Aug 17, 2021

Preparation of fibrils and Quality control

In 1 collection

The Michael J Fox Foundation Pff Standardization Consortium[1]

[1]MJFF 2017 Committee

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ABSTRACT

This is a consensus protocol developed through discussions with Laura Volpicelli-Daley, Caryl Sortwell, Kelvin Luk, Lindsey Gottler, and Virginia Lee. This protocol is intended for research purposes only, using specially-formulated monomeric alpha-synuclein protein available for purchase at Proteos, Inc as the result of efforts by The Michael J. Fox Foundation (MJFF). Each batch of the “Alpha-Synuclein Monomer Protein for Making Pre- Formed Fibrils” has undergone internal purification and quality control at Proteos in addition to external validation to confirm successful generation of pathogenic aSyn PFFs. See Reference section for methods and results from application of alpha-synuclein pre-formed fibrils (aSyn PFFs) in primary neuron cultures in vitro or in mice in vivo. This protocol is referenced in the Polinski et al 2018 paper entitled "Best Practices for Generating and Using Alpha-Synuclein Pre-Formed Fibrils to Model Parkinson's Disease in Rodents" (doi: 10.3233/JPD-171248).

ATTACHMENTS

dz3jbh9f7.pdf

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PROTOCOL CITATION

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COLLECTIONS

Protocol for Generation of Pre-Formed Fibrils from Alpha-Synuclein Monomer

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Protocol for Generation of Pre-Formed Fibrils from Alpha-Synuclein Monomer

MATERIALS TEXT

Reagents:

DPBS no calcium, no magnesium **Invitrogen - Thermo**

- * **Fisher Catalog #14190136**
- **25 mg/ml** - **30 mg/ml** purified recombinant aSyn protein.* This protocol is also applicable to aSyn containing an epitope or affinity tag (e.g. Myc or polyHis). However, additional studies may be required to ensure that the buffer is compatible with assembly of a particular modified protein.
- BCA kit (Thermoscientific, 23227) or Nanodrop device
- Thioflavin T (Sigma, T3516): 1 mM stock solution in sterile water

* = if using the "Alpha-Synuclein Monomer Protein for Making Pre-Formed Fibrils" purchased from Proteos, the starting protein concentration will be ~6-7 mg/mL. In this case, you will need to replace the 1x Dulbecco's PBS with 10x Dulbecco's PBS.

Equipment:

- Benchtop centrifuge
- Eppendorf Thermomixer R
- 37ºC incubator
- **Microcentrifuge lid locks (Fisher, 1415-1508)**
- Gel-Loading Tips, 1-200µL, Volume: 200µL; Length: 2.75 in.; O.D.: 0.6mm; Packaging: BP **Thermo Fisher Catalog #02707181**

Snap-Cap™ Microcentrifuge Tubes: Standard **Thermo**

- **Fisher Catalog #02681230**
- Tabletop ultracentrifuge (e.g. Beckman-Coulter, 361544)

Nunc™ 384-Well Polystyrene Microplates, black **Thermo**

- **Fisher Catalog #262260**
- Plate reader with excitation filter 450 emission filter 510
- **-80 °C** freezer

NOTE ON ENDOTOXINS: For the Proteos "Alpha-Synuclein Monomer Protein for Making PreFormed Fibrils", endotoxin content is reported on the datasheet. If aSyn monomers are being used as a control, endotoxin units (EUs) should be ≤ 0.5 EU/mL or < 0.05 EU/mg at 10mg/mL protein. The Pierce High Capacity Endotoxin Removal Kit is a reliable method for removing endotoxins. You will lose some sample in the process and should re-measure protein levels.

Step 1. Preparation of fibrils. (Timing ~30 min; 7 days for fibril formation)

1 Thaw aliquot of "Alpha-Synuclein Monomer Protein for Making PreFormed Fibrils" or other recombinant aSyn monomer **On ice** .

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2 10m

Centrifuge at 4 °C for 00:10:00 in benchtop centrifuge at highest speed (12000 x g - 15000 x g).

3 Retain only the supernatant with a pipette, avoiding any aSyn that may have pelleted. Determine the protein concentration of the sample.
METHOD 1 (most recommended): Measure protein by A280 on a nanodrop device. Use Beer's law to measure concentration (ε for synuclein = voo5960 M^{-1} cm^{-1} for human synuclein and 7450 M^{-1} cm^{-1} for mouse synuclein).
METHOD 2 (less recommended): Perform BCA protein assay on this material to determine final protein concentration. We recommend performing the assay at 3 dilutions of protein (in triplicate for each dilution) to obtain accurate measurements.

4 Assemble the pre-formed fibrils (PFFs) in 1.5 mL microcentrifuge tubes by diluting the monomeric protein into PBS for a final concentration of [M]5 mg/ml .

EXAMPLE: If the protein concentration is [M]25 mg/ml , add 100 µl protein to 400 µl PBS in a 1.5 mL tube. If the protein concentration is [M]6 mg/ml (in [M]10 Milimolar (mM) Tris, [M]50 Milimolar (mM) NaCl, pH7.6), add 44 µl of a [M]40 Milimolar (mM) phosphate, [M]230 Milimolar (mM) NaCl solution to 156 µl of the monomeric aSyn sample. This will result in a final buffer formulation of ~ [M]100 Milimolar (mM) NaCl, ~ [M]7.5 Milimolar (mM) Tris, and ~ [M]10 Milimolar (mM) phosphate with a pH of 7.2-7.6.
NOTE: Because activity can decline over time when the PFFs undergo freeze-thaw cycles, we recommend that reactions not exceed 500 µl per tube.

5 3s

Vortex tubes at high speed for 00:00:03 to mix contents.

6 Place microcentrifuge lid lock on lid of tube to prevent opening of lid. Label and date tube.

7 Place in orbital shaker (e.g. Eppendorf Thermomixer R) at 37 °C .

NOTE: It is recommended to place the entire shaker in a 37ºC incubator since most shakers do not heat the top of the tube, resulting in condensation.

8 1w

Shake for 168:00:00 at 1000 rpm . Solution should turn turbid during this period.

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9 

Pipet **25 µl** aliquots in small tubes using gel loading pipet tips.

10 Freeze aliquots on dry ice and store at **-80 °C** or store aliquots at **Room temperature** .

Step 2. Quality control to verify fibril formation 1h 37m

11 Thaw an aliquot at **Room temperature** and perform Thioflavin T assay toconfirm presence of amyloid fibrils:

11.1 Dilute **1 Milimolar (mM)** Thioflavin T stock in PBS to **25 Micromolar (µM)** final concentration (1:40 dilution).

11.2 

Pipet **95 µl** of the **25 Micromolar (µM)** Thioflavin T per well of 384 well plate.

11.3 

Pipet PFFs up and down to mix, add **2.5 µl** to wells with Thioflavin T.

11.4 For controls, include **2.5 µl** PBS alone and **2.5 µl** monomeric aSyn.

11.5  1h 2m

Incubate at **Room temperature** for **00:02:00** to **01:00:00** .

11.6 Read plate (excitation **450 nm** , emission **50 nm**).

> The presence of amyloid-like fibrils typically result in readings that are 20-100 fold higher than samples containing monomeric protein only for the human aSyn.

12 12. The presence of fibrils can also be assessed by sedimentation.

12.1 Dilute **2 µl** of **5 mg/ml** PFFs in **20 µl** PBS.

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12.2 30m

Spin in ultracentrifuge (e.g. TLA-100) at **100000 x g** for **00:30:00** at **25 °C** .

12.3 Remove supernatant, dilute in 5X Laemlli buffer.

12.4

Add **20 µl** PBS to pellet, pipet up and down several times until resuspended, dilute in 5X Laemlli buffer.

12.5 5m

Boil samples at **95 °C** for **00:05:00** .

12.6 Run equal volumes of supernatant and pellet fractions on 15% polyacrylamide gel.

12.7 Stain with coomassie brilliant blue to visualize bands.

Properly generated PFFs should result in equal amounts of protein in the supernatant and pellet fractions or greater amounts of protein in the pellet versus the supernatant fraction. If more protein is in the supernatant versus the pellet, PFF formation has been suboptimal.

13 Keep records of results of Thioflavin T and sedimentation assay to make batch to batch comparisons.

NOTE: Thioflavin T and sedimentation assays are basic biochemical analyses to verify general fibril formation. More quality control is recommended when testing a new protocol for generating PFFs or generating PFFs for the first time in one's lab. More quality control is also recommended before commencing a long-term in vivo study with aSyn PFFs. For examples for additional recommended quality control experiments, see the table in Page 1.

Purpose	Circumstances for Performing	Examples of Experiments	Anticipated Results
Verify Fibril Size	When using a protocol for generating PFFs for the first time. Prior to long-term *in vivo* studies.	Electron Microscopy	Majority of fragments are ≤ 50nm
		Dynamic Light Scattering	Majority of fragments are ≤ 50nm
Verify Seeding Capacity	When using a protocol for generating PFFs for the first time. Prior to long-term *in vivo* studies.	*In vitro* Seeding in Mouse Primary Neuron Cultures	pS129 pathology develops in primary neurons following aSyn PFF, but not aSyn monomer, incubation
Verify Fibril Formation	With each new batch of PFFs or prior to using frozen aliquots	Thioflavin T Assay	Readings 20-100 fold higher with human aSyn PFFs vs monomer
		Sedimentation Assay	Equal amounts of protein in solute and pellet or more protein in pellet vs solute
		Visual Appearance	Solution should appear turbid

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