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Production of α -synuclein preformed fibrils (PFF)

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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 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #P8340**

⊗ Superdex 200 increase 10/300G **GE Healthcare Catalog #45-002-570**

⊗ Amicon Ultra centrifugal filter **Merck Millipore (EMD Millipore) Catalog #n/a**

⊗ Hitrap Q Sepharose Fast Flow anion-exchange columns **GE Healthcare Catalog #450-002-58**

⊗ 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 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #M9942**

⊗ Donkey polyclonal anti-mouse Alexa fluor 488 **Jackson ImmunoResearch Laboratories, Inc. Catalog # Cat#715-545-151**

⊗ Donkey polyclonal anti-mouse CY3 **Jackson ImmunoResearch Laboratories, Inc. 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 H₂O (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 ClearColi™ 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 μ m 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 Coomassie 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 μ L 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 ($\rightarrow$ '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 ($\rightarrow$ '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 $\mu\text{g/mL}$ (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 μ g/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.

Protocol references

Volpicelli-Daley, L.A., Luk, K.C., and Lee, V.M. (2014). Addition of exogenous alpha-synuclein preformed fibrils to primary neuronal cultures to seed recruitment of endogenous alpha-synuclein to Lewy body and Lewy neurite-like aggregates. Nat Protoc 9, 2135-2146. 10.1038/nprot.2014.143.