

FEB 21, 2024

OPEN ACCESS



Protocol Citation: Tae-In Kam, Rong Chen, Valina L. Dawson, Ted Dawson 2024. Production of α -synuclein preformed fibrils (PFF). protocols.io https://protocols.io/view/productio n-of-synuclein-preformed-fibrilspff-c8vmzw46

License: This is an open access protocol distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

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

Created: Feb 07, 2024

Last Modified: Feb 21, 2024

PROTOCOL integer ID: 94861

Production of α-synuclein preformed fibrils (PFF)

 \checkmark Forked from Production of α-synuclein preformed fibrils (PFF)

Tae-In Kam^{1,2}, Rong Chen^{1,2}, Valina L. Dawson^{1,2,3,4}, Ted Dawson^{1,2,3,5}

¹Neuroregeneration and Stem Cell Programs, Institute for Cell Engineering, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA;

²Department of Neurology, Johns Hopkins University School of Medicine, Baltimore, MD 21205. USA:

³Department of Physiology, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA;

⁴Solomon H. Snyder Department of Neuroscience, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA;

 5 Department of Pharmacology and Molecular Sciences, and SJ Yan and HJ Mao Laboratory of Chemical Biology, Johns Hopkins University School of Medicine, Baltimore, MD 21205. USA



Eileen Ruth Torres Weill Cornell Medicine

ABSTRACT

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

Keywords: ASAPCRN, alphasynuclein, preformed fibrils, PFF, SNCA

MATERIALS

X 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 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 Regional 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

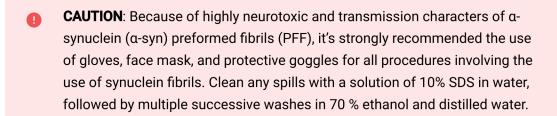
Equipment

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



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

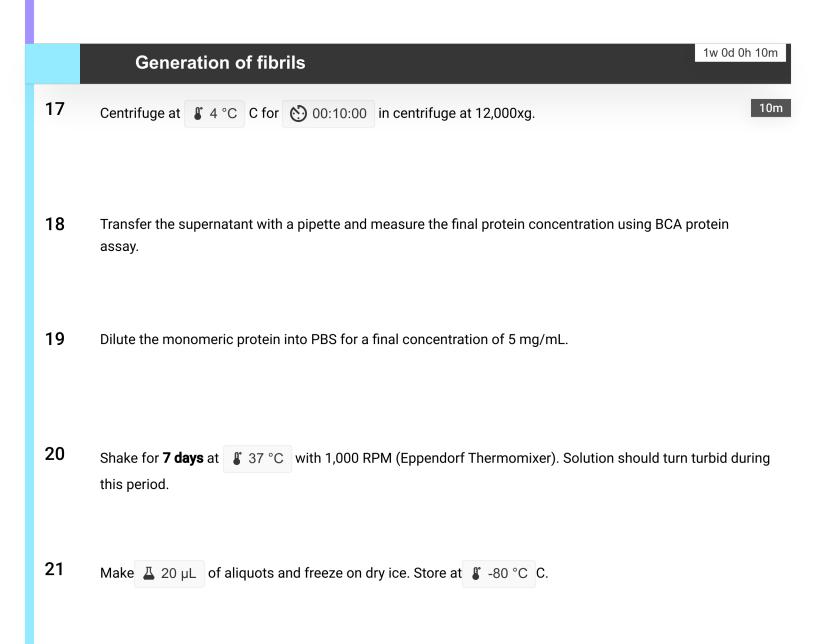
Generation of α-synuclein monomer

13h 5m

- 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.
- 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.

Boil for 00:15:00 to precipitate other proteins and then immediately incubate on 00:15:00 to coo. 15m 6 7 20m Spin at 6,000 g for 300:20:00 at \$4 °C C. 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 (100 kDa cutoff). 10 Filter the protein using a 0.22 µm syringe filter and load it onto a Superdex 200 column. 11 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 Store at 4 -80 °C until needed to generate fibrils

- 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.
- 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).
- Concentrate the fractions, aliquot, and store at \$\selline{\cup} -80 \circ \cap \circ \cir



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

22.1 Thioflavin T assay

10m

- 1. Prepare 1 mM Thioflavin T stock in PBS.
- 3. Incubate at room temperature for (5) 00:10:00 .
- 4. Measure the fluorescence at an excitation 450 nm and emission at 490 nm.

22.2 Sedimentation assay

1h

- 1. Centrifuge 4 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 $\; \underline{\mbox{\mbox{$\mbox{$\bot$}}}}\;$ 20 $\mu L \;$ 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 \perp 20 μ L of PBS (\rightarrow 'pellet' fraction).
- 5. Perform SDS-PAGE, followed by Coomassie staining.

23 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

- NOTE: All the steps here should be done in a fume hood or biosafety cabinet.
- Thaw sufficient aliquots of 5 mg/mL PFF at Room temperature immediately before use.

27

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.

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

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

1m

30 Quality control testing

30.1 Transmission electron microscopy (TEM)

2m 30s

- 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-HCI (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.

30.2 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.