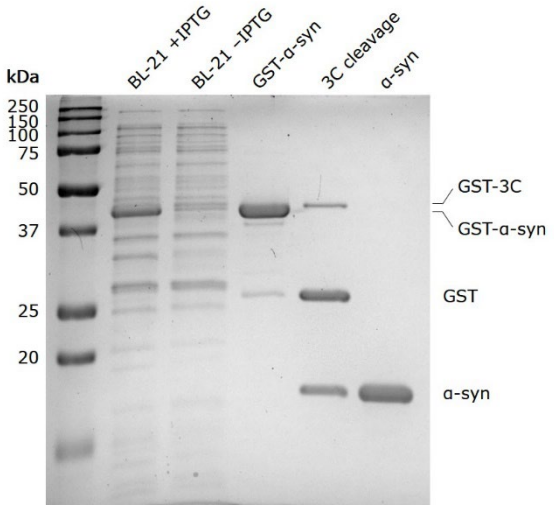


Figure 3. 14% polyacrylamide SDS-PAGE gel of GST-tagged α-synuclein (GST-α-syn), 3C protease cleavage of the GST tag (3C cleavage), and purified untagged α-synuclein (α-syn).

10. Determine the synuclein concentration, adjust to 5 mg/mL with sterile PBS (see Appendix 4.6 for procedure), and sterile passing 0.22 µm filter. Prepare 500 µL aliquots in 1.5-mL autoclaved microtubes and store at –80°C. Reserve 1 aliquot determine endotoxin levels (next step).

    - Depending on the downstream application, other aliquot volumes may be preferred (e.g. 20 µL, 50 µL).
    - Aliquots should be labelled with the batch number, name of protein, protein concentration, and date.

11. Determine the endotoxin concentration of reserved aliquot (see Appendix 4.10 for procedure).

    - When using this protocol, the endotoxin concentration is typically 100 to 1000 EU/mg of protein when α-synuclein is produced in BL-21 cells, or <100 EU/mg of protein when α-synuclein is produced in ClearColi cells.

3.3 Generation of α-synuclein PFFs

Materials:

- Recombinant monomeric α-synuclein (500-µL aliquot; 5 mg/mL)
- Parafilm
- 37°C digital heating shaking drybath or thermomixer
- Bioruptor Plus sonicator

Procedure:

1. Seal the 500-µL aliquot of monomeric α-synuclein with Parafilm. Incubate in a 37°C digital heating shaking drybath or thermomixer at 1000 rpm for 5 days.
2. Sonicate the PFFs on the following settings: 10 cycles, 30 seconds ON/30 seconds OFF, high power, 10°C water circulation.
    - Ensure that the tube holder is balanced with tubes containing equal volumes.
3. Depending on the downstream application, prepare 20- to 100-µL aliquots of PFFs in 1.5-mL tubes and store at −80°C.

3.4 Fluorescent labelling of α-synuclein PFFs

Materials:

- α-synuclein PFFs
- 1.5-mL tubes
- Aluminum foil
- Slide-A-Lyzer dialysis device and 50-mL conical tube
- Alexa-488, Alex-568, and/or Alexa-633 fluorescent dye (5 mg/mL in DMSO)
- 1x PBS
- Bradford Assay Kit and materials (see Appendix 4.6 for procedure)
- Orbital shaker at 4°C

Procedure:

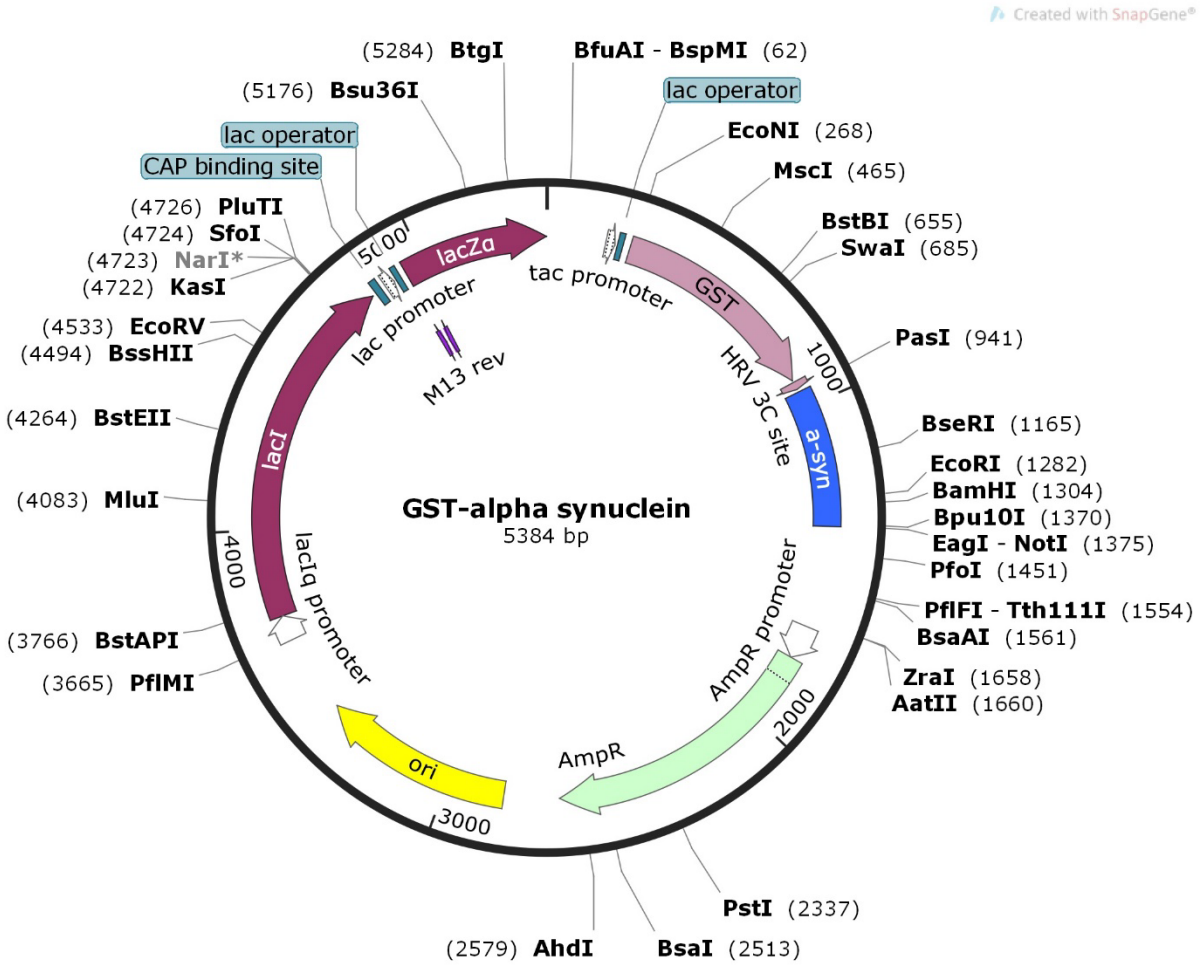
1. Add 5 µL of a fluorescent dye to 150 µL of α-synuclein PFFs and mix gently. Cover samples with aluminum foil to protect from light and incubate at room temperature for 20 minutes.
    - **IMPORTANT:** Do not vortex PFFs.
    - Incubation time may vary depending on the dye. Refer to the incubation time recommended by the manufacturer.
2. Add 1 mL of PBS to a dialysis device placed in the 50-mL conical tube. Let sit for 5 minutes to ensure the membrane does not leak (replace the dialysis device if leaking). Empty the dialysis device and remove from the tube.
3. Add 45 mL of PBS to the tube. Add the dye-PFFs mixture to the dialysis device and place the dialysis device in the tube. Cap the tube, place on the orbital shaker at 4°C, and shake at 150 rpm for at least 2 hours.
    - Ensure the membrane of the dialysis device is submerged in the PBS.
4. Remove the dialysis device from the tube to discard the PBS. Add 45 mL of fresh PBS to the tube and replace the dialysis device. Cap the tube, place on the orbital shaker at 4°C, and shake at 150 rpm for at least 2 hours.
5. Collect the labeled PFFs from the dialysis device. Prepare 20- to 50-µL aliquots in tube and store at –80°C. Reserve an aliquot of labelled PFFs to determine the protein concentration.
    - **IMPORTANT:** Store all aliquots of labelled PFFs at –80°C before determining protein concentration of reserved aliquot. Do not store PFFs on ice or at 4°C, as this causes dissociation and reduces activity.

4 Appendix

4.1 Human GST-α-synuclein plasmid

The plasmid used to express human GST-α-synuclein is pGEX-6-alpha synuclein (backbone plasmid: pGEX6P1) and was originally purchased from the University of Dundee MRC Protein Phosphorylation and Ubiquitination Unit (#DU30005).

Map of pGEX-6-alpha synuclein:



Sequence of insert:

GGATCTATGGATGTATTCATGAAAGGACTTTCAAAGGCCAAGGAGGGAGTTGTGGCTGCT
GCTGAGAAAACCAAACAGGGTGTGGCAGAAGCAGCAGGAAAGACAAAAGAGGGTGTTCTC
TATGTAGGCTCCAAAACCAAGGAGGGAGTGGTGCATGGTGTGGCAACAGTGGCTGAGAAG
ACCAAAGAGCAAGTGACAAATGTTGGAGGAGCAGTGGTGACGGGTGTGACAGCAGTAGCC
CAGAAGACAGTGGAGGGAGCAGGGAGCATTGCAGCAGCCACTGGCTTTGTCAAAAAGGA
CCAGTTGGGCAAGAATGAAGAAGGAGCCCCACAGGAAGGAATTCTGGAAGATATGCCTGT
GGATCCTGACAATGAGGCTTATGAAATGCCTTCTGAGGAAGGGTATCAAGACTATGAACCT
GAAGCCTAAGCGGCCGC

Properties of recombinant human α-synuclein (untagged):

Amino acid sequence	GPLGSMDVFMKGLSKAKEGVVAAAEKTKQGVAEAAGKTKE GVLYVGSKTKEGVVHGVATVAEKTKEQVTNVGGAVVTGVT AVAQKTVEGAGSIAAATGFVKKDQLGKNEEGAPQEGILEDM PVDPDNEAYEMPSEEGYQDYEPEA*
Molecular weight	14,871.61 DA
Theoretical pI	4.67

4.2 Mouse GST-α-synuclein plasmid

The plasmid used to express mouse GST-α-synuclein was created in the laboratory of Dr. Edward Fon (Montreal Neurological Institute, McGill University) by cloning the complete coding sequence for mouse α-synuclein into the pGEX6P1 backbone plasmid. The insert was generated by GeneArt® Life Technologies (construct #15AB4CPC).

Please refer to the plasmid map in **Appendix 4.1** for details on the pGEX6P1 backbone plasmid.

Sequence of insert:

GGATCCATGGACGTGTTCATGAAGGGCCTGAGCAAGGCCAAAGAGGGCGTGGTGGCCGC
TGCCGAAAAGACCAAGCAGGGCGTGGCCGAGGCCGCTGGCAAGACAAAAGAGGGGGTG
CTGTACGTGGGCAGCAAAACAAAAGAAGGCGTGGTGCACGGCGTGACCACCGTGGCCGA
AAAGACAAAAGAACAGGTCACAAACGTGGGCGGAGCCGTGGTGACAGGCGTGACAGCTG
TGGCCCAGAAAACCGTGGAAGGCGCTGGCAATATCGCCGCTGCCACCGGCTTCGTGAAG
AAAGACCAGATGGGCAAGGGCGAGGAAGGCTACCCCCAGGAAGGCATCCTGGAAGATAT
GCCCGTGGACCCCGGCAGCGAGGCCTACGAGATGCCCAGCGAAGAGGGCTACCAGGACT
ACGAGCCCGAGGCCTGACTCGAGCGGCCGC

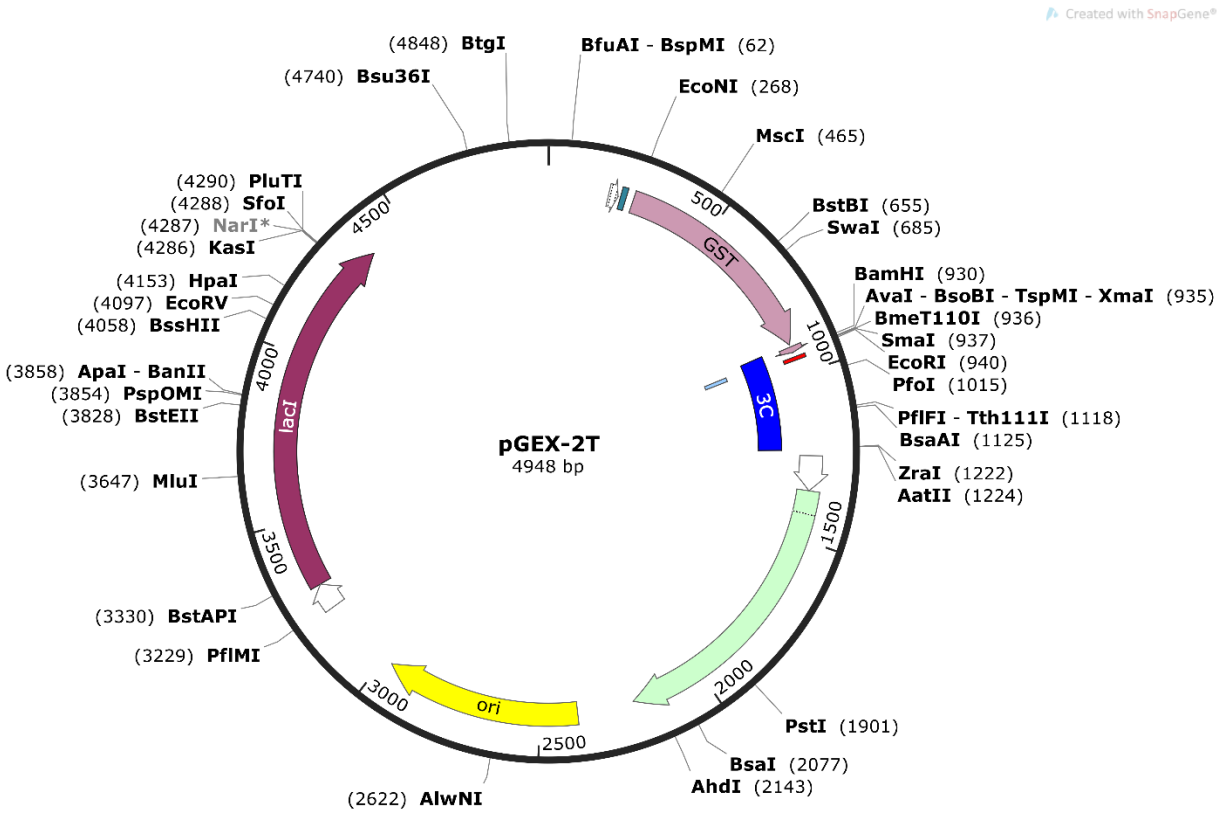
Properties of recombinant mouse α-synuclein (untagged):

Amino acid sequence	GPLGSMDVFMKGLSKAKEGVVAAAEKTKQGVAEAAGKTKE GVLYVGSKTKEGVVHGVTTVAEKTKEQVTNVGGAVVTGVTA VAQKTVEGAGNIAAATGFVKKDQMGKGEEGYPQEGILEDM PVDPGSEAYEMPSEEGYQDYEPEA
Molecular weight	14,896.68Da
Theoretical pI	4.74

4.3 GST-3C plasmid

The plasmid used to express GST-3C protease was created by cloning the coding sequence for human rhinovirus (HRV) 3C protease into the pGEX-2T backbone plasmid.

Map of pGEX-2T-3C:



Properties of recombinant HRV 3C protease (untagged):

Amino acid sequence	AFRPCNVNTKIGNAKCCPFVCGKAVTFKDRSTCSTYNLSSS LHHILEEDKRRRQVVDVMSAIFQGPISLDAPPPPAIADLLQSV RTPRVIKYCQIIMGHPAECQVERDLNIANSIIAIIANIISIAGIIFVI YKLFCSLQGPYSGEPKPKTKVPERRVVAQGPEEEFGRSILK NNTCVITTGNGKFTGLGIHDRILIIPTHADPGREVQVNGVHTK VLDSYDLYNRDGVKLEITVIQLDRNEKFRDIRKYIPETEDDYP ECNLALSANQDEPTIIKVGDVVSYGNILLSGNQTARMLKYNY PTKSGYCGGVLYKIGQILGIHVGGNGRDGFSAMLLRSYFTG QIKVNKHATECGLPDIQTIHTPSKTKLQPSVFYDVFPGSKEPA VLTDNDPRLEVNFKEA
Molecular weight	44351.97
Theoretical pI	8.50
Extinction coefficient	23100 M^{-1} cm^{-1}, at 280 nm measured in water (assuming all pairs of Cys residues form cystines)

4.4 Preparation of LB-ampicillin agar plates

Materials:

- 1-L glass bottle with cap
- Petri dishes
- LB broth
- Agar
- Ampicillin (100 mg/mL)

Procedure:

1. Add 12.5 g LB broth and 7.5 g agar to the 1-L glass bottle. Add ddH_2O to the bottle to make a total volume of 500 mL and autoclave on the liquid cycle.
2. After the autoclave cycle is complete, allow the LB agar medium to cool to approximately 42°C. Add 500 µL of ampicillin (final concentration of 100 µg/mL) and mix well.
3. Immediately pour the LB-ampicillin agar medium into the Petri dishes (approximately 20 mL per dish). Let the plates sit undisturbed at room temperature for 30 minutes to allow the medium to solidify.
4. Transfer the plates into a sealed bag and store at 4°C.

4.5 Preparation and recovery of bacterial glycerol stocks

Materials:

- Overnight bacterial culture
- 50% glycerol in ddH_2O
- LB agar selection plate (containing appropriate antibiotic)
- 37°C bacterial incubator

Procedure:

1. Add 500 µL of bacterial culture to 500 µL of 50% glycerol in a cryovial. Mix gently. Store at –80°C.
2. To recover bacteria from the stock, use a 200 µL tip to scrape the surface of the frozen solution and streak onto a warm LB agar selection plate. Store the remaining stock at –80°C. Incubate plate at 37°C overnight.
    - Do not allow the glycerol stock to thaw during handling.
3. Colonies on a plate may be used to grow a bacterial culture.

4.6 Measurement of protein concentration using Bradford Assay Kit

Materials:

- Bradford Assay kit
- Albumin Standard (BSA)
- Samples of unknown concentration
- Blank solution (sample solvent)
- 1.5-mL microcentrifuge tubes
- Clear 96-well plate
- Coomassie Reagent
- Infinite 200 PRO series plate reader

Procedure:

1. Prepare standards by diluting the contents of 1 Albumin Standard (BSA) ampule into several clean microcentrifuge tubes (2000 µg/mL–25 µg/mL).
2. Pipette 5 µL of blank, standard, or unknown sample in duplicate into a clear 96-well plate.
    - Blanks should be run in duplicate.
3. Add 250 µL of the Coomassie Reagent to each well and mix properly. Incubate plate for 10 minutes at room temperature.
4. Measure the absorbance at 595 nm using a plate reader.
5. Subtract the average measurements for the blank duplicates from all other individual standards or sample measurements.
6. Prepare a standard curve by plotting the average blank-corrected measurement for each BSA standard versus its concentration in µg/mL. Use the standard curve to determine the protein concentration of each unknown sample.

4.7 Measurement of optical density

Materials:

- LB medium
- Bacterial culture
- Spectrophotometer and cuvettes
- Samples of unknown optical density

Procedure:

1. Add 1 mL of LB medium to a disposable polystyrene spectrophotometer cuvette.
2. Place cuvette in spectrophotometer and measure the absorbance at a wavelength of 600 nm. This is the blank measurement.
3. Add 1 mL of bacterial culture to another cuvette and measure the absorbance at a wavelength of 600 nm.
4. Subtract the blank absorbance value from the sample absorbance value to find the optical density.

4.8 SDS-PAGE and gel staining

Materials:

- Protein samples
- Protein molecular weight standard
- Gel casting module (casting stand, casting frame, comb, short plate, spacer plate)
- Filter paper
- 100% EtOH
- Tetra cell (electrode assembly, tank, lid with power cables, buffer dam)
- PowerPac
- Coomassie stain solution
- Orbital shaker
- Solutions:

Solution	Components
4% stacking gel solution (15 mL)	• 1.98 mL of 30% acrylamide/bis-acrylamide • 3.78 mL of 0.5 M Tris-HCl, pH 6.8 • 150 µL of 10% SDS • 9 mL ddH_2O • 15 µL TEMED (add just before pouring gel) • 75 µL of 10% APS (add just before pouring gel)

Solution	Components
14% separating gel solution (15 mL)	• 7.0 mL of 30% acrylamide/bis-acrylamide • 3.75 mL of 1.5 M Tris-HCl, pH 8.8 • 150 µL of 10% SDS • 4.03 mL ddH_2O • 7.5 µL TEMED (add just before pouring gel) • 75 µL of 10% APS (add just before pouring gel)
4x SDS loading buffer	• 200 mM Tris • 8% SDS • 40% glycerol • 0.4% bromophenol blue • 400 mM DTT • Dilute to 1x when preparing loading samples
10x SDS-PAGE running buffer	• ddH_2O • 1% SDS • 250 mM Tris • 1.92 M glycine • Dilute 1:10 to prepare a 1x solution
Destain solution	• 50% ddH_2O • 40% Methanol • 10% Acetic acid

Procedure:

1. Assemble glass cassette sandwich in the casting frame and secure on casting stand.
2. Add 10% APS and TEMED to the separating gel solution and fill the cassette sandwich up to the point that will be approximately 1 cm below the teeth of the comb when inserted. Overlay the gel solution with 100% EtOH. Leave separating gel undisturbed for 45 to 60 minutes to allow polymerization.
3. Pour off the alcohol overlay and rinse the top of the gel with ddH_2O. Dry the area above the gel with filter paper. Partially insert the comb between the glass plates.
4. Add 10% APS and TEMD to the stacking gel solution and fill the cassette sandwich. Insert the comb completely. Leave stacking gel undisturbed for 30 to 45 minutes to allow polymerization.
5. Remove the comb and rinse wells ddH_2O.
6. Prepare protein samples in 1x SDS loading buffer so that 1 µg to 5 µg of protein in a volume of 10 µL to 20 µL can be loaded per well.

7. Set the electrode assembly to the open position on a clean, flat surface. Place the gel cassette in the electrode assembly.

    - Two cassettes are required to create a functioning assembly. When using one or three gels, use the buffer dam (included with the cell) to complete the assembly.

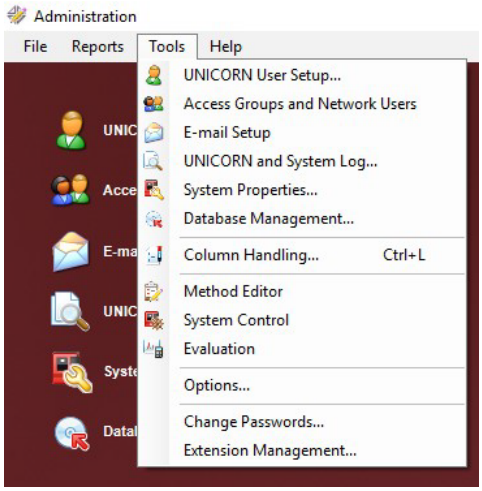
8. Place the assembly into the tank. Fill the buffer chambers with 1x SDS-PAGE running buffer.

    - Approximately 200 mL of buffer is required for the inner buffer chamber and approximately 550 mL (for 1–2 gels) or 800 mL (for 3–4 gels) in the outer buffer chamber.

9. Remove the comb from the gel to allow the sample wells to fill with running buffer.
10. Gently load 5 µL of protein ladder into the first well from the left. Load the remaining wells with 10 to 20 µL of samples.
11. Secure the lid on the tank and connect the power cables to the PowerPac. Set the power supply to constant voltage at 150 V. Run gel for approximately 1.5 hours or until the loading dye front and/or molecular weight standards reach the desired position on the gel.
12. Disassemble the gel apparatus. Open the gel cassettes and transfer the gel to a container containing 50 mL ddH_2O. Place the container with the gel on an orbital shaker set to 50 rpm and wash for 5 minutes. Discard the wash. Repeat this step 2 more times.
13. Add 30 mL of Coomassie stain solution to the gel. Shake until the blue protein bands become visible (approximately 30 minutes).
14. Discard the stain solution and rinse gel with ddH_2O.
15. Add 50 mL destain solution to the gel. Shake until the gel background is clear and only the blue protein bands are visible (2 hours to overnight).

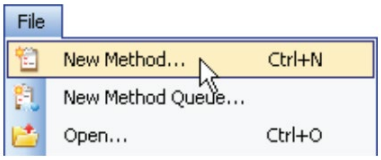
4.9 Instructions for using UNICORN 7.0.2 software on the ÄKTA pure L protein purification system

1. Turn on the ÄKTA pure L system and open the UNICORN software.
2. In the **Administration** window, under the **Tools** menu, select **Method Editor** from the **Tools** menu.

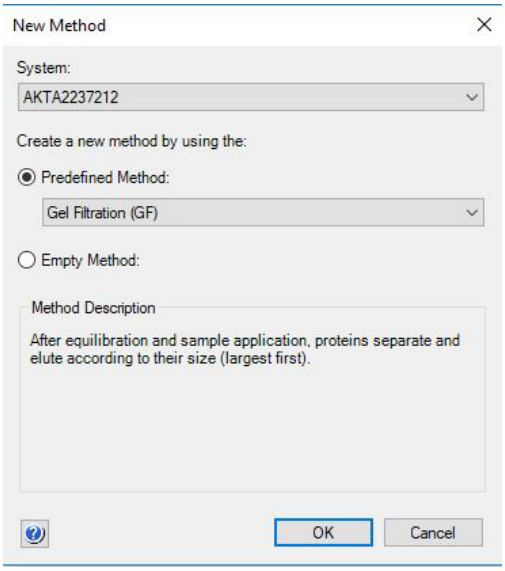


3. Create a new method to equilibrate the Superdex 200 16/600 column.

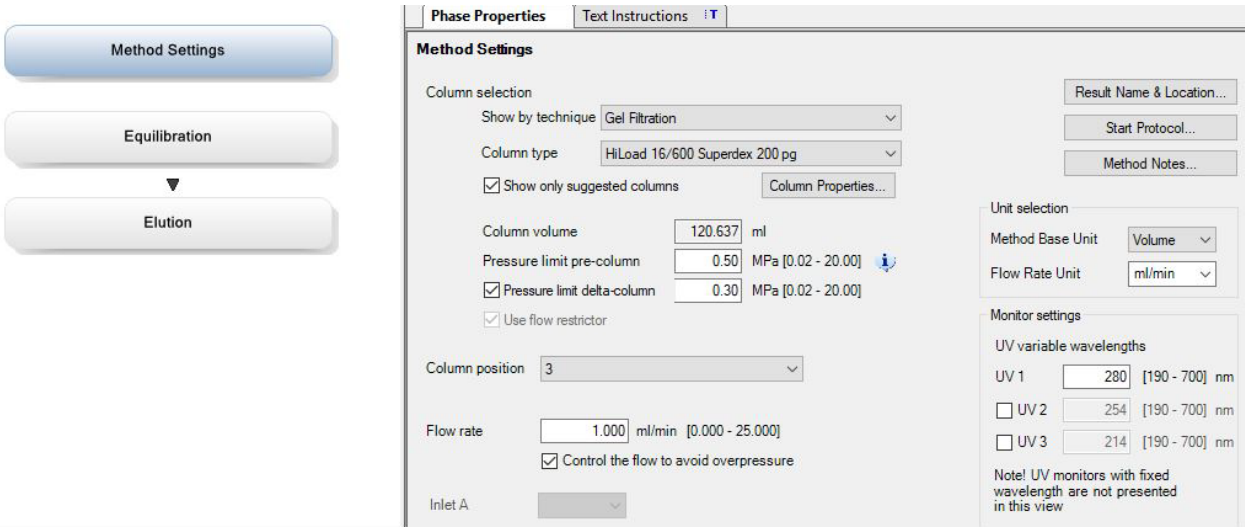
   - In the **Method Editor** window, select the **New Method** icon or by selecting **New Method** from the **File** menu.



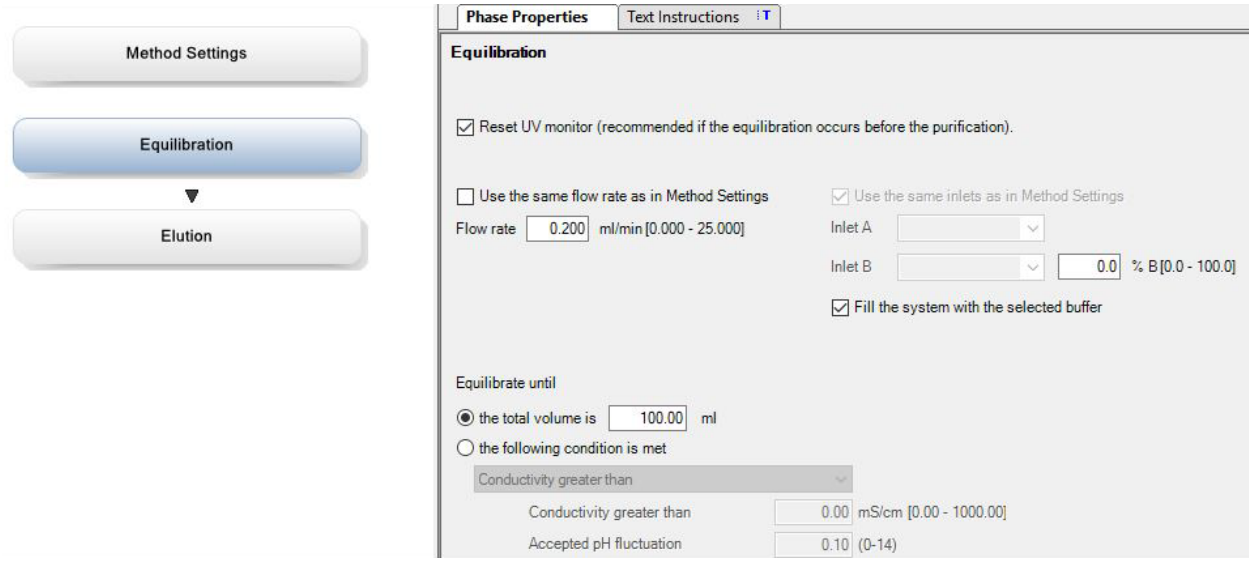
- In the **New Method** window, select **Gel Filtration (GF)** under **Predefined Method** and then select **OK**. A **Method Navigator** window will open.



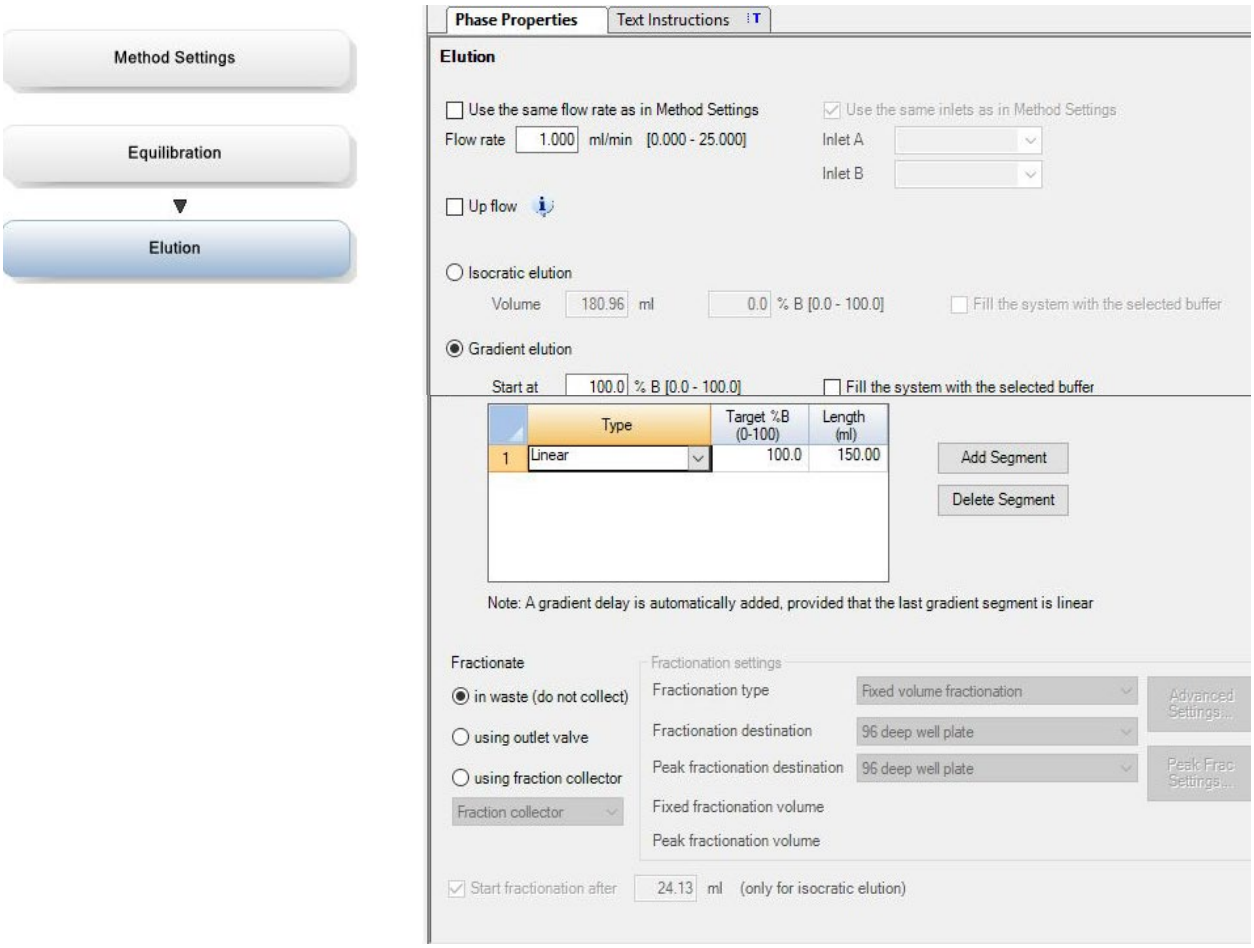
- In the **Method Navigator** window, select **Method Settings** and then enter the parameters shown in the image:



- In the **Method Navigator** window, select **Equilibration** and then enter the parameters shown in the image:



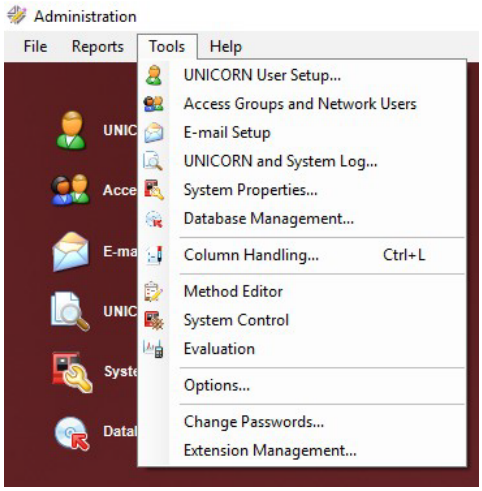
- In the **Method Navigator** window, select **Elution** and then enter the parameters shown in the image:



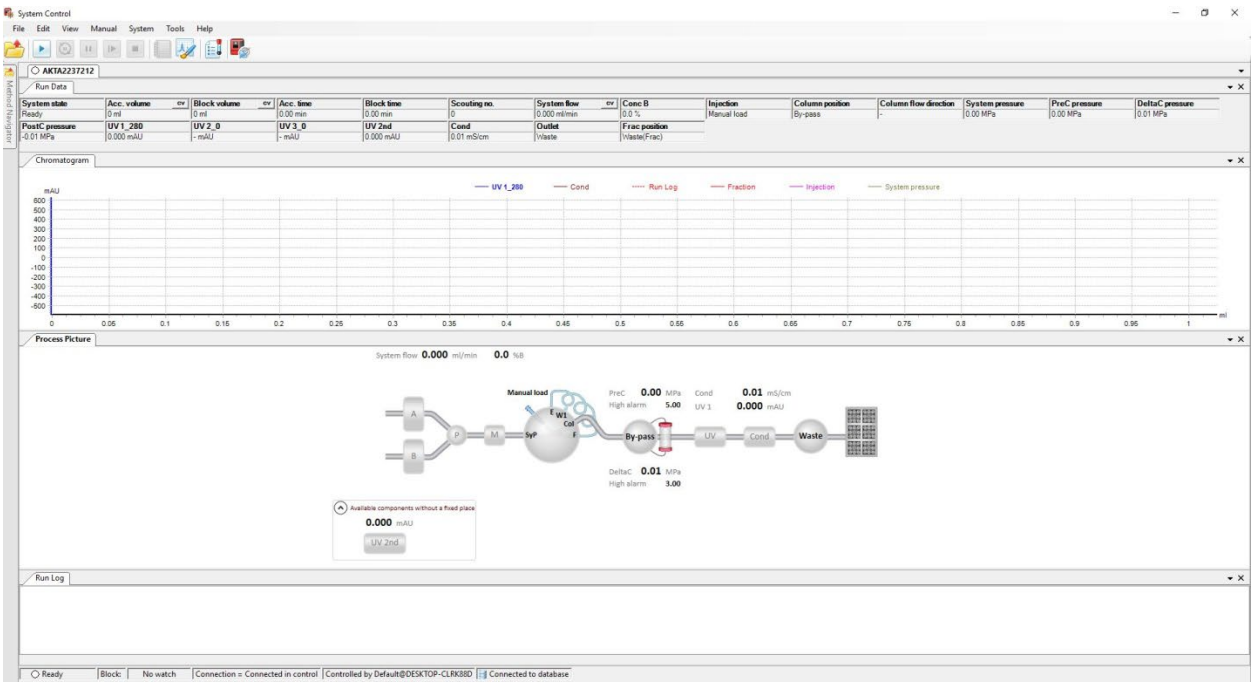
- Save the method with unique file name.

4. Run the equilibration method.

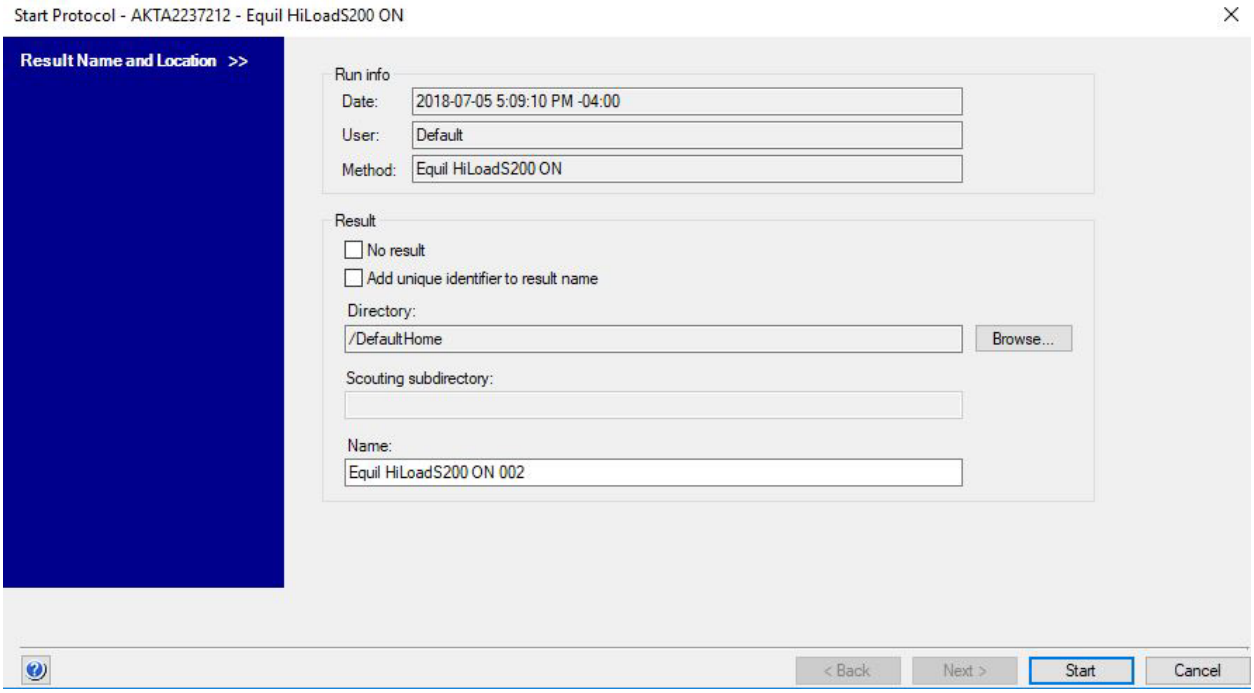
   - Place line A1 into a flask containing ddH_2O and line B1 into a flask containing filtered, sonicated PBS. Seal the tops of the flasks with Parafilm.
   - In the **Administration** window, open the **System Control** from the **Tools** menu.



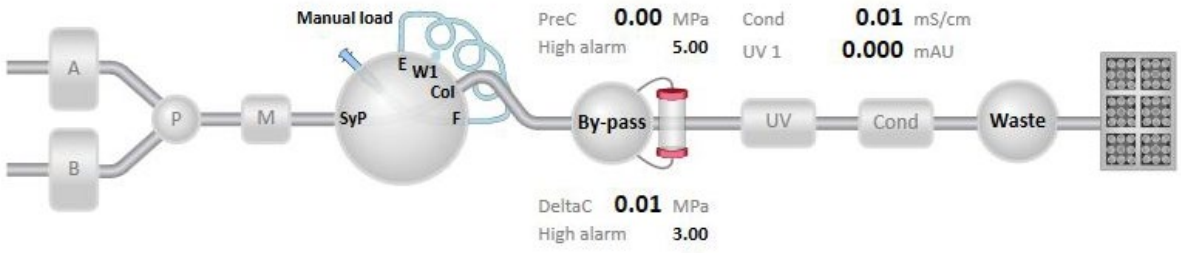
   - In the **System Control** window, select **Open** from the **File** menu. The **Method Navigator** window will open.



- In the **Method Navigator** window, select your equilibration method. Select **Start** to run the method. The method takes approximately 11 hours to run (can be run overnight).



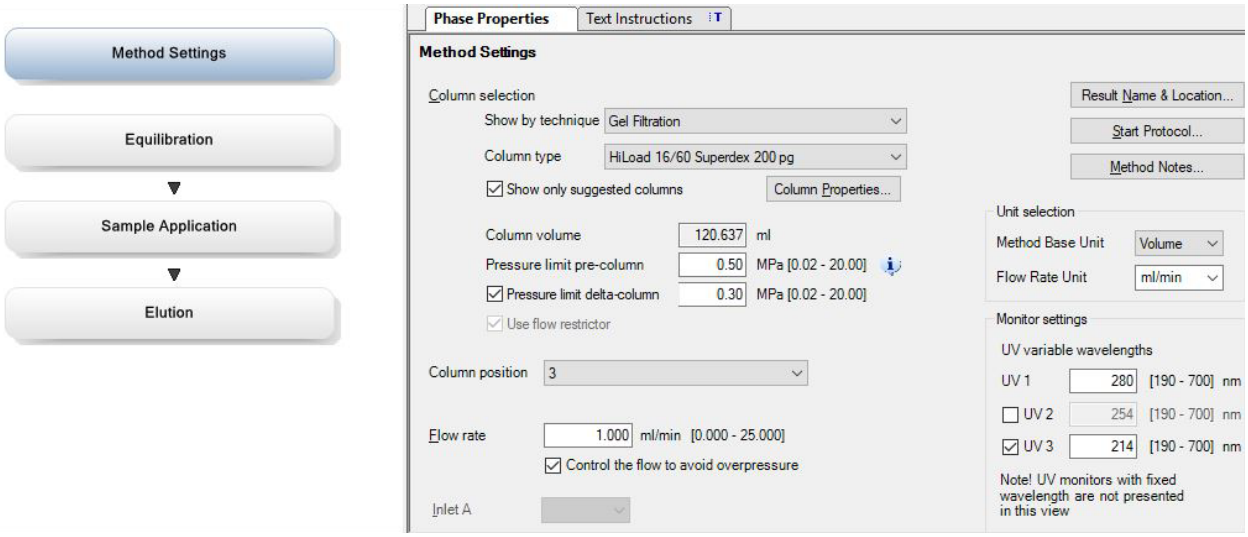
- Switch line A1 from the flask containing water to the flask containing PBS.
- In the **System Control** window, right-click on the **A** in the **Process Picture**. Select **Start pump A wash** to fill the line with PBS.



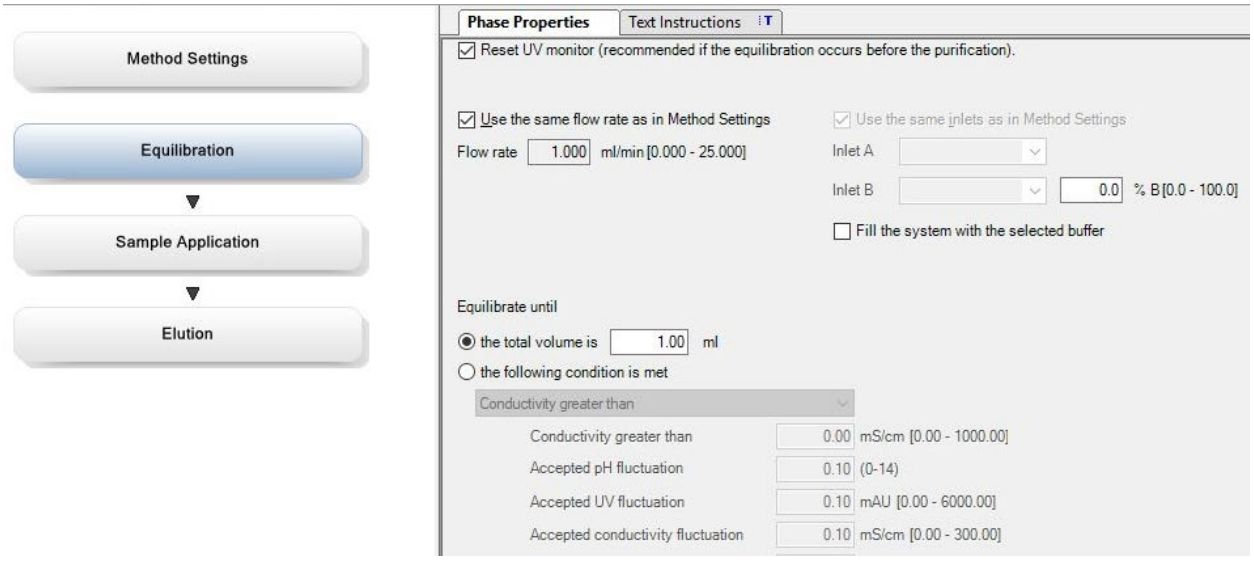
5. Create a new method to run the sample.

- In the **Method Editor** window, select the **New Method** icon or by selecting **New Method** from the **File** menu.
- In the **New Method** window, select **Gel Filtration (GF)** under **Predefined Method** and then select **OK**. A **Method Navigator** window will open.

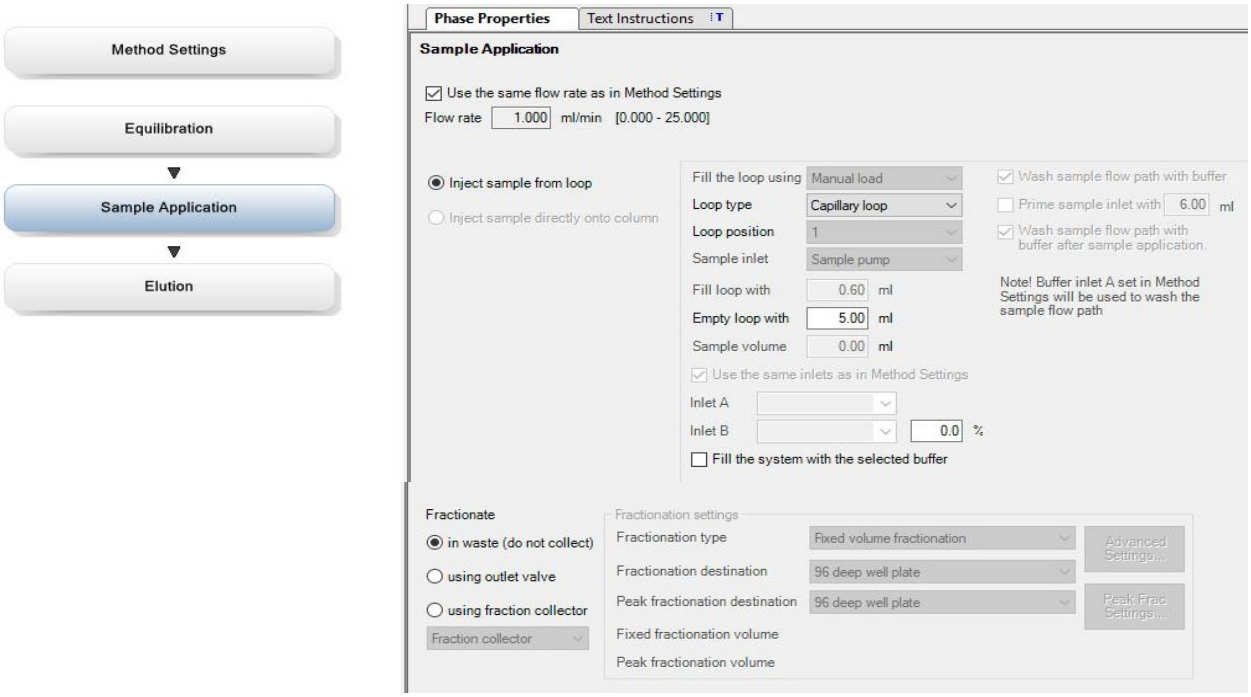
- In the **Method Editor** window, select **Methods Settings** and then enter the parameters shown in the image:



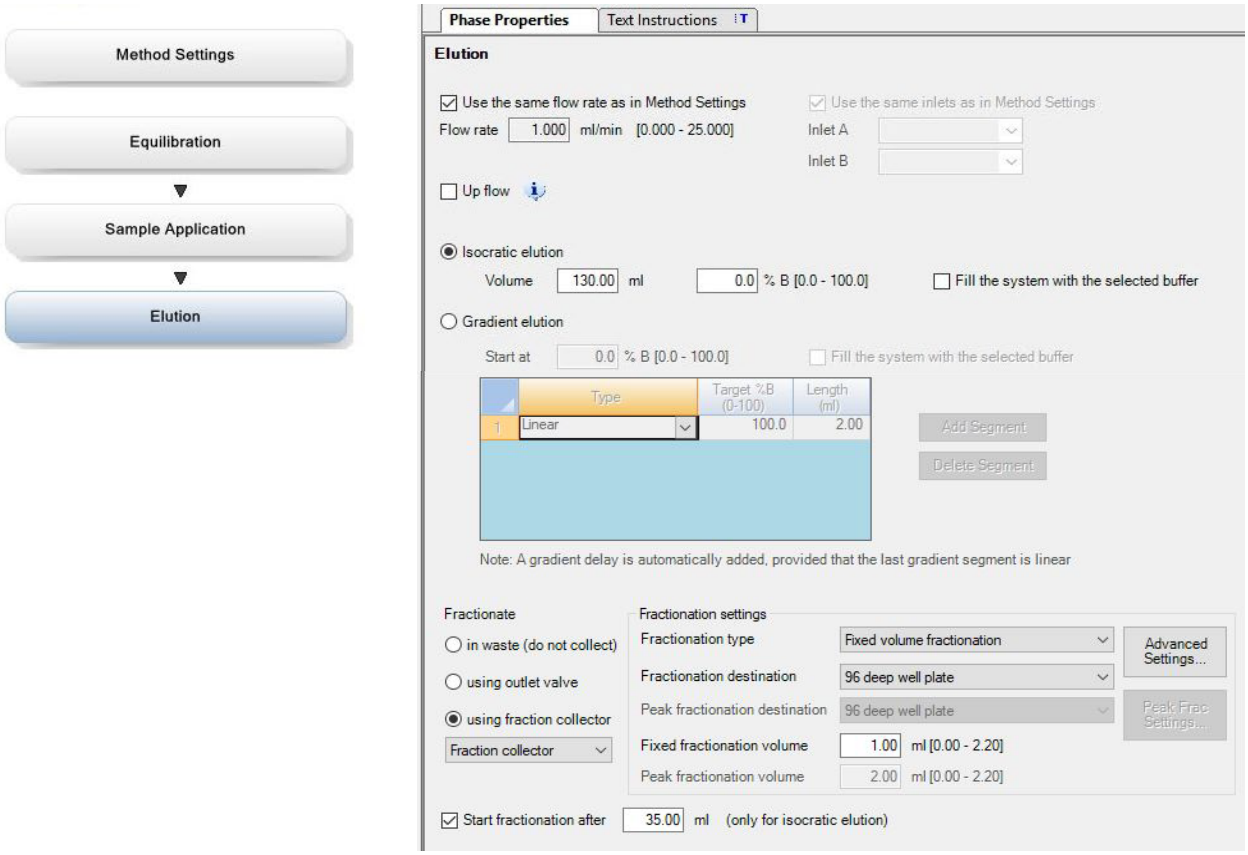
- In the **Method Editor** window, select **Equilibration** and then enter the parameters shown in the image:



- In the **Method Editor** window, select **Sample Application** and then enter the parameters shown in the image:



- In the **Method Editor** window, select **Elution** and then enter the parameters shown in the image:



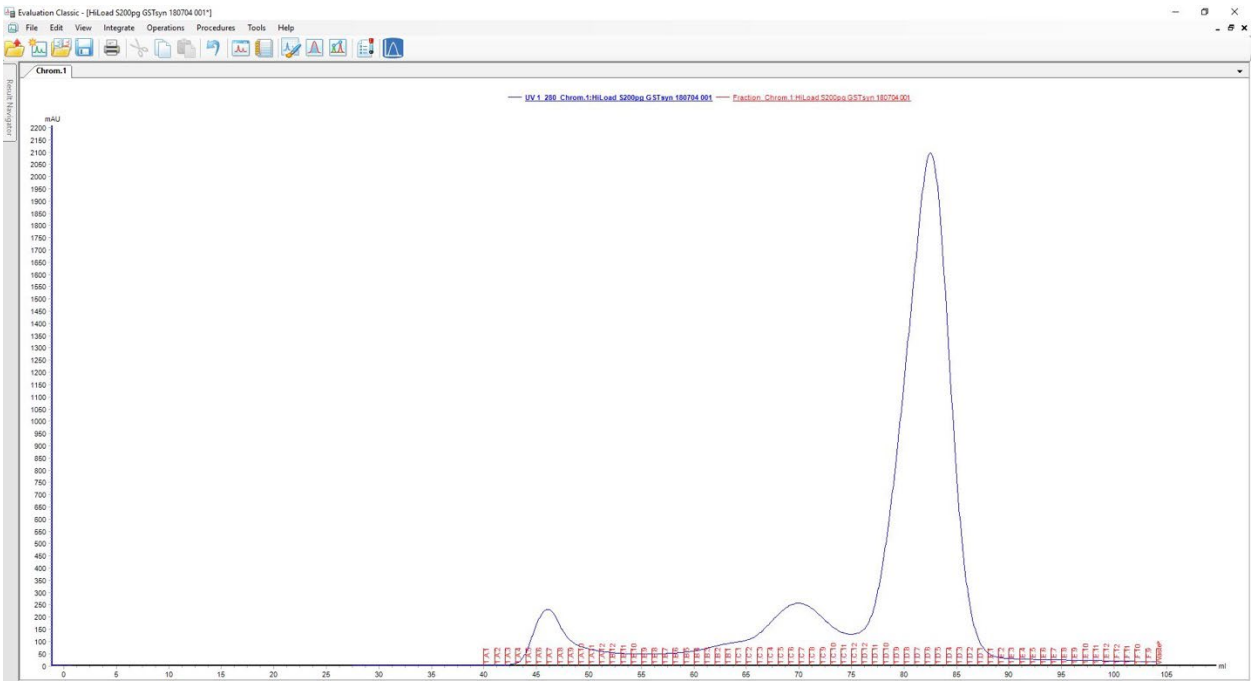
- Save method with a unique file name.

6. Run the sample method.

- Place a 96 deepwell plate in the fraction collector.
- Clean the sample loop by injecting 10 mL of ddH_2O, and then prime the sample loop by injecting 10 mL of PBS.
- Inject the sample.
- In the **System Control** window, select **Open** from the **File** menu. The **Method Navigator** window will open.
- In the **Method Navigator** window, select your sample method and rename the file name to the sample name. Select **Start** to run the method. The method takes approximately 2 hours to run.

7. In the **Evaluation** window, visualize the sample fractionation. Collect and combine all fractions from the desired peak.

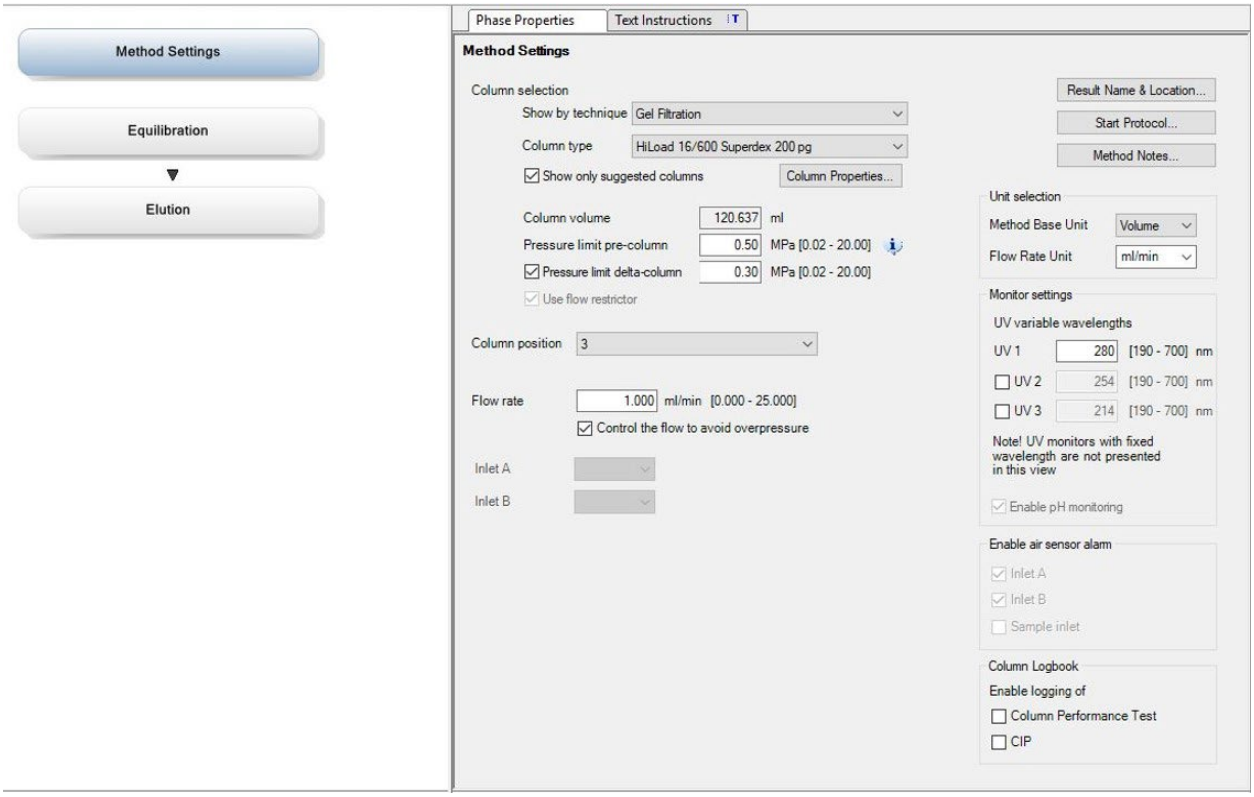
    - In the example shown below, fractions D1–D11 should be collected.



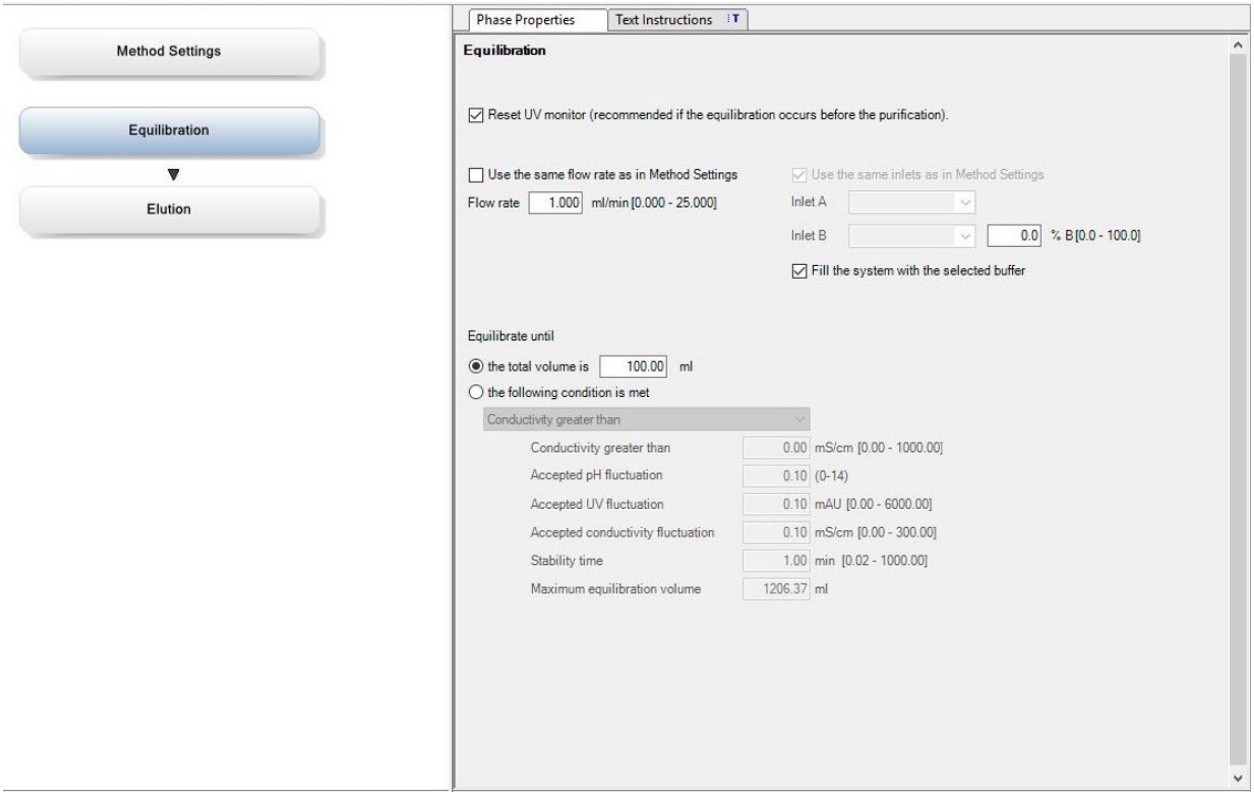
8. Create a new method to clean the column.

    - In the **Method Editor** window, select the **New Method** icon  or by selecting **New Method** from the **File** menu.
    - In the **New Method** window, select **Gel Filtration (GF)** under **Predefined Method** and then select **OK**. A **Method Navigator** window will open.

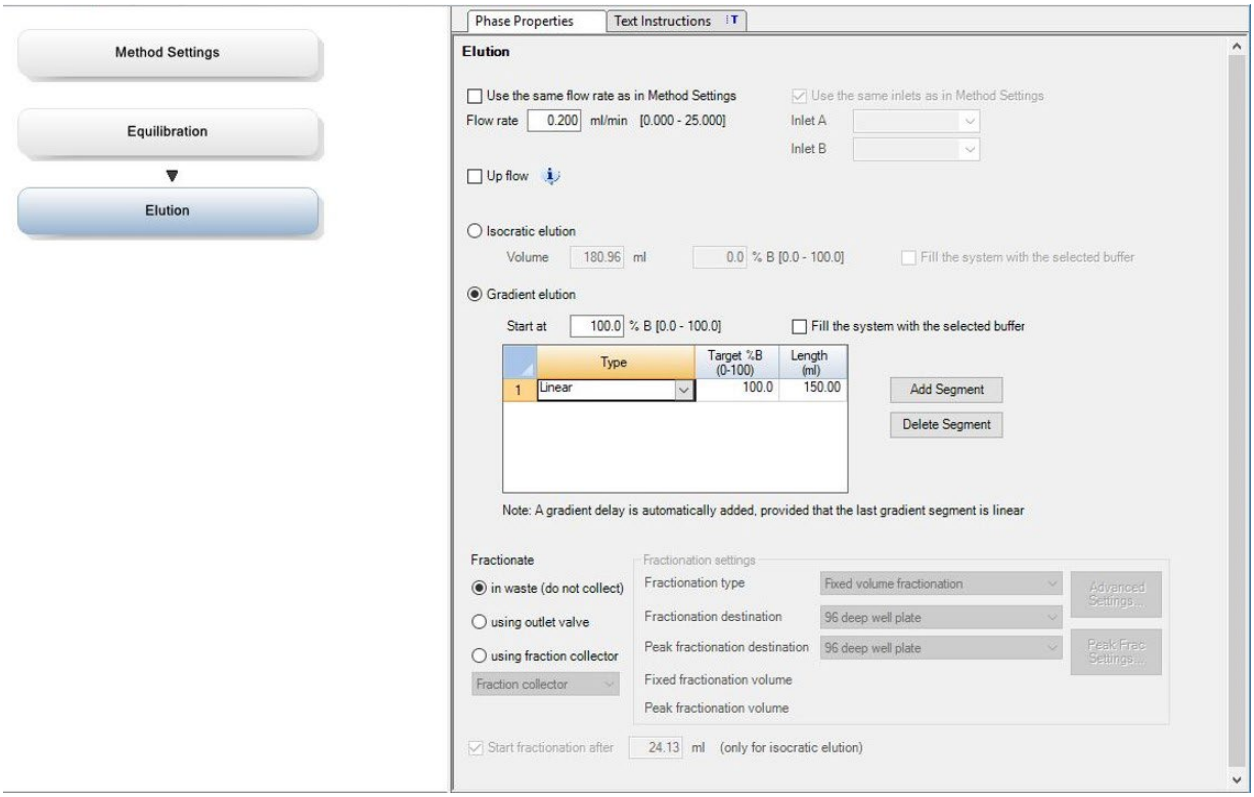
- In the **Method Editor** window, select **Methods Settings** and then enter the parameters shown in the image:



- In the **Method Editor** window, select **Equilibration** and then enter the parameters shown in the image:



- In the **Method Editor** window, select **Elution** and then enter the parameters shown in the image:



9. Run the cleaning method.

- Place line A1 into the flask containing ddH_2O and line B1 into 20% EtOH.
- Clean the sample loop by injecting 10 mL of PBS, then 10 mL of ddH_2O, and finally 10 mL of 20% EtOH.
- In the **Systems Control** window, select **Open** from the **File** menu. The **Method Navigator** window will open.
- In the **Method Navigator** window, select your cleaning method and rename the file name to the date. Select Start to run the method. The method takes approximately 15 hours to run.

4.10 Measurement of endotoxin concentration using LAL chromogenic endotoxin quantification kit

Materials:

- LAL chromogenic endotoxin quantification kit
- Sample of unknown concentration
- 25% acetic acid (Stop Reagent)
- 1.5-mL microcentrifuge tubes
- Clear 96-well plate
- Infinite 200 PRO series plate reader
- Heating block at 37°C
- Vortex mixer
- Timer

Procedure:

1. Reconstitute the vial of Endotoxin Standard by adding 1 mL of room temperature endotoxin-free water. Vortex the solution vigorously for at least 15 minutes on a vortex mixer before use.

    - The amount of the Endotoxin Standard is printed on the vial label (endotoxin units [EU]). For example, a vial of 26 EU, when reconstituted with 1 mL of endotoxin-free water, will yield a concentration of 26 EU/mL.
    - Reconstituted stock solution is stable at 2°C to 8°C for up to 4 weeks. Prior to subsequent use, warm the solution to room temperature and vigorously mix for 15 minutes (endotoxin adheres to sides of the glass vial).

2. Prepare standards by diluting the reconstituted Endotoxin Standard in endotoxin-free water in several clean microcentrifuge tubes (1 EU/mL–0.1 EU/mL).

    - Vigorously vortex each standard for at least 1 minute before use.

3. Reconstitute the LAL reagent immediately before use by adding 1.4 mL of endotoxin-free water. Swirl gently to dissolve the powder.

    - Avoid foaming; do not vortex the solution.
    - If more than one vial is required, pool two or more vials before use.
    - Reconstituted LAL reagent is stable at –20°C or colder for up to 1 week if frozen immediately after reconstitution. Thaw to room temperature and gently swirl the reagent to mix before adding to samples. Once thawed, the reagent may be used only one time and cannot be refrozen.

4. Reconstitute the Chromogenic Substrate by adding 6.5 mL of endotoxin-free water.

    - Reconstituted Chromogenic Substrate is stable at 2°C to 8°C for up to 4 weeks. Prior to use, warm up the appropriate volume of substrate solution required for the assay to 37°C.

5. Place the 96-well plate on the heating block for 10 minutes to equilibrate to 37°C.
6. Pipette 50 µL of blank (endotoxin-free water), standard, or unknown sample in duplicate into the plate wells.
7. Add 50 µL of LAL reagent to each well and start the timer. Remove the plate from the heating block and gently tap to mix. Replace the plate on the heating block and cover with the lid. Incubate for 10 minutes.
8. When the timer reaches 10 minutes, add 100 µL of Chromogenic Substrate to each well. Remove the plate from the heating block and gently tap to mix. Replace the plate on the heating block and cover with the lid. Incubate for 6 minutes.

    - Add the Chromogenic Substrate in the same well order and with the same speed as the LAL reagent was added.

9. When the timer reaches 16 minutes, add 100 µL of Stop Reagent to each well. Remove the plate from the heating block and gently tap to mix.
10. Measure the absorbance at 405 to 410 nm using a plate reader.
11. Subtract the average measurements for the blank duplicates from all other individual standards or sample measurements.
12. Prepare a standard curve by plotting the average blank-corrected measurement for each endotoxin standard versus its concentration in EU/mL. Use the standard curve to determine the endotoxin concentration of each unknown sample.