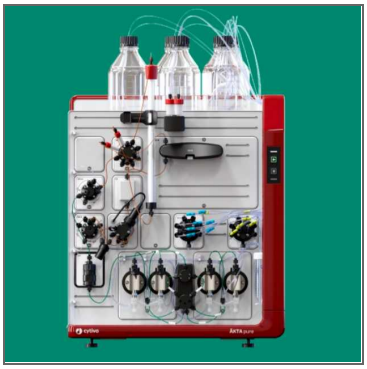

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Protocol status: Working
We use this protocol and it's working

Created: May 21, 2022

a-Synuclein protein expression and purification

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ABSTRACT

Protocol for recombinant a-synuclein purification, useful as monomer template for seeded-amplification assays (like RT_QUIC or PMCA) . Recommendations are to store the protein always on ice while not running and do not stop purification when it is started.

MATERIALS

1. BL21-CodonPlus (DE3)-RIL Chemical Competent Cells (Agilent #230245-41)
2. Thermo Scientific™ Low Protein Binding Collection Tubes (1.5 mL) PI90411

Nalgene™ Rapid-Flow™ Sterile Single Use Vacuum Filter Units **Thermo Fisher Scientific Catalog #565-0010**

SnakeSkin Dialysis Tubing 3.5K MWCO 35 **Thermo Fisher Scientific Catalog #88244**

Endotoxin detection kit LAL **Genscript Catalog #95045-024**

ToxinEraser™ Endotoxin Removal Kit **Genscript Catalog #89233-330**

ToxinEraser™ Endotoxin Removal Resin **Genscript Catalog #L00402**

HiPrep Q HP anion exchange chromatography column **Cytiva Catalog #29018182**

MilliporeSigma™ Amicon™ Ultra-15 Centrifugal Filter Units **Catalog #MilliporeSigma™ UFC901024**

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PROTOCOL MATERIALS

Nalgene™ Rapid-Flow™ Sterile Single Use Vacuum Filter Units **Thermo Fisher Scientific Catalog #565-0010**

Materials, Step 21

SnakeSkin Dialysis Tubing 3.5K MWCO 35 **Thermo Fisher Scientific Catalog #88244**

Materials, Step 22

Endotoxin detection kit LAL **Genscript Catalog #95045-024**

Materials, Step 43

ToxinEraser™ Endotoxin Removal Kit **Genscript Catalog #89233-330**

Materials, Step 41

ToxinEraser™ Endotoxin Removal Resin **Genscript Catalog #L00402**

Materials, Step 41

HiPrep Q HP anion exchange chromatography column **Cytiva Catalog #29018182**

Materials, Step 24

MilliporeSigma™ Amicon™ Ultra-15 Centrifugal Filter Units **Catalog #MilliporeSigma™ UFC901024**

Materials, Step 35

Transformation

1d

1 Thaw down an aliquot of plasmid construct (pRK172) encoding WT-human-a-synuclein [M] 0.3 mg/r... 15m

On ice

2 Thaw down On ice an aliquot of BL21 (DE3) RIL competent E Coli cells 15m

3 Add 1 µL of plasmid construct to the thawed competent cells and gently mix by flicking the bottom of the tube with a finger a few times

Safety information

do not resuspend

3.1 Incubate the reaction mix On ice 15m

4 Perform heat-shock transformation 42 °C in water bath incubator with manually shaking at 100 rpm, 00:00:45 1m

Equipment

Precision™ General Purpose Water Bath	NAME
Water Bath	TYPE
Thermo Scientific	BRAND
TSGP10	SKU

5 Immediately transfer the tube on ice and incubate for 1 min. 1m

6 Add 1000 µL of SOC media to a chilled reaction 10s

7 Incubate the bacteria 200 rpm, 37°C, 00:30:00 30m

Equipment	
ThermoMixer® C	NAME
ThermoMixer	TYPE
Eppendorf	BRAND
5382000023	SKU

7.1 Prepare sterile 10cm LB agar plate containing [M] 0.1 mg/mL of ampicillin

8 Centrifuge at 500 x g, 10°C, 00:03:00 and discard the supernatant leaving 50 µL of media 3m

8.1 Spread 50 ul of cell suspension onto a selection plate and incubate overnight at 37 °C in bacterial incubator 10m

Equipment	
Isotemp™ Microbiological Incubator, 178 L, Stainless Steel	NAME
Microbiological Incubator	TYPE
Fisherbrand	BRAND
15-103-0513	SKU

Protein expression 12h

9 Pick one colony and transfer into 10 mL LB media with [M] 0.1 mg/mL of ampicillin start in the morning (9:00 am)

9.1 Incubate the bacteria 250 rpm, 37°C, 05:00:00 until it reaches OD 0.2-0.3 5h

Equipment	
Natural convection incubator	NAME
Bacterial shaker	TYPE
Innova	BRAND
M1335-0000	SKU

10 Transfer a starter culture to 2X2L flasks filled with 0.5L LB media with [M] 0.1 mg/mL of ampicillin 5 mL to each flask

11 Incubate the culture at the same conditions until it reaches OD 0.8 (use nanodrop or cuvette) (reaches optimal density at 6-7 pm) 5h

12 Induce protein expression by adding [M] 0.05 millimolar (mM) IPTG, incubate at 18 °C for 12:00:00 overnight 12h

Note

To cool down the grown culture, transfer the flasks into ice-bath and incubate until it reaches desired temperature

Cell lysis

11m 45s

13 Collect the pellets by centrifugation (JA14 rotor) at 5000 x g x g at 4 °C for 00:10:00. 10m
Used 250 ml Beckman tubes
Usually get 10-12 g from 2L

14 Add to pellets 80 ml of lysis buffer (total): [M] 10 millimolar (mM) ThisHCl pH 7.6, [M] 750 millimolar (mM) NaCl, [M] 1 millimolar (mM) EDTA, [M] 1 millimolar (mM) PMSF (add just before using, have aliq frozen [M] 0.1 Molarity (M)), protease inhibitors (use MAXI version, need only one tablet);

15 Carefully resuspend the pellets to homogenize the solution

15.1 Heat up 1 L of water in a high temperature resistant glass beaker (turn heat to the max on the magnetic stirrer)

16 While waiting on water to get to the boiling point sonicate the lysates (use thick prob-tip) for 10m 00:01:00, 30%, 00:00:15 ON 00:00:30 OFF of amplitude then go to next falcon, had 3 falcons (repeat 3 times, avoid overheating)

17 After sonication samples need to get boiled thereby put the falcon tubes into glass beaker and boil f 25m 00:25:00. Use tweezers to pull out the tubes

18 Transfer boiled homogenates into new 50 mL falcon tubes; chill down suspensions at room tempera 20m for 20 min

19 Prepare 4 L of buffer [M] 10 millimolar (mM) TrisHCl pH 7.6, [M] 50 millimolar (mM) NaCl, [M] 1 millimolar (mM) EDTA, [M] 1 millimolar (mM) PMSF for dialysis

20 Centrifuge the homogenates at 20000 x g for 01:00:00 at 4 °C 1h

21 Filter the supernatant using 0.45 um filter unit

Nalgene™ Rapid-Flow™ Sterile Single Use Vacuum Filter Units Thermo Fisher Scientific Catalog #565-0010

22 Transfer filtered supernatant into dialysis bag which is: SnakeSkin Dialysis tubing, 3.5K MWCO, 35 mm dry I.D., 35 feet.

Measure the dialysis tube taking into consideration that 5 cm length of tube holds 48 mL of the sample (plus 2.5cm at each end for closure). Clip the tube using green clips, make sure it does not leak.

Place the dialysis bag into 4 L plastic beaker filled with dialysis buffer, incubate overnight on magnetic plate on the slow mode (Chromatography fridge)

SnakeSkin Dialysis Tubing 3.5K MWCO 35 Thermo Fisher Scientific Catalog #88244

Equipment

ÄKTA pure 25 L1	NAME
ÄKTA pure chromatography system	TYPE
Cytiva	BRAND
29018225	SKU

Protein purification (anion-exchange chromatography)

23 After a night of dialysis (4 °C slow mixing) collect the suspension into 100 mL glass bottle (filter the sample before running on the column, 0.22 um filter).

24 Column - HiPrep Q HP 16/10 column 1x20 ml (stored in 70% ethanol);

HiPrep Q HP anion exchange chromatography column **Cytiva Catalog #29018182**

24.1 Wash the column 2V of miliQ degassed water

24.2 Wash the column with 2V of **STARTING BUFFER** [M] 10 millimolar (mM) TrisHCl pH 7.6, [M] 50 millimolar (mM) NaCl

24.3 Activate with 1V of [M] 10 millimolar (mM) TrisHCl pH 7.6, [M] 1 Molarity (M) NaCl

24.4 Equilibrate with 3V of starting buffer

25 Load 80 mL of suspension and then washed with 100 ml [M] 50 millimolar (mM) NaCl [M] 10 millimolar (mM) TrisHCL, 300 mL of gradient elution (0-100%), 2 ml/min flow rate. Collected samples using fraction collector 2, every fraction 4 ml (use 10 ml glass tubes)

26 Place supernatant into channel A1 (was previously use for starting buffer, do not generate bubbles)

27 Place starting buffer in channel A2 (clean the tubing using the program mode)

28 Place elution buffer in channel B1 ([M] 10 millimolar (mM) TrisHCl pH 7.6 , [M] 1 Molarity (M) NaCl)
Collected samples using fraction collector 2, every fraction 4 ml (use 10 ml glass tubes);

29 Analyze the fractions eluted at 250-350 mM salt (20 RFU conductivity) though SDS-PAGE (stain with Coomassie).
Combine 10 µL *of each fraction with* 10 µL *of 2X laemmli buffer and analyze fractions by SDS-PAGE with 4–20% gradient gels, followed by coomassie staining/destaining*

30 Measure A280/260 for the fractions containing single a-syn band, avoid collecting samples with A280/260 > 0.85

31 Combine the evaluated factions and measure total protein concentration using nanodrop.

32 Dialyze with 4 L of [M] 10 millimolar (mM) TrisHCl pH 7.6 , [M] 50 millimolar (mM) NaCl (follow instruction for dialysis)

Further purification

33 Repeat section 'Protein purification (anion-exchange chromatography)' for the further fractionation of the purified preparation go to step #23

Protein concentration

10m

34 Concentrate dialyzed protein sample to approximately [M] 30 mg/mL aliquot
Prepare the ultra-concentration system

35 Use 50 mL ultra centrifugation units with 3K cutoff

MilliporeSigma™ Amicon™ Ultra-15 Centrifugal Filter Units **Catalog #MilliporeSigma™ UFC901024**

36 Wash off the unit with miliQ water through centrifugation at 5000 x g at 4 °C for 00:05:0 5m , JA10 rotor

37 Load first 15 mL of the sample into ultracentrifugation unit (max load of the unit is approx. 15 mL)

38 Centrifuge at 5000 x g at 4 °C for 00:05:00 , JA10 rotor 5m

39 Resuspend concentrated sample, add more of protein sample and concentrate until the total volume is ~ 5 mL

40 Store at -80 °C . Yield should be approximately 80 mg per 2 L culture

Endotoxin removal

41 Follow instructions for ToxinEraser™ Endotoxin Removal Kit **Genscript Catalog #89233-330** with modifications
For a more successful endotoxin removal, add 1 mL of
ToxinEraser™ Endotoxin Removal Resin **Genscript Catalog #L00402** before the regeneration process

42 Collect the eluate into 5 mL endotoxin-free tube and save 2 aliquots (10 µL and 50 µL) for protein concentration and endotoxin measurements

Endotoxin quantification

43 Follow instructions for Endotoxin detection kit LAL Genscript

Endotoxin detection kit LAL
Genscript Catalog #95045-024