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Expression and purification of untagged asynuclein

In 1 collection

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ABSTRACT

This protocol details the generation of untagged a-synuclein.

ATTACHMENTS

dh34biqa7.pdf

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PROTOCOL CITATION

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COLLECTIONS

Expression and purification of untagged asynuclein and recombinant a-synuclein pre-formed fibril generation

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PARENT PROTOCOLS

Part of collection
Expression and purification of untagged asynuclein and recombinant a-synuclein pre-formed fibril generation

MATERIALS TEXT

Materials:

1. Expression:

1a. Transformation Reagents:

- [M]**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

1b. 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 (100x **15 mm**)
- 1000x stock of antibiotic (in our case [M]**100 mg/ml** of ampicillin in 50% ethanol)

1c. 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
- [M]**1 Milimolar (mM)** Isopropyl Œ≤-D-1-thiogalactopyranoside
- 1000x stock of antibiotic

2. Purification:

2a. Cell Lysis:

- Centrifuge capable of handling **1 L** total volume at **9.000 x g**
- 2x **250 mL** centrifuge tubes
- Hot Plate
- **2 L** Erlenmeyer Flask

2b. Ion Exchange Chromatography (IEX):

- Ion Exchange Buffer A: [M]**20 Milimolar (mM)** TRIS, [M]**25 Milimolar (mM)** NaCl, [M]**1 Milimolar (mM)** EDTA, pH**8**
- Ion Exchange Buffer B: [M]**20 Milimolar (mM)** TRIS, [M]**1 Molarity (M)** NaCl, [M]**1 Milimolar (mM)** EDTA, pH**8**

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- **5 mL HiTrap Q HP columns (GE Life Sciences, 17516301)**
- ÄKTAprime plus FPLC system

2c. Size Exclusion (SEC):

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

Expression 40m

1 10m

Thaw **20 µl** - **50 µl** of BL21(DE3) competent E. coli **On ice** for ~ **00:10:00** or until melted.

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 

Once the cells are completely thawed, add **1 µl** - **5 µl** of **10 pg** - **100 ng** of pET21a-alpha-synuclein and gently mix by inverting the tube.

Our lab has had consistent success using ~50-ng of pET21a-alpha-synuclein DNA for transformation.

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3 30m

Following the addition of the plasmid, incubate the cell and plasmid mixture **On ice** for about **00:30:00** .

This time can be reduced to a minimum of **00:02:00** , but the transformation efficiency significantly decreases with each halving of the time.

4 10s

Then, heat-shock the cell and plasmid mixture for exactly **00:00:10** in a **42 °C** water bath.

5 5m

Remove the mixture from the water bath and place it **On ice** for **00:05:00** , then add **950 µl** - **980 µl** of SOC medium to the tube.

SOC medium is ideal, however LB medium can also be used but will result in a roughly two fold loss of transformation efficiency.

6 1h

Incubate the bacteria at **37 °C** for **01:00:00** , while shaking at ~ **250 rpm** . After incubation, repeatedly invert the tube to mix the culture well and then plate **100 µl** onto selection plates. Incubate the plate(s) upside-down overnight at **37 °C** .

Note: 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.
Note: 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).

1h

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On the following day, choose a single colony to inoculate in ~ **180 mL** of LB medium at **37 °C** **Overnight** (pre-culture).

8 4h

The next day, add a 1:20 dilution of the pre-culture to LB medium and grow at **37 °C** until OD_{600} = 0.5-0.6. Once an OD_{600} = 0.5-0.6 is reached, induce the expression of αS with **1 Milimolar (mM)** IPTG and let the culture grow at **37 °C** for ~ **04:00:00** .

The amount of IPTG to be used for the induction (typically **0.1 Milimolar (mM)** - **1 Milimolar (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 °C** - **30 °C**) because overexpressed αS does not tend to form inclusion bodies in E. coli due to its high solubility in the bacterial[17].

9 40m

Then, spin the culture at **9000 x g** for **00:20:00** (**Room temperature**). After the spin, remove the supernatant and freeze the pellet **Overnight** at **-20 °C** . Freezing the pellet in this fashion already lyses most of the bacteria.

Purification 1h

10

This protocol is written for an ÄKTAprime plus FPLC system equipped with a **280 undetermined** 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.

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 Milimolar (mM)** TRIS, **25 Milimolar (mM)** NaCl, **1 Milimolar (mM)** EDTA, pH **8**) and vortex until the pellet is completely resuspended, taking extra care not to leave any pellet in solution.

If cells are spun in a **180 mL** centrifuge tube, approximately **60 mL** of IEX buffer A should be enough to fully submerge the pellet.

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11 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.

12 15m
3. After the first visible signs of boiling, allow the mixture to boil for an additional **00:15:00** to denature and precipitate proteases and other protein contaminants.

13 45m

4. After boiling, let the lysate cool down to **4 °C** , transfer it to **250 mL** centrifuge bottles and spin it at **20000 x g** for **00:45:00** (**4 °C**). 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.

It is highly recommended to incubate the lysate mixture at **4 °C** after cooling on the benchtop to avoid damaging centrifuge tubes upon high-speed centrifugation.

14

Ion Exchange Chromatography (IEX):

1. Wash the FPLC system with IEX Buffer A at **5 ml/min** .

15 1h
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. After the columns are attached, equilibrate them with IEX Buffer A at **1 ml/min** for **00:40:00** or until the conductivity reaches a steady measurement for ~ **00:20:00** .

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.

16 20m

3. Following equilibration, load the cell lysate mixture onto the columns **Overnight** at a max flow rate of **1 ml/min** .

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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.

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 mL** - **200 mL** .

18 5. After the UV absorbance plateaus, elute the protein with a gradient, from 0% to 100%, of IEX Buffer B (**20 Milimolar (mM)** TRIS, **1 Molarity (M)** NaCl, **1 Milimolar (mM)** EDTA, pH **8**) over **75 mL** and collect fractions every **5 mL** .

19 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.

For a lab-scale expression (typically 1- or 2-L expression), 2x 5 mL HiTrap columns should be enough to allow for complete aS 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 aS to the columns. If aS is detected in the flow-through fraction, increase the number of columns in the system to increase the overall binding capacity.

20 7. Pool the fractions (usually around a conductivity of ~25 mSi when adopting this protocol and purification system) containing the greatest amount of αS and (eventually) concentrate down to **10 mL** , which will then be loaded on a size-exclusion column.

21

Size-Exclusion Chromatography (SEC):

1. Wash the FPLC system with **50 Milimolar (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.

22 2. Attach a **10 mL** loop to the six-way valve of the FPLC system and flush it with **20 mL** of **50 Milimolar (mM)** ammonium acetate to remove any aggregated protein or built-up waste.

23 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.

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

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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).

24 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 Milimolar (mM)** ammonium acetate are run through the loop, switch the valve position back to “Load”.

25 5. Flush a total of **350 mL** of Ammonium Acetate buffer through the FPLC, collecting 14x **50 mL** fractions between **110 mL** and **180 mL** corresponding to a molecular weight of 60 kDa.

26 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 (ε(aS)=0.412 mL·mg-1·cm-1). Usually purity of >95% can be obtained.

27 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 **-80 °C**) the samples.

28 8. After lyophilization, seal the tubes with Parafilm, to prevent moisture from entering, and store at **4 °C** for short-term storage (1-2 weeks) or **-20 °C** for long-term storage.

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