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## **EDDU Protocols**

# **Production of Recombinant $\alpha$ -Synuclein Monomers and Preformed Fibrils (PFFs)**

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# 1 Introduction

## 1.1 Objectives

This protocol describes how to:

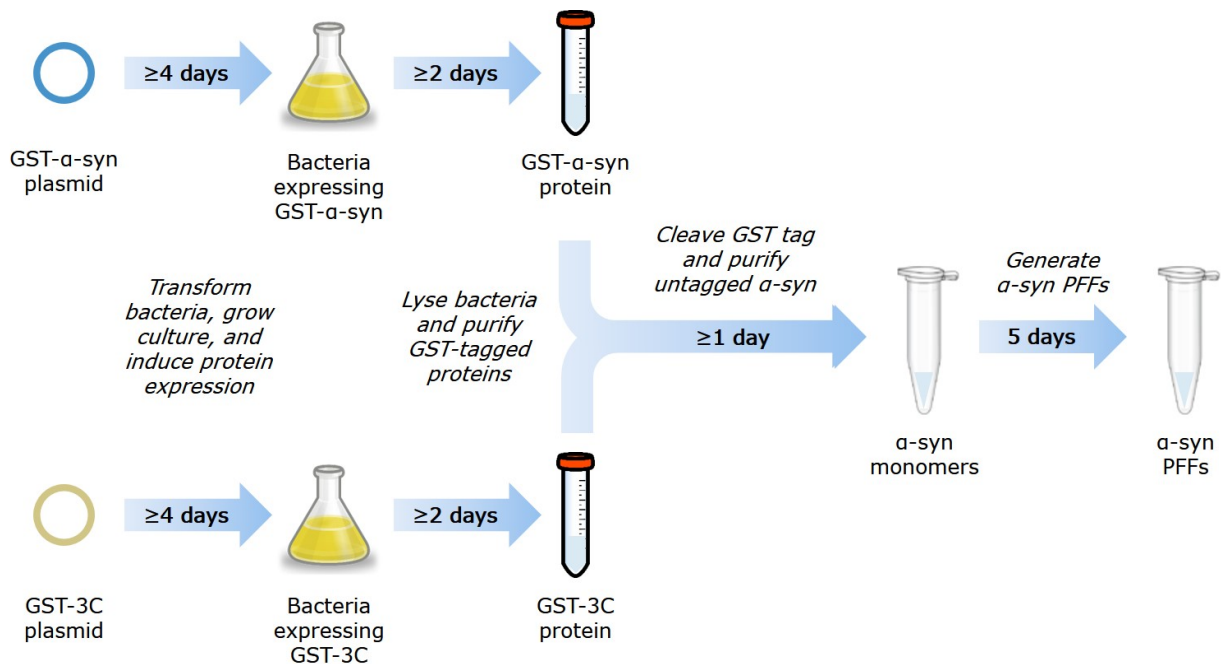
- Produce and purify recombinant human and mouse  $\alpha$ -synuclein in monomeric form from transformed bacteria
- Generate  $\alpha$ -synuclein preformed fibrils (PFFs) from recombinant monomeric  $\alpha$ -synuclein
- Fluorescently label  $\alpha$ -synuclein PFFs

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

The recombinant monomeric  $\alpha$ -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  $\alpha$ -synuclein (GST- $\alpha$ -syn) and 3C enzyme (GST-3C), cleavage of GST tags and purification of untagged  $\alpha$ -synuclein, and generation of  $\alpha$ -synuclein PFFs.**

Untagged  $\alpha$ -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  $\alpha$ -synuclein for marmosets).

### 1.3 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 deionized, distilled water (ddH<sub>2</sub>O; MiliQ water) to prepare all media and buffers. Do not use distilled water (dH<sub>2</sub>O) because it is too acidic for bacterial and protein work.
- 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  $\alpha$ -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  $\alpha$ -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  $\alpha$ -synuclein PFFs, refer to Bousset et al. 2016. *J. Parkinson Dis.* (6) 143–151.
- Store  $\alpha$ -synuclein PFFs at  $-80^{\circ}\text{C}$  and thaw at room temperature. Do not store PFFs on ice or at  $4^{\circ}\text{C}$ , as this causes dissociation and reduces activity. Avoid repeated freeze-thaw cycles.
- Do not vortex  $\alpha$ -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 <i>E. coli</i> 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- $\alpha$ -synuclein plasmid, human	NA	NA	100 ng/ $\mu$ L	2 ng/ $\mu$ L	-20°C
GST-3C plasmid	NA	NA	100 ng/ $\mu$ L	2 ng/ $\mu$ L	-20°C
GST- $\alpha$ -synuclein plasmid, mouse	NA	NA	100 ng/ $\mu$ L	2 ng/ $\mu$ L	-20°C
Isopropyl- $\beta$ -D-thiogalactoside (IPTG)	Biobasic	IB0168	0.5 M	300 $\mu$ M	Stock: -20°C
LAL chromogenic endotoxin quantification kit <sup>†</sup>	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 $\mu$ g/mL	Stock: -20°C Working: 4°C
Methanol (Certified ACS)	Fisher Scientific	A412-1	100%	40%	RT
Paraformaldehyde (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

<b>Item</b>	<b>Supplier</b>	<b>Catalogue #</b>
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- $\alpha$ -synuclein and GST-3C enzyme

#### Materials:

- BL21 (DE3) competent *E. coli* cells
- Plasmid(s):
  - Human GST- $\alpha$ -synuclein expression plasmid (100 ng/ $\mu$ L)
  - Mouse GST- $\alpha$ -synuclein expression plasmid (100 ng/ $\mu$ L)
  - GST-3C enzyme expression plasmid (100 ng/ $\mu$ L)
- 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- $\mu$ m 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<sub>2</sub>O
- Glutathione sepharose bead slurry

- Solutions:

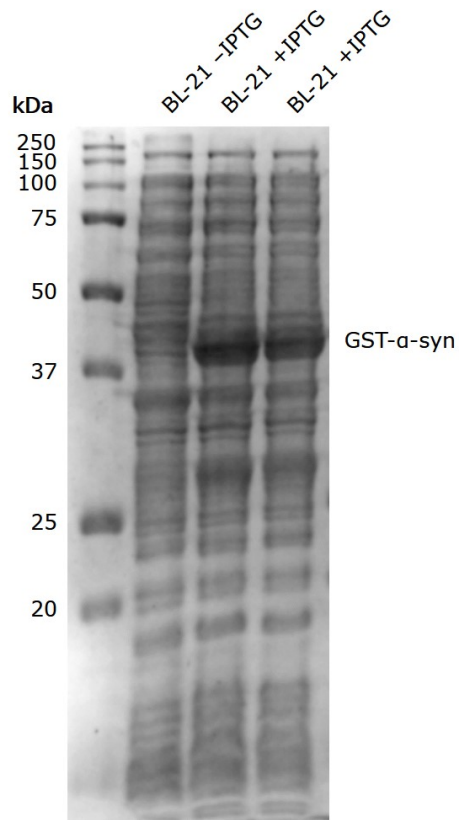
Solution	Components
LB medium (autoclaved)	<ul style="list-style-type: none"> <li>• 1 L ddH<sub>2</sub>O</li> <li>• 25 g LB broth</li> </ul>
Resuspension buffer (80 mL; prepare fresh and chill on ice before use)	<ul style="list-style-type: none"> <li>• ddH<sub>2</sub>O</li> <li>• 25 mM Tris, pH 8</li> <li>• 400 mM NaCl</li> <li>• 5% glycerol</li> <li>• 0.5% Triton X</li> <li>• 5 mM PMSF</li> <li>• 0.5 mg/mL benzamidine</li> <li>• 0.5 µg/mL leupeptin</li> <li>• 0.5 µg/mL aprotinin</li> <li>• 1 mM DTT</li> </ul>
Wash buffer (500 mL; chill on ice)	<ul style="list-style-type: none"> <li>• ddH<sub>2</sub>O</li> <li>• 50 mM Tris, pH 8</li> <li>• 400 mM NaCl</li> <li>• 5% glycerol</li> <li>• 1 mM DTT (add fresh before use)</li> </ul>
Elution buffer (20 mL; prepare fresh and chill on ice before use)	<ul style="list-style-type: none"> <li>• Wash buffer</li> <li>• 20 mM glutathione (once dissolved adjust pH to 8)</li> </ul>

- 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 cells on ice for 10 minutes.
2. Add 1  $\mu\text{L}$  (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  $\mu\text{L}$  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  $\mu\text{L}$  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  $\mu\text{g}/\text{mL}$ .
7. Use a sterile 20  $\mu\text{L}$  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  $\mu\text{g}/\text{mL}$ .
  - 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 ( $\text{OD}_{600}$ ) until it reaches 0.6 (see Appendix 4.7 for procedure).
  - It usually takes 3 to 5 hours or longer for the  $\text{OD}_{600}$  to reach 0.6.
10. Add IPTG to the culture to a final concentration of 300  $\mu\text{M}$  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 level of GST- $\alpha$ -synuclein, run 100  $\mu$ L of the supernatant (cleared lysate) on a 14% polyacrylamide SDS-PAGE gel (see Appendix 4.8 for procedure).
  - Recombinant GST- $\alpha$ -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- $\alpha$ -synuclein (GST- $\alpha$ -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 bead slurry to two clean 50-mL tubes. Wash the beads by adding 30 mL of ddH<sub>2</sub>O 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- $\mu$ m 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 ten 10-mL or five 20-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 15 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<sub>2</sub>O. 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 3000 rpm at 4°C for 30 minutes.
24. Add 10 mL of PBS and centrifuge at 3000 rpm 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 remove the GST tag from the GST-α-synuclein protein. Reserve 100 μL of GST-α-synuclein protein to analyze by SDS-PAGE (see Section 3.2, step 9).
  - **IMPORTANT:** Do not store purified protein overnight; proceed directly to cleavage reaction. The purified protein is sensitive to degradation and should not be stored to perform cleavage reaction at a later time.

## 3.2 Cleavage of GST- $\alpha$ -synuclein and purification of untagged $\alpha$ -synuclein

### Materials:

- Recombinant GST- $\alpha$ -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<sub>2</sub>O
- Solutions:

Solution	Components
Wash buffer (500 mL; chill on ice)	<ul style="list-style-type: none"><li>• ddH<sub>2</sub>O</li><li>• 50 mM Tris, pH 8</li><li>• 400 mM NaCl</li><li>• 1 mM DTT (add fresh before use)</li></ul>
Elution buffer (20 mL; prepare fresh and chill on ice before use)	<ul style="list-style-type: none"><li>• Wash buffer</li><li>• 20 mM glutathione (once dissolved adjust pH to 8)</li></ul>

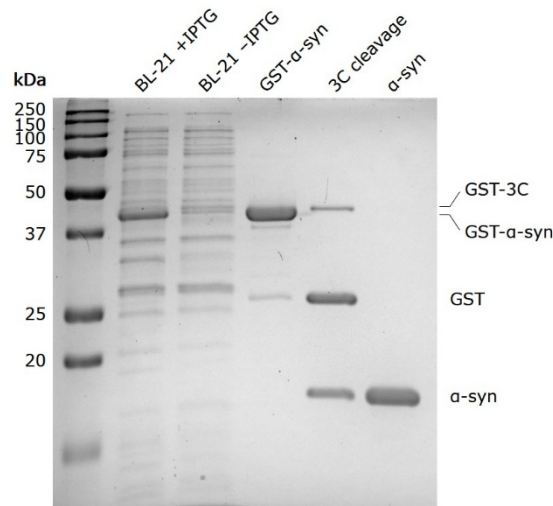
- Bradford Assay kit and materials (see Appendix 4.6 for procedure)
- Thermo Scientific Heraeus Megafuge 40R Refrigerated Centrifuge
- Ultrasonic cleaning bath
- ÄKTA pure L protein purification system with UNICORN 7.0.2 software
- LAL chromogenic endotoxin quantification kit and materials (see Appendix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

### Procedure:

1. Add GST-3C enzyme to GST- $\alpha$ -synuclein at a 1:50 mass-to-mass ratio. Incubate reaction at 4°C overnight.
  - For example, add 0.3 mg of GST-3C enzyme (150  $\mu$ L of 2 mg/mL stock solution) to 15 mg of GST- $\alpha$ -synuclein protein (3 mL of 5 mg/mL stock solution).

2. 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.
3. Set up a GStap 4B column in the support stand. Place a vessel (e.g. beaker) under the column to collect flow-through. Wash column with 20 mL of ddH<sub>2</sub>O to remove EtOH, and then wash with 20 mL of cold wash buffer.
4. Reserve 100 µL of reaction to analyze by SDS-PAGE. Inject the remaining reaction into the GStap 4B column and collect the eluent in a 50-mL tube.
  - 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<sub>2</sub>O, and then 10 mL of 20% EtOH. Store at 4°C until the expiry date on the product label.
5. Transfer eluents to an Amicon Ultra-15 Centrifugal Filter Unit. Centrifuge at 3000 rpm at 4°C for 30 to 45 minutes to concentrate the eluent to ≤4 mL.
6. 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.
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  $\alpha$ -synuclein, run 20  $\mu$ L of protein on a 14% polyacrylamide SDS-PAGE gel alongside the 3C cleavage reaction (from step 3) and GST- $\alpha$ -synuclein (from Section 3.1; see Appendix 4.8 for procedure).
  - Recombinant untagged  $\alpha$ -synuclein will run at approximately 15 kDa on a 14% polyacrylamide SDS-PAGE gel (**Figure 3**). The calculated molecular weight of  $\alpha$ -synuclein is 14.87 kDa based on its amino acid sequence.



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

10. Determine the protein concentration and adjust to 5 mg/mL with sterile PBS (see Appendix 4.6 for procedure). Prepare 500  $\mu$ L aliquots in 1.5-mL autoclaved microtubes and store at  $-80^{\circ}\text{C}$ . Reserve 1 aliquot determine endotoxin levels (next step).
  - Depending on the downstream application, other aliquot volumes may be preferred (e.g. 20  $\mu$ L, 50  $\mu$ L).
  - 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  $\alpha$ -synuclein is produced in BL-21 cells, or  $<100$  EU/mg of protein when  $\alpha$ -synuclein is produced in ClearColi cells.

### 3.3 Generation of $\alpha$ -synuclein PFFs

#### Materials:

- Recombinant monomeric  $\alpha$ -synuclein (500- $\mu$ L aliquot; 5 mg/mL)
- Parafilm
- 37°C digital heating shaking drybath or thermomixer
- Bioruptor Plus sonicator

#### Procedure:

1. Seal the 500- $\mu$ L aliquot of monomeric  $\alpha$ -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- $\mu$ L aliquots of PFFs in 1.5-mL tubes and store at -80°C.

### 3.4 Fluorescent labelling of $\alpha$ -synuclein PFFs

#### Materials:

- $\alpha$ -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:

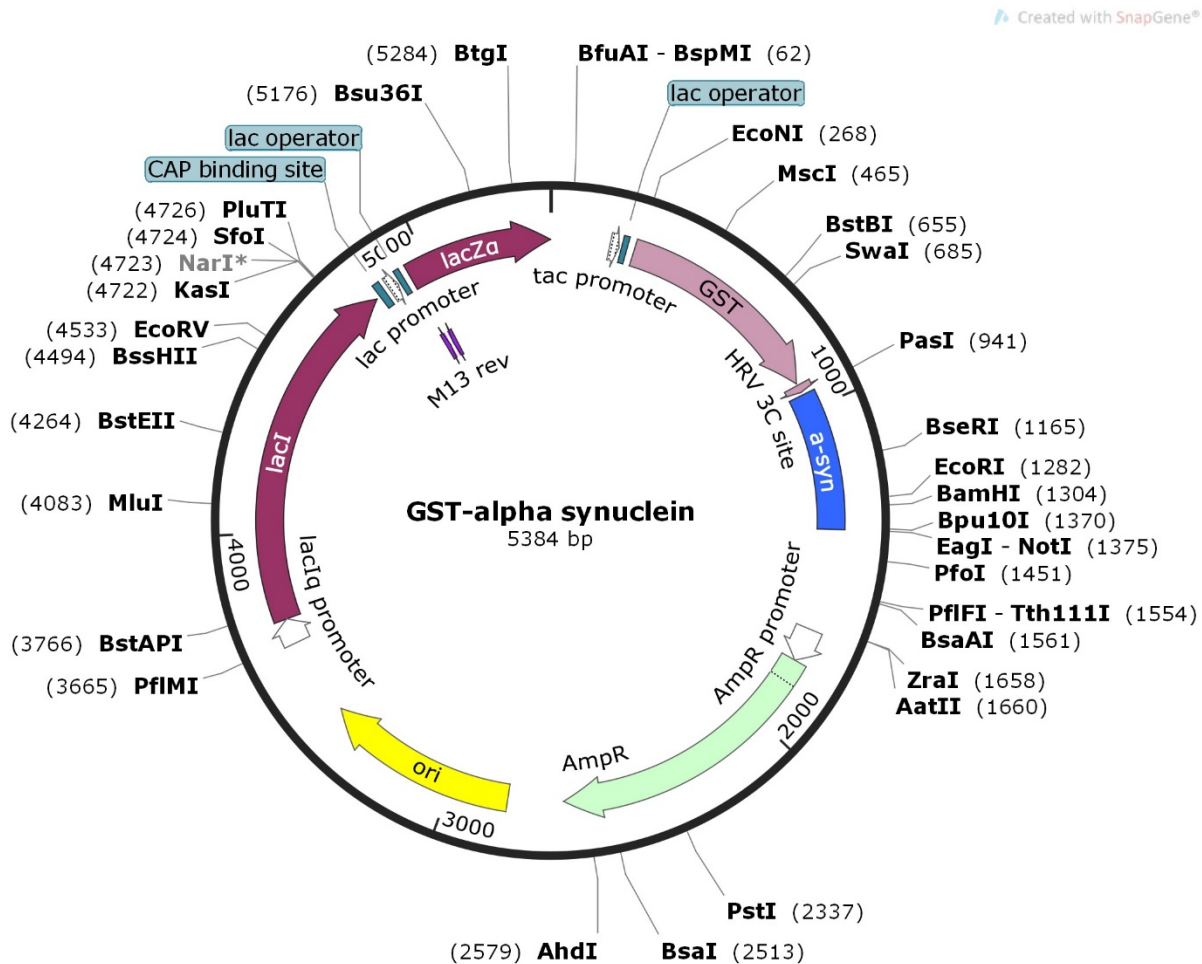
1. Add 5  $\mu$ L of a fluorescent dye to 150  $\mu$ L of  $\alpha$ -synuclein PFFs and mix gently. Cover samples with aluminum foil to protect from light and incubate at room temperature for 20 minutes.
  - **IMPORTANT:** Do not vortex PFFs.
  - Incubation time may vary depending on the dye. Refer to 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- $\mu$ L aliquots in tube and store at  $-80^{\circ}\text{C}$ . Reserve an aliquot of labelled PFFs to determine the protein concentration.
  - **IMPORTANT:** Store all aliquots of labelled PFFs at  $-80^{\circ}\text{C}$  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- $\alpha$ -synuclein plasmid

The plasmid used to express human GST- $\alpha$ -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  
GCTGAGAAAACCAAACAGGGTGTGGCAGAAGCAGCAGGAAAGACAAAAGAGGGTGTTCCTC  
TATGTAGGCTCCAAAACCAAGGAGGGAGTGGTGCATGGTGTGGCAACAGTGGCTGAGAAG  
ACCAAAGAGCAAGTGACAAATGTTGGAGGAGCAGTGGTGACGGGTGTGACAGCAGTAGCC  
CAGAAGACAGTGGAGGGAGCAGGGAGCATTGCAGCAGCCACTGGCTTTGTCAAAAAGGA  
CCAGTTGGGCAAGAATGAAGAAGGAGCCCCACAGGAAGGAATTCTGGAAGATATGCCTGT  
GGATCCTGACAATGAGGCTTATGAAATGCCTTCTGAGGAAGGGTATCAAGACTACGAACCT  
GAAGCCTAAGCGGCCGC

Properties of recombinant human  $\alpha$ -synuclein (untagged):

<b>Amino acid sequence</b>	GPLGSMDVFMKGLSKAKEGVVAAAETKQGVAAEAAGKTKE GVLYVGSKTKEGVVHGVATVAEKTKEQVTNVGGAVVTGVT AVAQKTVEGAGSIAAATGFVKKDLGKNEEGAPQEGILED M PVDPDNEAYEMPSEEGYQDYEPEA*
<b>Molecular weight</b>	14,871.61 DA
<b>Theoretical pI</b>	4.67
<b>Extinction coefficient</b>	5960 M <sup>-1</sup> cm <sup>-1</sup> , at 280 nm measured in water

## 4.2 Mouse GST- $\alpha$ -synuclein plasmid

The plasmid used to express mouse GST- $\alpha$ -synuclein was created in the laboratory of Dr. Edward Fon (Montreal Neurological Institute, McGill University) by cloning the complete coding sequence for mouse  $\alpha$ -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:

AGATACCCACAGCCCTCACGCACCGCACCTCCAACCAACCCGTCCCCTCCCTAGGAAGAG  
GAGCGAAGGCACGAGGCAGGCGAGGGGCGGGGAGAGGGCGCTGACAAATCAGCTGCGGG  
GGCGACGTGAAGGAGCCAGGGAGCCAGAGCGCCCGGCAGCAGGCAGCAGACGGCAGGA  
GACCAGCAGGTGTTCCCCCTGCCCTGCCCTTGCCCTCTTTTCATTGAAATTAGATTGG  
GGAAAACAGGAAGAATCGGAGTTCCTCAGAAGCCTAGGGAGCCGTGTGGAGCAAAAATAC  
ATCTTTAGCCATGGATGTGTTTCATGAAAGGACTTTCAAAGGCCAAGGAGGGAGTTGTGGCT  
GCTGCTGAGAAAACCAAGCAGGGTGTGGCAGAGGCAGCTGGAAAGACAAAAGAGGGAGT  
CCTCTATGTAGGTTCCAAAATAAGGAAGGAGTGGTTCATGGAGTGACAACAGTGGCTGA  
GAAGACCAAAGAGCAAGTGACAAATGTTGGAGGAGCAGTGGTACTGGTGTGACAGCAGT  
CGCTCAGAAGACAGTGGAGGGAGCTGGGAATATAGCTGCTGCCACTGGCTTTGTCAAGAA  
GGACCAGATGGGCAAGGGTGGAGGGGGTACCCACAGGAAGGAATCCTGGAAGACATGC  
CTGTGGATCCTGGCAGTGAGGCTTATGAAATGCCTTCAGAGGAAGGCTACCAAGACTATG  
AGCCTGAAGCCTAAGAATGTCATTGCACCCAATCTCCTAAGATCTGCCGGCTGCTCTTCCA  
TGCGGTACAAGTGCTCAGTTCCAATGTGCCAGTCATGACCTTTTCTCAAAGCTGTACAGT  
GTGTTTCAAAGTCTTCCATCAGCAGTGATCGGCGTCCTGTACCTGCCCTCAGCATCCCGG  
TGCTCCCCTCTCACTACAGTGAAAACCTGGTAGCAGGGTCTTGTGTGCTGTGGATATTGTT  
GTGGCTTCACACTTAAATTGTTAGAAGAACTTAAAACACCTAAGTACTACCACTTATTTCT  
AAATCTTCATCGTTTTCTTTTTGTTGCTGTTCTTAAGAAGTTGTGATTTGCTCCAAGAGTTTT  
AGGTGTCCTGAATGACTCTTTCTGTCTAAGAATGATGTGTTGTGAAATTTGTTAATATATATT  
TTAAATATGTGAGCATGAGACTATGCACCTATAAATATTAATTTATGAATTTTACAGTTTT  
GTGATGTGTTTTATTAACCTTGTGTTTGTATATAAATGGTGGAAAATAAAATAAAATATTATCC  
ATTGCAAAATCAAAAAAAAAAAAAAAAAAAAA

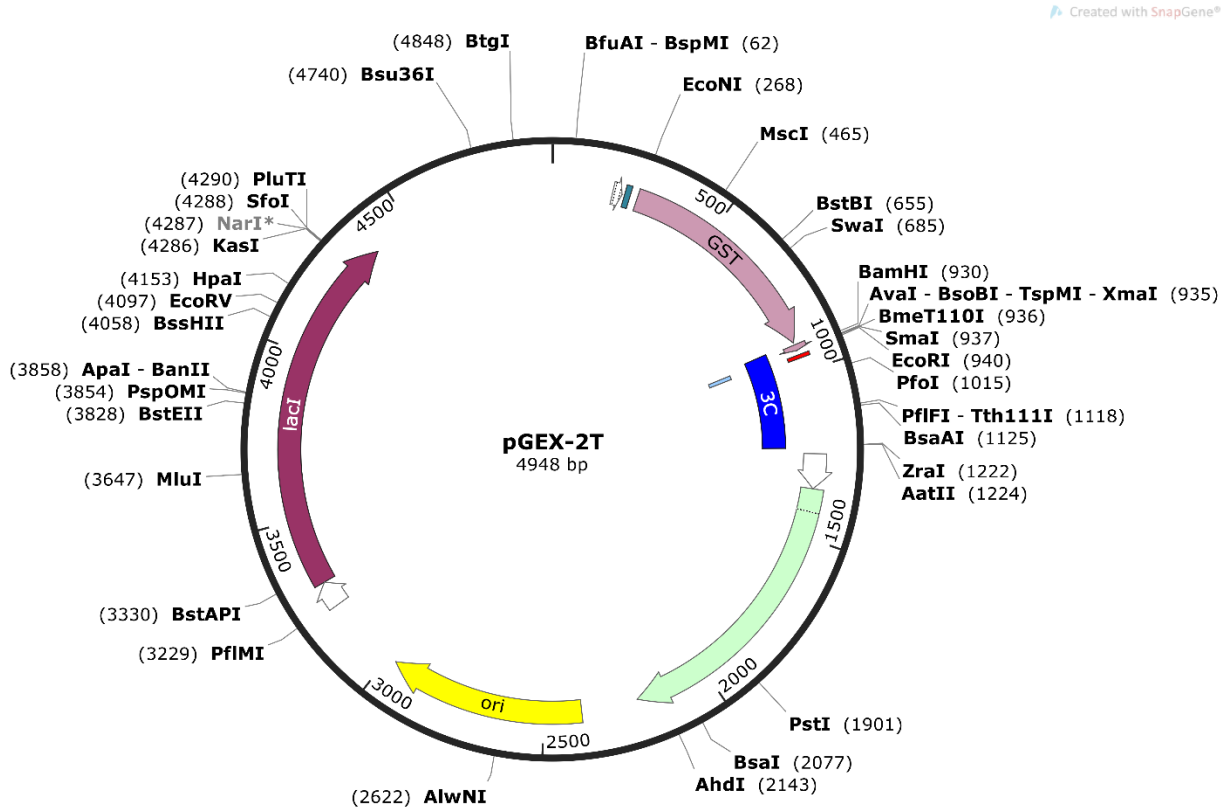
Properties of recombinant mouse  $\alpha$ -synuclein (untagged):

<b>Amino acid sequence</b>	MDVFMKGLSKAKEGVVAAAEKTKQGVAEAAAGKTKEGVLYV GSKTKEGVVHGVTTVAEKTKQVTVNGGAVVTGVTAVAQK TVEGAGNIAAATGFVKKDQMGKGEEGYPQEGILEDMPVDP GSEAYEMPSEEGYQDYEPEA
<b>Molecular weight</b>	14,485.22 Da
<b>Theoretical pI</b>	4.74
<b>Extinction coefficient</b>	7450 M <sup>-1</sup> cm <sup>-1</sup> , at 280 nm measured in water

### 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):

<b>Amino acid sequence</b>	AFRPCNVNTKIGNAKCCPFVCGKAVTFKDRSTCSTYNLSSS LHHILEEDKRRRQVVDVMSAIFQGPISLDAPPPPAIADLLQSV RTPRVIKYCQIIMGHPAECQVERDLNIANSIIAIIANIISIAGIIFVI YKLFCSLQGPYSGEPKPKTKVPERRVVAQGPEEEFGRSILK NNTCVITGNGKFTGLGIHDRILIIPHTADPGREVQVNGVHTK VLDSYDLYNRDGVKLEITVIQLDRNEKFRDIRKYIPETEDDYP ECNLALSANQDEPTIIVKVDVSYGNILLSGNQTARMLKYN PTKSGYCGGVLYKIGQILGIHVGGNGRDGFSAMLLRSYFTG QIKVNHATECGLPDIQTIHTPSKTKLQPSVDFYDVFPGSKEPA VLTDNDPRLEVNFKEA
<b>Molecular weight</b>	44351.97
<b>Theoretical pI</b>	8.50
<b>Extinction coefficient</b>	23100 M <sup>-1</sup> cm <sup>-1</sup> , 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<sub>2</sub>O 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°.

## 4.5 Preparation and recovery of bacterial glycerol stocks

### Materials:

- Overnight bacterial culture
- 50% glycerol in ddH<sub>2</sub>O
- LB agar selection plate (containing appropriate antibiotic)
- 37°C bacterial incubator

**Procedure:**

1. Add 500  $\mu\text{L}$  of bacterial culture to 500  $\mu\text{L}$  of 50% glycerol in a cryovial. Mix gently. Store at  $-80^{\circ}\text{C}$ .
2. To recover bacteria from the stock, use a 200  $\mu\text{L}$  tip to scrape the surface of the frozen solution and streak onto a warm LB agar selection plate. Store the remaining stock at  $-80^{\circ}\text{C}$ . Incubate plate at  $37^{\circ}\text{C}$  overnight.
  - Do not allow the glycerol stock to thaw during handling.
3. Colonies on 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  $\mu\text{g}/\text{mL}$ –25  $\mu\text{g}/\text{mL}$ ).
2. Pipette 5  $\mu\text{L}$  of blank, standard, or unknown sample in duplicate into a clear 96 well plate.
  - Blanks should be run in duplicate.
3. Add 250  $\mu\text{L}$  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 standard or sample measurements.
6. Prepare a standard curve by plotting the average blank-corrected measurement for each BSA standard versus its concentration in  $\mu\text{g}/\text{mL}$ . 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 to 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)	<ul style="list-style-type: none"><li>• 1.98 mL 30% acrylamide/bis-acrylamide</li><li>• 3.78 mL 0.5 M Tris-HCl, pH 6.8</li><li>• 150 µL 10% SDS</li><li>• 9 mL ddH<sub>2</sub>O</li><li>• 15 µL TEMED (add just before pouring gel)</li><li>• 75 µL 10% APS (add just before pouring gel)</li></ul>
14% separating gel solution (15 mL)	<ul style="list-style-type: none"><li>• 7.0 mL 30% acrylamide/bis-acrylamide</li><li>• 3.75 mL 1.5 M Tris-HCl, pH 8.8</li><li>• 150 µL 10% SDS</li><li>• 4.03 mL ddH<sub>2</sub>O</li><li>• 7.5 µL TEMED (add just before pouring gel)</li><li>• 75 µL 10% APS (add just before pouring gel)</li></ul>

Solution	Components
4x SDS loading buffer	<ul style="list-style-type: none"> <li>• 200 mM Tris</li> <li>• 8% SDS</li> <li>• 40% glycerol</li> <li>• 0.4% bromophenol blue</li> <li>• 400 mM DTT</li> <li>• Dilute to 1x when preparing loading samples</li> </ul>
10x SDS-PAGE running buffer	<ul style="list-style-type: none"> <li>• ddH<sub>2</sub>O</li> <li>• 1% SDS</li> <li>• 250 mM Tris</li> <li>• 1.92 M glycine</li> <li>• Dilute 1:10 to prepare a 1x solution</li> </ul>
Destain solution	<ul style="list-style-type: none"> <li>• 50% ddH<sub>2</sub>O</li> <li>• 40% Methanol</li> <li>• 10% Acetic acid</li> </ul>

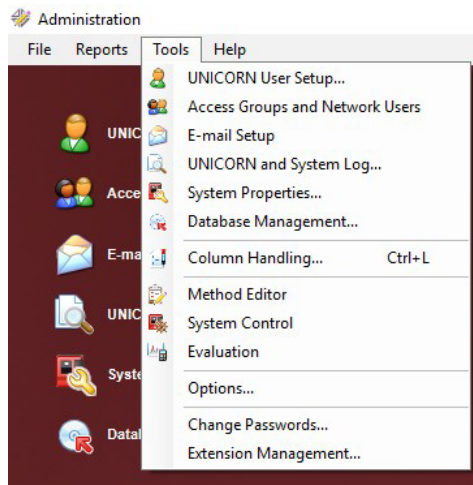
**Procedure:**


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<sub>2</sub>O. 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<sub>2</sub>O.
6. Prepare protein samples in 1x SDS loading buffer so that 1 to 5 µg of protein in a volume of 10 to 20 µL can be loaded per well.

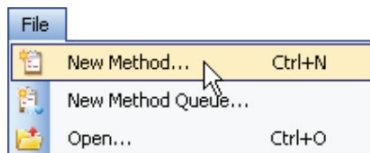
7. Set the electrode assembly to the open position on a clean, flat surface. Place the gel cassette in the electrode assembly.
  - Two cassettes are required to create a functioning assembly. When using 1 or 3 gels, use the buffer dam (included with the cell) to complete the assembly.
8. Place the assembly 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  $\mu$ L of protein ladder into the first well from the left. Load the remaining wells with 10 to 20  $\mu$ L 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<sub>2</sub>O. 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<sub>2</sub>O.
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.

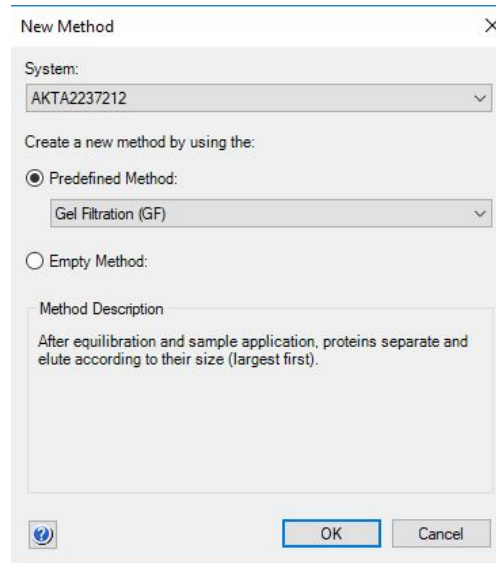


3. Create a new method to equilibrate the Superdex 200 16/600 column.
  - In the **Method Editor** window, select the **New Method** icon  or by selecting **New Method** from the **File** menu.

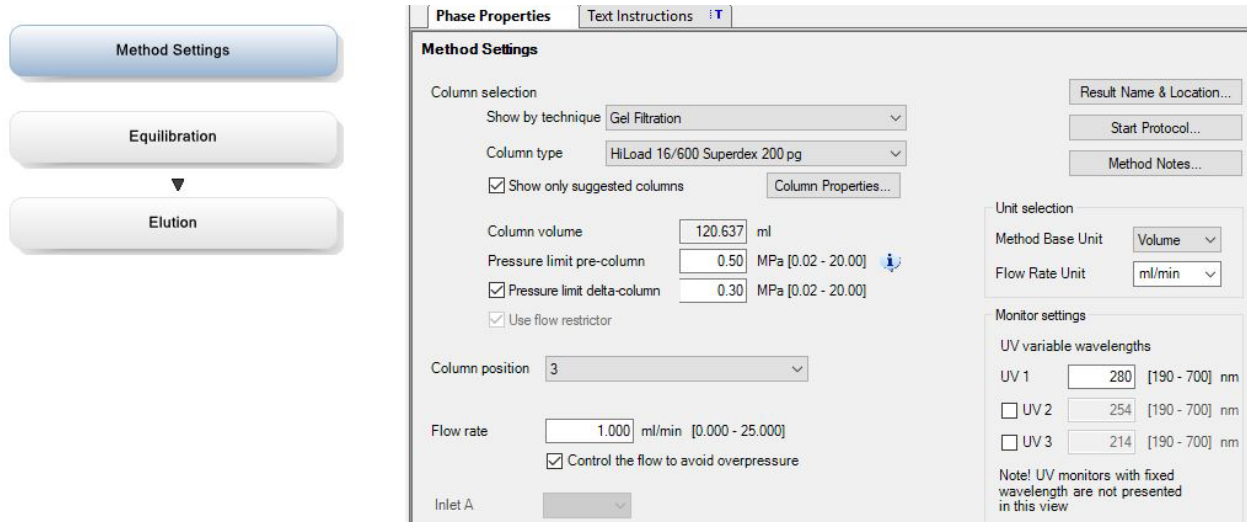




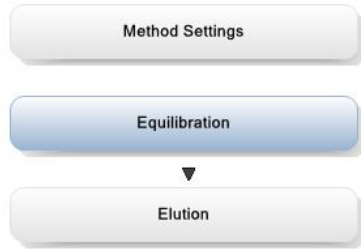
- In the **New Method** window, select **Gel Filtration (GF)** under **Predefined Method** and then select **OK**. A **Method Navigator** window will open.



- 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:



**Phase Properties** | Text Instructions | T

**Equilibration**

Reset UV monitor (recommended if the equilibration occurs before the purification).

Use the same flow rate as in Method Settings       Use the same inlets as in Method Settings

Flow rate  ml/min [0.000 - 25.000]      Inlet A    
 Inlet B   % B [0.0 - 100.0]

Fill the system with the selected buffer

Equilibrate until

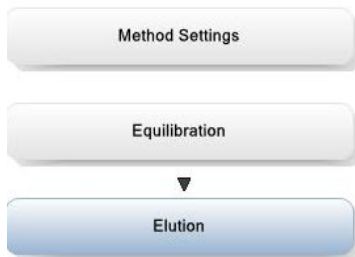
the total volume is  ml

the following condition is met

Conductivity greater than  mS/cm [0.00 - 1000.00]

Accepted pH fluctuation  (0-14)

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



**Phase Properties** | Text Instructions | T

**Elution**

Use the same flow rate as in Method Settings       Use the same inlets as in Method Settings

Flow rate  ml/min [0.000 - 25.000]      Inlet A    
 Inlet B

Up flow ⓘ

Isocratic elution

Volume  ml       % B [0.0 - 100.0]       Fill the system with the selected buffer

Gradient elution

Start at  % B [0.0 - 100.0]       Fill the system with the selected buffer

	Type	Target %B (0-100)	Length (ml)
1	Linear	100.0	150.00

Note: A gradient delay is automatically added, provided that the last gradient segment is linear

Fractionate

in waste (do not collect)

using outlet valve

using fraction collector

Fraction collector

Fractionation settings

Fractionation type

Fractionation destination

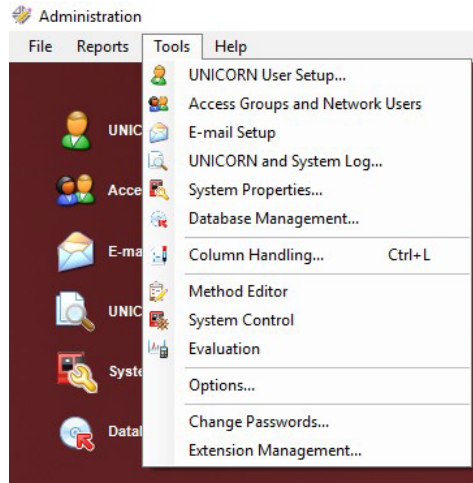
Peak fractionation destination

Fixed fractionation volume

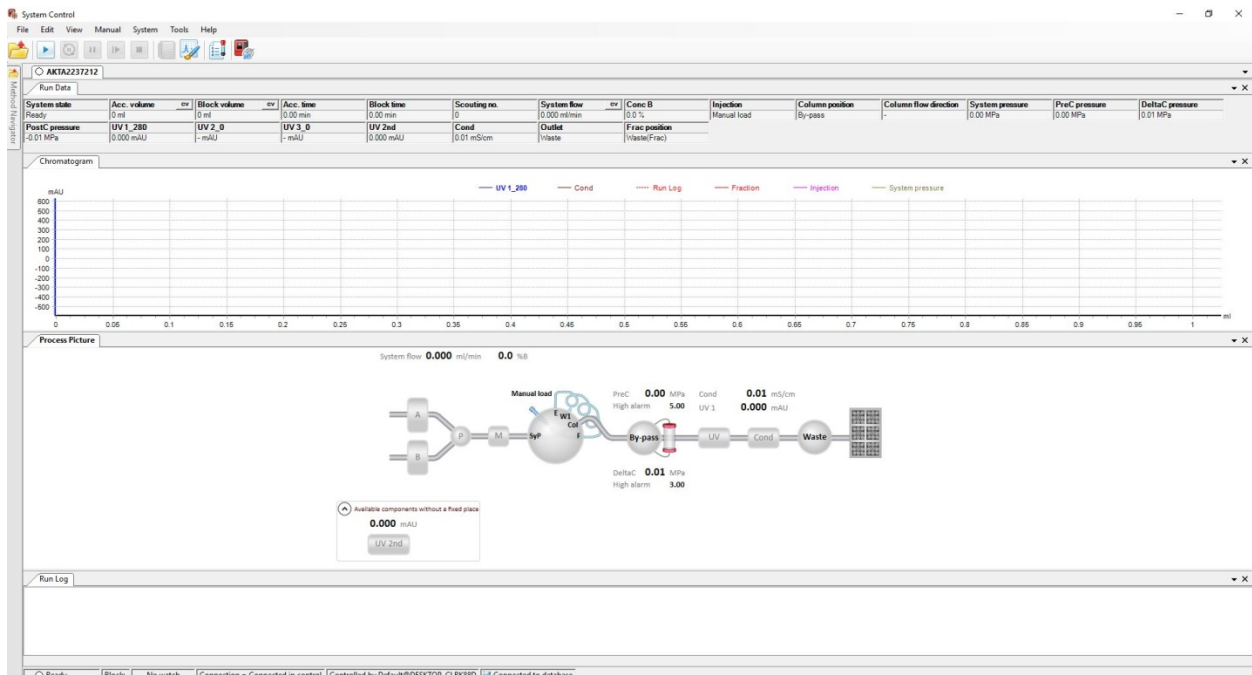
Peak fractionation volume

Start fractionation after  ml (only for isocratic elution)

- Save the method with unique file name.
4. Run the equilibration method.
- Place line A1 into a flask containing ddH<sub>2</sub>O 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).

Start Protocol - AKTA2237212 - Equil HiLoadS200 ON

Result Name and Location >>

**Run info**

Date:

User:

Method:

**Result**

No result

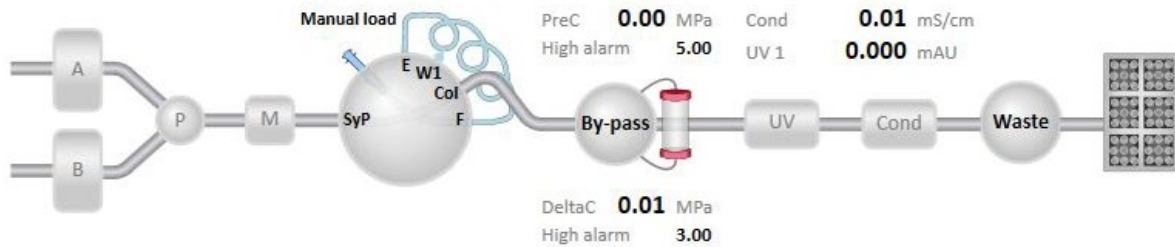
Add unique identifier to result name

Directory:

Scouting subdirectory:

Name:

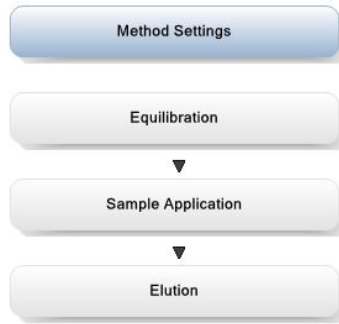
- Switch line A1 from the flask containing water to the flask containing PBS.
- In the **System Control** window, right click on the **A** in the **Process Picture**. Select **Start pump A wash** to fill the line with PBS.



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:



Phase Properties | Text Instructions | IT

**Method Settings**

Column selection

Show by technique: Gel Filtration

Column type: HiLoad 16/60 Superdex 200 pg

Show only suggested columns | Column Properties...

Column volume: 120.637 ml

Pressure limit pre-column: 0.50 MPa [0.02 - 20.00]

Pressure limit delta-column: 0.30 MPa [0.02 - 20.00]

Use flow restrictor

Column position: 3

Flow rate: 1.000 ml/min [0.000 - 25.000]

Control the flow to avoid overpressure

Inlet A: [dropdown]

Result Name & Location... | Start Protocol... | Method Notes...

Unit selection

Method Base Unit: Volume

Flow Rate Unit: ml/min

Monitor settings

UV variable wavelengths

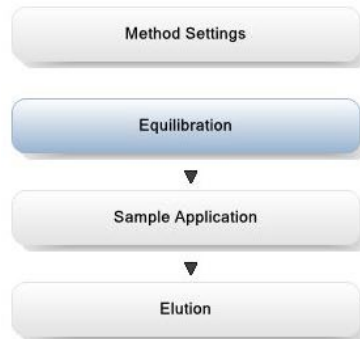
UV 1: 280 [190 - 700] nm

UV 2: 254 [190 - 700] nm

UV 3: 214 [190 - 700] nm

Note! UV monitors with fixed wavelength are not presented in this view

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



Phase Properties | Text Instructions | IT

Reset UV monitor (recommended if the equilibration occurs before the purification).

Use the same flow rate as in Method Settings |  Use the same inlets as in Method Settings

Flow rate: 1.000 ml/min [0.000 - 25.000]

Inlet A: [dropdown]

Inlet B: [dropdown] 0.0 % B [0.0 - 100.0]

Fill the system with the selected buffer

Equilibrate until

the total volume is 1.00 ml

the following condition is met

Conductivity greater than [dropdown]

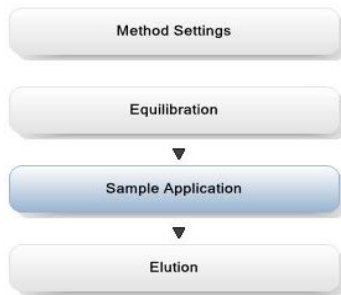
Conductivity greater than: 0.00 mS/cm [0.00 - 1000.00]

Accepted pH fluctuation: 0.10 (0-14)

Accepted UV fluctuation: 0.10 mAU [0.00 - 6000.00]

Accepted conductivity fluctuation: 0.10 mS/cm [0.00 - 300.00]

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



Phase Properties | Text Instructions | T

### Sample Application

Use the same flow rate as in Method Settings  
 Flow rate  ml/min [0.000 - 25.000]

Inject sample from loop  
 Inject sample directly onto column

Fill the loop using   
 Loop type   
 Loop position   
 Sample inlet   
 Fill loop with  ml  
 Empty loop with  ml  
 Sample volume  ml

Wash sample flow path with buffer  
 Prime sample inlet with  ml  
 Wash sample flow path with buffer after sample application.  
 Note! Buffer inlet A set in Method Settings will be used to wash the sample flow path

Use the same inlets as in Method Settings  
 Inlet A   
 Inlet B  %  
 Fill the system with the selected buffer

### Fractionate

in waste (do not collect)  
 using outlet valve  
 using fraction collector

Fractionation settings  
 Fractionation type   
 Fractionation destination   
 Peak fractionation destination   
 Fixed fractionation volume  
 Peak fractionation volume

Advanced Settings...  
 PeakFrac Settings...



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

The screenshot shows the 'Phase Properties' window in the 'Elution' tab. The configuration is as follows:

- Use the same flow rate as in Method Settings
- Use the same inlets as in Method Settings
- Flow rate: 1.000 ml/min [0.000 - 25.000]
- Inlet A: [Dropdown]
- Inlet B: [Dropdown]
- Up flow
- Isocratic elution
  - Volume: 130.00 ml
  - 0.0 % B [0.0 - 100.0]
  - Fill the system with the selected buffer
- Gradient elution
  - Start at: 0.0 % B [0.0 - 100.0]
  - Fill the system with the selected buffer

Type	Target %B (0-100)	Length (ml)
1 Linear	100.0	2.00

Buttons: Add Segment, Delete Segment

Note: A gradient delay is automatically added, provided that the last gradient segment is linear

Fractionate options:

- in waste (do not collect)
- using outlet valve
- using fraction collector

Fraction collector: [Dropdown]

Fractionation settings:

- Fractionation type: Fixed volume fractionation
- Fractionation destination: 96 deep well plate
- Peak fractionation destination: 96 deep well plate
- Fixed fractionation volume: 1.00 ml [0.00 - 2.20]
- Peak fractionation volume: 2.00 ml [0.00 - 2.20]

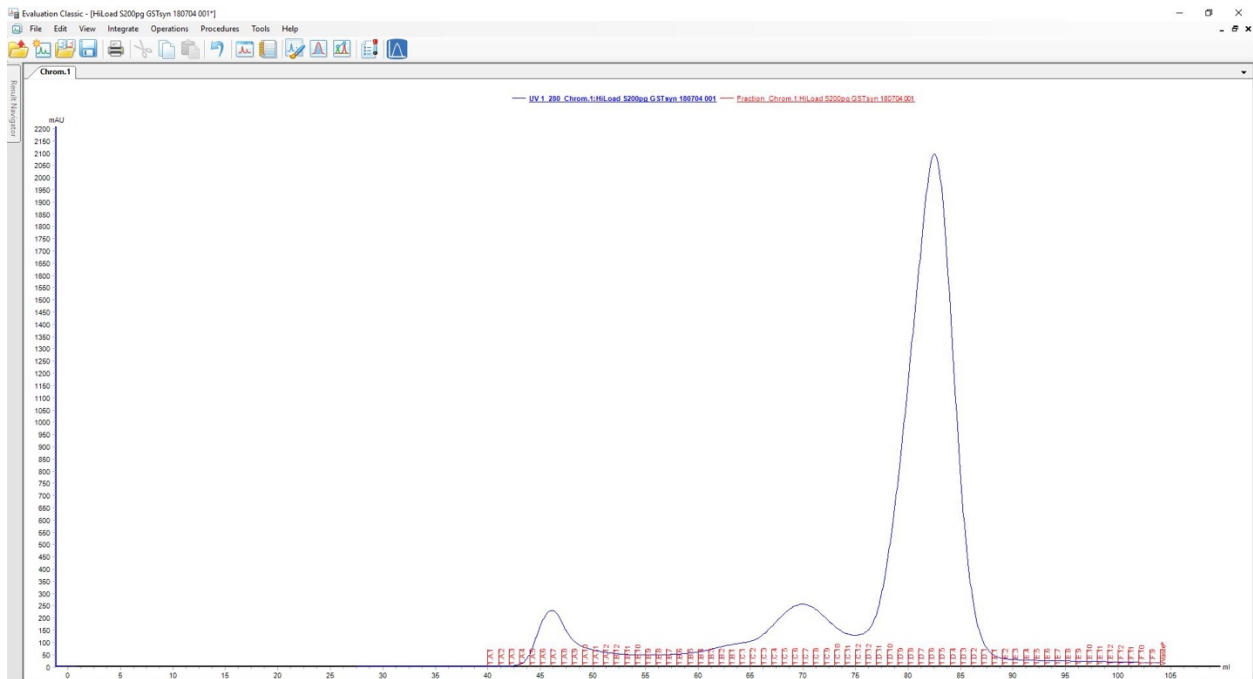
Start fractionation after 35.00 ml (only for isocratic elution)

Buttons: Advanced Settings..., Peak Frac Settings...


- 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<sub>2</sub>O, 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:

The screenshot shows the **Method Editor** window with the **Method Settings** tab selected. The window is divided into three main sections: a left sidebar with buttons for **Method Settings**, **Equilibration**, and **Elution**; a central **Method Settings** panel; and a right sidebar with additional options.

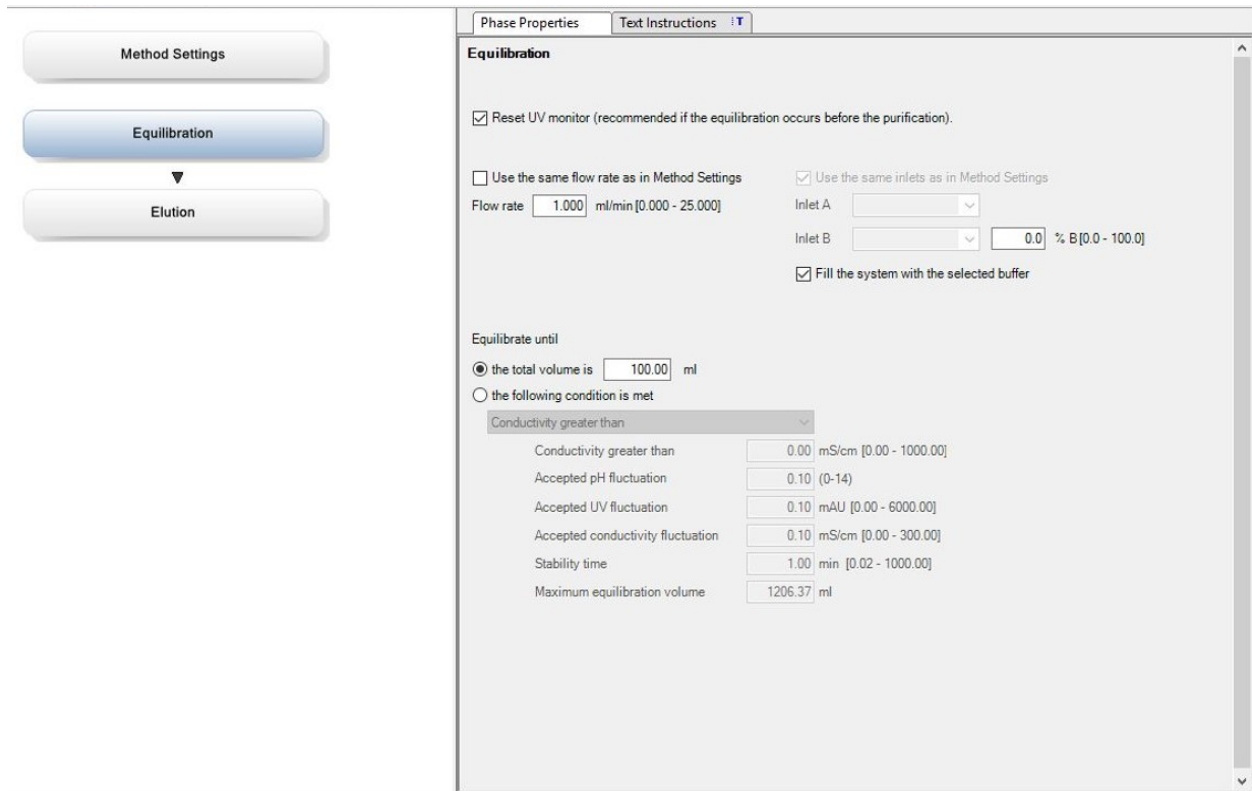
**Method Settings Panel:**

- Column selection:**
  - Show by technique: Gel Filtration
  - Column type: HiLoad 16/600 Superdex 200 pg
  - Show only suggested columns
  - Column volume: 120.637 ml
  - Pressure limit pre-column: 0.50 MPa [0.02 - 20.00]
  - Pressure limit delta-column: 0.30 MPa [0.02 - 20.00]
  - Use flow restrictor
- Column position:** 3
- Flow rate:** 1.000 ml/min [0.000 - 25.000]
  - Control the flow to avoid overpressure
- Inlet A:** [Dropdown]
- Inlet B:** [Dropdown]

**Right Sidebar:**

- Unit selection:**
  - Method Base Unit: Volume
  - Flow Rate Unit: ml/min
- Monitor settings:**
  - UV variable wavelengths:
    - UV 1: 280 [190 - 700] nm
    - UV 2: 254 [190 - 700] nm
    - UV 3: 214 [190 - 700] nm
  - Note! UV monitors with fixed wavelength are not presented in this view
  - Enable pH monitoring
- Enable air sensor alarm:**
  - Inlet A
  - Inlet B
  - Sample inlet
- Column Logbook:**
  - Enable logging of:
    - Column Performance Test
    - CIP

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



Method Settings

Equilibration

Elution

Phase Properties Text Instructions

### Equilibration

Reset UV monitor (recommended if the equilibration occurs before the purification).

Use the same flow rate as in Method Settings

Flow rate  ml/min [0.000 - 25.000]

Use the same inlets as in Method Settings

Inlet A

Inlet B   % B [0.0 - 100.0]

Fill the system with the selected buffer

Equilibrate until

the total volume is  ml

the following condition is met

Conductivity greater than

Conductivity greater than	<input type="text" value="0.00"/>	mS/cm [0.00 - 1000.00]
Accepted pH fluctuation	<input type="text" value="0.10"/>	(0-14)
Accepted UV fluctuation	<input type="text" value="0.10"/>	mAU [0.00 - 6000.00]
Accepted conductivity fluctuation	<input type="text" value="0.10"/>	mS/cm [0.00 - 300.00]
Stability time	<input type="text" value="1.00"/>	min [0.02 - 1000.00]
Maximum equilibration volume	<input type="text" value="1206.37"/>	ml

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

Method Settings

Equilibration

▼

Elution

Phase Properties | Text Instructions | T

**Elution**

Use the same flow rate as in Method Settings       Use the same inlets as in Method Settings

Flow rate: 0.200 ml/min [0.000 - 25.000]      Inlet A: [ ]  
 Inlet B: [ ]

Up flow [ ]

Isocratic elution  
 Volume: 180.96 ml      0.0 % B [0.0 - 100.0]       Fill the system with the selected buffer

Gradient elution  
 Start at: 100.0 % B [0.0 - 100.0]       Fill the system with the selected buffer

	Type	Target %B (0-100)	Length (ml)
1	Linear	100.0	150.00

Add Segment  
Delete Segment

Note: A gradient delay is automatically added, provided that the last gradient segment is linear

**Fractionate**

in waste (do not collect)       using outlet valve       using fraction collector

Fractionation settings:

Fractionation type: Fixed volume fractionation [ ]      Advanced Settings...  
 Fractionation destination: 96 deep well plate [ ]  
 Peak fractionation destination: 96 deep well plate [ ]      Peak Frao Settings...  
 Fixed fractionation volume: [ ]  
 Peak fractionation volume: [ ]

Start fractionation after: 24.13 ml (only for isocratic elution)

## 9. Run the cleaning method.

- Place line A1 into the flask containing ddH<sub>2</sub>O and line B1 into 20% EtOH.
- Clean the sample loop by injecting 10 mL of PBS, then 10 mL of ddH<sub>2</sub>O, 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 sufficient substrate solution 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 standard 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.