Production of α-synuclein preformed fibrils (PFF)

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

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ABSTRACT

This protocol outlines the procedure to **produce preformed fibrils (PFF).**
It has been adapted from Volpicelli-Daley et al., 2014

MATERIALS

- 1X PBS **Quality Biological Catalog #114-058-101**
- ClearColi BL21(DE3) Electrocompetent cells **Lucigen Catalog #60810**
- Protease Inhibitor Cocktail **Sigma Aldrich Catalog #P8340**
- Superdex 200 increase 10/300G **Ge Healthcare Catalog #45-002-570**
- Amicon Ultra centrifugal filter **Emd Millipore Catalog #n/a**
- Hitrap Q Sepharose Fast Flow anion-exchange columns **Ge Healthcare Catalog #450-002-58**

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Keywords: ASAPCRN, alpha-synuclein, preformed fibrils, PFF, SNCA

- Ni Sepharose 6 Fast Flow **Ge Healthcare Catalog #17-5318-06**
- ToxinSensor Chromogenic LAL Endotoxin Assay Kit **Genscript Catalog # L00350**
- PD-10 columns **Ge Healthcare Catalog #17085101**
- Pierce BCA protein assay **Thermo Scientific Catalog #23227**
- 400 mesh carbon coated copper grids **SPI supplies Catalog #3540C-CF**
- Mouse anti-pSer129-α-synuclein **BioLegend Catalog #825701**
- Mouse anti-MAP2 **Sigma Aldrich Catalog #M9942**
- Donkey polyclonal anti-mouse Alexa fluor 488 **Jackson Immunoresearch Catalog # Cat#715-545-151**
- Donkey polyclonal anti-mouse CY3 **Jackson Immunoresearch Catalog #715-165-151**
- Primary cultured neuron (mouse cortical neuron) on DIV 7. **Catalog #n/a**

High-salt buffer :750 mM Nacl, 10 mM Tris (pH 7.6) and 1 mM EDTA with protease inhibitors including 1 mM PMSF.
Coomassie stain: 0.2% (wt/vol) Coomassie Brilliant Blue R250 and 50% (vol/vol) methanol; dissolve the dye, add 10% (vol/vol) acetic acid, and then bring it to the final volume with water. This solution can be stored indefinitely at room temperature.

SDS-PAGE (12%): 4.9mL H20 (autoclaved), 2.5mL Tris HCl pH 8.8, 120uL SDS 20%, 2.5mL Bisacrylamide, 60uL APS, 5uL TEMED

Equipment
Branson Digital sonifier, Danbury, CT, USA
Eppendorf Thermomixer
Phillips CM 120 TEM (80 kV) with an AMT ER-80 charge-coupled device (8 megapixel).
Philips EM 410 TEM with a Soft Imaging System Megaview III digital camera.

SAFETY WARNINGS

CAUTION: Because of highly neurotoxic and transmission characters of α-synuclein (α-syn) preformed fibrils (PFF), it's strongly recommended the use of gloves, face mask, and protective goggles for all procedures involving the use of synuclein fibrils. Clean any spills with a solution of 10% SDS in water, followed by multiple successive washes in 70 % ethanol and distilled water.

Step 3. Preparation of fibrils for neuronal treatment or injection. The steps here should be done
in a fume hood or biosafety cabinet.

Generation of α-synuclein monomer

13h 5m

1 Transform α-synuclein plasmids (full length human α-synuclein cloned into pRK172 vector) into ClearColiTM BL21-competent E. coli, that have been genetically modified so that LPS does not trigger LPS-mediated immune response. From the small scale culture in LB medium, make a bacteria cell stock and keep at -80 °C.

2 Prepare starter culture by adding a cell stock to LB medium.

3 Add starter culture to a large culture medium with ampicillin, followed by incubation Overnight at 37 °C with shaking.

4 Resuspend the pellet in high-salt buffer (750 mM Nacl, 10 mM Tris (pH 7.6) and 1 mM EDTA with protease inhibitors including 1 mM PMSF.

5 Break the bacterial cells using a high-pressure homogenizer, micro-fluidizer.

6 Boil for 00:15:00 to precipitate other proteins and then immediately incubate on On ice to cool. 15m

7 Spin at 6,000 g for 00:20:00 at 4 °C C. 20m

8 Use the supernatant for further dialysis with 10 mM Tris (pH 7.6), 50 mM NaCl and 1 mM EDTA.

9 Concentrate the protein through Amicon Ultra centrifuge filter (3.5 kDa cutoff).

10 Filter the protein using a 0.22 µm syringe filter and load it onto a Superdex 200 column.

11 Collect samples and check each fraction by SD-PAGE, followed by Coomassie staining.

12 Collect the pure fractions with an appropriate α-synuclein bands (~15 kDa) and dialyze with 10 mM Tris (pH 7.6), 25 mM NaCl, and 1 mM EDTA.

13 Apply protein to a Hi-Trap Q HP anion-exchange column (gradient ranging from 25mM NaCl to 1 M NaCl) and collect fractions, followed by SDS-PAGE and Coomasie staining.

14 **Generate endotoxin-free α-synuclein:** remove the bacterial endotoxins using Toxineraser endotoxin

removal kit (GeneScript), and measure the level of endotoxin using ToxinSensor Chromogenic LAL Endotoxin Assay Kit (GenScript).

15 Dialyze with 10 mM Tris (pH 7.6) and 50 mM NaCl.

16 Concentrate the fractions, aliquot, and store at -80 °C °C.

Generation of fibrils

1w 0d 0h 10m

17 Thaw aliquot of recombinant α-synuclein monomer on On ice.

18 Centrifuge at 4 °C C for 00:10:00 in centrifuge at 12,000xg. 10m

19 Transfer the supernatant with a pipette and measure the final protein concentration using BCA protein assay.

20 Dilute the monomeric protein into PBS for a final concentration of 5 mg/mL.

21 Vortex tubes for 00:00:03 to mix contents and seal the microcentrifuge lid with a parafilm to prevent opening of lid. 3s

22 Shake for **7 days** at 37 °C with 1,000 RPM (Eppendorf Thermomixer). Solution should turn turbid during this period.

23 Make 20 µL of aliquots and freeze on dry ice. Store at -80 °C C.

24 **Validation of fibril formation before move to the next step (e.g. Thioflavin T, sedimentation assay)**

24.1 **Thioflavin T assay** 10m

1. Prepare 1 mM Thioflavin T stock in PBS.

Add 5 µL of α-synuclein PFF into 95 µL of 25 µM Thioflavin T. (Use 5 µL of PBS alone and 5 µL of monomeric α-synuclein as a control.)

3. Incubate at room temperature for 00:10:00 .

4. Measure the fluorescence at an excitation 450 nm and emission at 490 nm.

24.2 **Sedimentation assay** 1h

1. Centrifuge 20 µL of PFFs at 100,000 g for 00:30:00 at room temperature.

2. Transfer the supernatant to a new tube (→ 'soluble' fraction).

3. Resuspend the pellet in 20 µL of PBS, and centrifuge it again at 100,000 g for 00:30:00 at room temperature.

4. Discard the supernatant and resuspend the pellet in 20 µL of PBS (→ 'pellet' fraction).

5. Perform SDS-PAGE, followed by Coomassie staining.

25 **NOTE:**

- Freeze/thawing can compromise the activity of PFF. Please prevent thawing of unused aliquots.
- Sterile components are used to assemble reactions to prevent microbial contamination.

Preparation of fibrils for neuronal treatment or injection 4m

26 **NOTE:** All the steps here should be done in a fume hood or biosafety cabinet.

27 Thaw sufficient aliquots of 5 mg/mL PFF at Room temperature immediately before use.

28 Dilute PFF to 100 µg/mL (for primary neuronal culture experiment) or 2 mg/ml (for intrastriatal injection) by adding PFF to a sterile microcentrifuge tube containing the appropriate volume of sterile PBS.

29 Seal the microcentrifuge with a parafilm and make a small hole for sonication.

30 Sonicate (Branson Digital Sonifier SFX 150 from Emerson) at amplitude 20% for a total of 60 pulses (0.5 seconds on/off cycle). Pause briefly between every 10-12 pulses to prevent solution from heating up excessively and to avoid frothing.

31 Allow sonicated PFF solution to settle for 00:01:00. PFF suspension is now ready for use. 1m

32 **Quality control testing**

32.1 **Transmission electron microscopy (TEM)** 2m 30s

1. Adsorb α-synuclein PFF (prepare the samples before and after sonication) to glow discharged 400 mesh carbon coated copper grids for 00:02:00.
2. Quickly transfer the grids through three drips of Tris-HCl (50 mM pH 7.4), rinse, and then float upon two consecutive drops of 0.75% uranyl formate for 00:00:30 each.
3. Aspirate the stained solution and allow the grid to dry before imaging.
4. Plate on a Phillips CM 120 TEM operating at 80 kV and capture the images with an ER-80 CCD.

32.2 **Immunofluorescence with phosphorylated α-synuclein (Ser129) antibody** 30s

1. Add 1 µg/mL of alpha-synuclein PFF into primary cultured neurons on DIV7.
2. Incubate the neurons for a further 10-14 days with replacing a half of the fresh medium every 3

days.

3. Fix the neurons and perform double-staining immunofluorescence using p-α-syn (Biolegend) and MAP2 (Sigma) antibodies at 4 °C Overnight

4. Visualize p-α-syn aggregates formed from endogenous alpha-synuclein with a confocal microscope.