Khurana Lab BRIGHAM HEALTH BRIGHAM AND WOMEN'S HOSPITAL HARVARD MEDICAL SCHOOL	SOP-VK	**Expression and purification of untagged a-synuclein**	Author: Alain	Revision: 1.0	Issued 4.21.2020
			Approved:		Revised

1. Purpose

Generation of untagged a-synuclein.

2. Materials

2.1. Expression

2.1.1. Transformation Reagents

- 50 ng/µL pET21a-alpha-synuclein
- 50 µL BL21(DE3) E. coli
- 1 mL SOC medium: 20 g/L typtone, 5 g/L yeast extract, 4.8 g/L MgSO4, 3.6 g/L dextrose, 0.5 g/L NaCl, 0.186 g/L KCl
- Incubator
- Water Bath

2.1.2. Selection Plates

- 37 g of mixed LB/Agar powder (5 g peptone, 10 g peptone from casein, 10 g NaCl, 12 g Agar)
- 1 L of MilliQ water
- Autoclave
- Sterile Dishes (100x15 mm)
- 1000x stock of antibiotic (in our case 100 mg/mL of ampicillin in 50% ethanol)

2.1.3. Inoculation and Induction

- 250-mL Erlenmeyer Flask
- LB medium (10 g/L casein digest peptone, 10 g/L NaCl, 5 g/L yeast extract, 1.5 g/L TRIS-HCl)
- 37 C Incubator
- 2-L Erlenmeyer Flask
- 1mM Isopropyl Œ≤-D-1-thiogalactopyranoside
- 1000x stock of antibiotic

2.2. Purification

2.2.1. Cell Lysis

- Centrifuge capable of handling 1-L total volume at 9,000xg
- 2x 250-mL centrifuge tubes
- Hot Plate

- 2-L Erlenmeyer Flask

2.2.2. Ion Exchange Chromatography (IEX)

- Ion Exchange Buffer A: 20 mM TRIS, 25 mM NaCl, 1 mM EDTA, pH=8.00
- Ion Exchange Buffer B: 20 mM TRIS, 1 M NaCl, 1 mM EDTA, pH=8.00
- 5-mL HiTrap Q HP columns (GE Life Sciences, 17516301)
- ÄKTAprime plus FPLC system

2.2.3. Size Exclusion (SEC)

- 50 mM ammonium acetate, pH=7.40
- 13-mL HiPrep 26/60 Sephacryl S-200 HR (GE Life Sciences, 17119501)
- ÄKTAprime plus FPLC system

3. Methods

3.1. Expression

1. Thaw 20-50 µL of BL21(DE3) competent *E. coli* on ice for ~10 minutes or until melted (**Note 1**).
2. Once the cells are completely thawed, add 1-5 µL of 10 pg-100 ng of pET21a-alpha-synuclein (**Note 2**) and gently mix by inverting the tube.
3. Following the addition of the plasmid, incubate the cell and plasmid mixture on ice for about 30 minutes (**Note 3**).
4. Then, heat-shock the cell and plasmid mixture for exactly 10 seconds in a 42°C water bath.
5. Remove the mixture from the water bath and place it on ice for 5 minutes, then add 950-980 µL of SOC medium to the tube (**Note 4**).
6. Incubate the bacteria at 37°C for 60 minutes, while shaking at ~250 rpm. After incubation, repeatedly invert the tube to mix the culture well and then plate 100 µL onto selection plates (See Materials 2.2). Incubate the plate(s) upside-down overnight at 37°C (**Notes 5 and 6**).
7. On the following day, choose a single colony to inoculate in ~180 mL of LB medium at 37ºC overnight (pre-culture)**.**
8. The next day, add a 1:20 dilution of the pre-culture to LB medium and grow at 37 ºC until OD_{600} = 0.5-0.6. Once an OD_{600} = 0.5-0.6 is reached, induce the expression of αS with 1 mM IPTG and let the culture grow at 37ºC for ~4 hours (**Note 7**).
9. Then, spin the culture at 9,000xg for 20 minutes (RT). After the spin, remove the supernatant and freeze the pellet overnight at -20ºC. Freezing the pellet in this fashion already lyses most of the bacteria.

3.2. Purification

This protocol is written for an ÄKTAprime plus FPLC system equipped with a 280-nm UV and conductivity detectors. Although other FPLC systems will largely follow the same protocol, it is advised to always refer to the manufacturer's specifications.

3.2.1. Cell Lysis

1. Pre-heat a hot plate and a 2-L Erlenmeyer Flask to the minimum temperature to initiate boiling. While the hotplate and flask are reaching the right temperature (enough to bring the culture to a boil but not too high that it will cause charring), submerge the frozen pellet in IEX Buffer A (20 mM TRIS, 25 mM NaCl, 1 mM EDTA, pH=8.00) and vortex until the pellet is completely resuspended, taking extra care not to leave any pellet in solution (**Note 8**).
2. Pour the resuspended cell suspension into the pre-heated 2-L Erlenmeyer flask. Increase the hotplate temperature and let the mixture rise to an even boil.
3. After the first visible signs of boiling, allow the mixture to boil for an additional 15 minutes to denature and precipitate proteases and other protein contaminants.
4. After boiling, let the lysate cool down to 4ºC (**Note 10**), transfer it to 250-mL centrifuge bottles and spin it at 20,000g for 45 minutes (4ºC). Filtrate the supernatant through a 0.22-μm or 0.45-μm filter. Bring the supernatant up to approximately 300 mL with IEX buffer A.

3.2.2. Ion Exchange Chromatography (IEX)

1. Wash the FPLC system with IEX Buffer A at 5 mL/min.
2. Connect the columns (2x 5-mL HiTrap Q HP columns) in the presence of a flow rate of 0.3 mL/min. to ensure that no air enters the column (**Note 11**). After the columns are attached, equilibrate them with IEX Buffer A at 1 mL/min. for 40 minutes or until the conductivity reaches a steady measurement for ~20 minutes.
3. Following equilibration, load the cell lysate mixture onto the columns overnight at a max flow rate of 1mL/min.
4. Wash the column with IEX Buffer A until a steady (around 40 mL) plateau in the 280-nm absorbance is achieved, in order to remove any weakly bound contaminants (**Note 12**).
5. After the UV absorbance plateaus, elute the protein with a gradient, from 0% to 100%, of IEX Buffer B (20 mM TRIS, 1 M NaCl, 1 mM EDTA, pH=8.00) over 75 mL and collect fractions every 5 mL.
6. Analyze all the fractions and the flow-through (the unbound lysate fraction which passes through the column and is collected in the FPLC waste during the lysate loading step) via Coomassie-stained SDS-PAGE or Western Blot (αS usually elutes between 25 and 35 mS) to determine the fractions with the highest concentration of αS (**Note 13**).
7. The fractions (usually around a conductivity of ~25 mSi when adopting this protocol and purification system) containing the greatest amount of αS can be pooled and (eventually) concentrated down to 10mL, which will then be loaded on a size-exclusion column.

3.2.3. Size-Exclusion Chromatography (SEC)

1. Wash the FPLC system with 50 mM ammonium acetate, pH=7.40 (5 mL/min.). Attach the SEC column (HiPrep 26/60 Sephacryl S-200 HR) in the presence of a buffer flow rate of 0.2 mL/min., to ensure that no air enters the column.

2. Attach a 10-mL loop to the six-way valve of the FPLC system and flush it with 20 mL of 50 mM ammonium acetate to remove any aggregated protein or built-up waste.
3. Inject the pooled IEX fractions in the loop, making sure not to inject air bubbles into the system or column. The valve position must be set to "Load" when injecting the sample onto the loop. (**Note 14**).
4. At the beginning of the run, switch the valve position to "Inject". At this point, the pooled IEX fractions will be injected onto the SEC column. After 25 mL of 50 mM ammonium acetate are run through the loop, switch the valve position back to "Load".
5. Flush a total of 350 mL of Ammonium Acetate buffer through the FPLC, collecting 14x 5-mL fractions between 110 mL and 180 mL corresponding to a molecular weight of 60 kDa.
6. Analyze the fraction purity via Coomassie-stained SDS-PAGE and pool 5-6 of the purest fractions. Measure the absorbance of these fractions at 280 nm ($\varepsilon(\alpha S)$=0.412 $mL \cdot mg^{-1} \cdot cm^{-1}$). Usually purity of >95% can be obtained.
7. Aliquot the pooled fractions into 1-mg aliquots, then flash-freeze them using liquid nitrogen or a dry ice/ethanol bath and lyophilize (or store frozen at -80ºC) the samples.
8. After lyophilization, seal the tubes with Parafilm, to prevent moisture from entering, and store at 4ºC for short-term storage (1-2 weeks) or -20ºC for long-term storage.

4. Notes

1. If more than 50 μL of cells are to be thawed, the remaining aliquot can be frozen using a 100% ethanol and dry ice bath.
2. Our lab has had consistent success using ~50-ng of pET21a-alpha-synuclein DNA for transformation.
3. This time can be reduced to a minimum of 2 minutes, but the transformation efficiency significantly decreases with each halving of the time.
4. SOC medium is ideal, however LB medium can also be used but will result in a roughly twofold loss of transformation efficiency.
5. When first plating the transformants, is it recommended that different volumes of the culture (e.g. 50 μL, 100 μL, 200 μL) are plated to ensure that at least 5-6 single colonies grow on each plate, but not so many that picking one becomes challenging.
6. If the plasmid contains resistance for ampicillin, the outgrowth step is not necessary[6]. However, if different resistance genes are present in the expression vector, it is necessary to perform this step to allow for cell recovery and expression of the antibiotic resistance gene(s).
7. The amount of IPTG to be used for the induction (typically 0.1-1 mM) depends on the cells, construct and culture conditions. It is recommended to perform a quick optimization, when setting up the expression protocol, in order to determine the optimal amount of IPTG. Also, it is not necessary to grow the cells at a lower temperature (e.g. 20-30ºC) because overexpressed αS does not tend to form inclusion bodies in *E. coli* due to its high solubility in the bacterial[17].
8. If cells are spun in a 250-mL centrifuge tube, approximately 60 mL of IEX buffer A should be enough to fully submerge the pellet.
9. If the pellet is not fully resuspended after generous vortexing, a 5-mL pipette can be used to break up the remaining clumps.
10. It is highly recommended to incubate the lysate mixture at 4ºC after cooling on the benchtop to avoid damaging centrifuge tubes upon high-speed centrifugation.

11. If columns are stored in 20% ethanol it is recommended to wash the columns with MilliQ before equilibrating the column with buffer. This will ensure that salts will not precipitate in the column.
12. The wash step can also be performed using low percentages of IEX buffer B (e.g. 5-10%), in order to have more stringent wash conditions. However, this can also wash off small amounts of αS and should thus be tested by checking the waste of the washing step by Coomassie-stained SDS-PAGE or WB. In total, the volume of IEX Buffer A used during the wash step should be between 150-200 mL.
13. For a lab-scale expression (typically 1- or 2-L expression), 2x 5 mL HiTrap columns should be enough to allow for complete αS binding. If a larger expression is required, it is recommended to increase the number of HiTrap columns in the system or switch to a column(s) with a larger bed volume(s). Always analyze the flow-through fraction to ensure complete binding of αS to the columns. If αS is detected in the flow-through fraction, increase the number of columns in the system to increase the overall binding capacity.
14. Injecting small amounts of air into the columns is unavoidable (especially when working with a 10-mL sample loop). Air bubbles, when eluting, will give off characteristic spikes in the UV absorbance trace[20]. On the other hand, if large amounts of air are injected onto the column (i.e. resin discoloration from the presence of air is visible in the bed), generously flush the system and column with buffer. This applies to both IEX columns and SEC columns, but SEC columns are much more sensitive to the presence of air in their resin because of their resin packing being critical for resolution (and cracks or lacunae can disrupt it completely and require column re-packing).

Khurana Lab BRIGHAM HEALTH BRIGHAM AND WOMEN'S HOSPITAL HARVARD MEDICAL SCHOOL	SOP-VK	**Recombinant a-synuclein pre-formed fibril generation**	Author: Alain	Revision: 1.0	Issued 4.21.2020
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5. Purpose

Generation of a-synuclein pre-formed fibrils.

6. Materials

- lyophilized monomeric synuclein
    - Alternatively: aliquots of untagged alpha-synuclein in PBS, 0,5 mg/mL or 5mg/ml
- Sterile dPBS
- 1,5ml Protein LoBind tubes (Eppendorf)
- Parafilm

1. Reconstitute 1mg of lyophilized monomeric synuclein with 100ul cold sterile DPBS on ice (do not pipet, close tube immediately).
2. Transfer tubes on wet ice to 4C cold room and rotate on tube rotator for 10min.
3. Centrifuge for 10min at 15,000g at 4C.
4. Transfer supernatant to clean Protein-LoBind tube.
5. Prepare 5ul aliquots of serial dilutions (1:10, 1:25, 1:50, 1:100) and measure concentration with Nanodrop (A280 - MW=14,5 kDa; Extinction coefficient ε for human α-syn = 5,960 $M^{-1}cm^{-1}$)
6. Dilute down to final concentration of 5mg/ml, use 100ul (100-500ul) aliquots for reproducible results
7. Take 1-2ul aliquot of monomer and dilute to 1ul/ml for electron microscopy, flash-freeze in dry ice – ethanol slurry
8. Seal tubes with parafilm if no tube lock
9. Place the tube in an orbital thermomixer with a heated lid for 7 days at 37 °C, shaking at 1,000rpm.
    a. NOTE: At the end of the 7 days, the contents of the tube should appear turbid. The thermomixer must have a lid to prevent condensation formation on the tube lids.
10. Gently flick the tube to resuspend a-syn fibrils
    a. Fibrils for quality control steps can be stored at RT overnight
11. Prepare aliquots for QC assays
    a. 5ul – thioflavin T binding assay
    b. 2ul for TEM
12. For long-term storage (12-18 months), flash-freeze fibrils in liquid nitrogen and store at -80C.

7. References

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