

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

OVERVIEW

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 “Human 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 1-3 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*.

OPTIMAL CONDITIONS FOR PFF GENERATION

Ionic strength and pH are very important to monitor when generating aSyn PFFs. These two parameters may affect PFF formation and should be analyzed prior to PFF induction. The pH should be ~7.0 and the salt concentration should be approximately 100mM NaCl. Proper sonication is extremely important. The parameters of this protocol are designed for use with the Proteos protein and sonication with the instruments listed. For optimal seeding, fibrils should be 50nm or smaller in length after sonication. For information on other sonication parameters, see Reference 8.

STORAGE OF PFFS

PFFs should be aliquoted into single-use tubes. Aliquots of aSyn PFFs may be stored at room temp or -80°C. PFFs should NOT be stored at 4°C. If aliquots are kept at room temp, ensure that sterile components were used to assemble reactions to prevent microbial contamination. Sodium azide can also be added as a preservative if compatible with downstream application but may result in cell toxicity. If aliquots are stored at -80°C, precautions must be made to avoid unnecessary freeze-thaw—keep samples in a box towards the back of the freezer and minimize the amount of time the box is open at room temp. Freeze-thaw cycles will degrade PFFs by dissociating the fibrils and/or causing non-specific aggregates. Properly-stored PFFs have been shown to lose pathogenicity after 1-1.5 years at -80°C. For *in vivo* studies, it is highly recommended to use freshly-made PFFs.

RECOMMENDED QUALITY CONTROL MEASURES FOR PFFS

Various biochemical and biophysical quality control experiments are recommended to ensure proper aSyn PFF formation. The necessity for each experiment is dictated by experience with the PFF generation protocol and the designated use of the PFFs.

Purpose	Circumstances for Performing	Examples of Experiments	Anticipated Results
Verify Fibril Size	When using a protocol for generating PFFs for the first time.	Electron Microscopy	Majority of fragments are ≤ 50 nm
	Prior to long-term <i>in vivo</i> studies.	Dynamic Light Scattering	Majority of fragments are ≤ 50 nm
Verify Seeding Capacity	When using a protocol for generating PFFs for the first time. Prior to long-term <i>in vivo</i> studies.	<i>In vitro</i> 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

Pre-injection handling of α -Synuclein Pre-formed fibrils (pffs)

Procedure Overview:

- Step 1: Generation of fibrils
- Step 2: Quality Control
- Step 3: Preparation of fibrils for intracerebral injection

NOTE: We *strongly recommend* appropriate laboratory attire and the use of gloves, face mask (VWR, 414004-670), and protective goggles for all procedures involving the use of synuclein fibrils. Use bench paper that can be thrown in biohazard trash when protocols are completed. Clean any spills with a solution of 10% SDS in water, followed by multiple successive washes in water.

Step 1. Preparation of fibrils. (Timing ~30 min; 7 days for fibril formation)⁴⁻⁶

Reagents:

- 1X Dulbecco's PBS without Calcium and Magnesium (Invitrogen 14190136)*
- 25-30mg/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 "Human 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
- 37°C incubator
- Microcentrifuge lid locks (Fisher, 1415-1508)
- Gel loading tips (Fisher, 02707181)
- 0.25 mL tubes for aliquots (Fisher, 02681230)
- Tabletop ultracentrifuge (e.g. Beckman-Coulter, 361544)
- 384 well plate for thioflavin T assay (Fisher 262260)
- Plate reader with excitation filter 450 emission filter 510
- -80 °C freezer

NOTE ON ENDOTOXINS: For the Proteos "Human Alpha-Synuclein Monomer Protein for Making Pre-Formed 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. Please note you will lose some sample in the process and should re-measure protein levels after cleanup. Endotoxins bind poorly to aSyn PFFs and the high speed centrifugation step will remove a large portion of the endotoxins present.

Protocol:

1. Thaw aliquot of “Human Alpha-Synuclein Monomer Protein for Making Pre-Formed Fibrils” or other recombinant aSyn monomer on ice.
2. Centrifuge at 4°C for 10 minutes in benchtop centrifuge at highest speed (12,000-15,000 xg).
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 = 1005960 $M^{-1} cm^{-1}$ 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 5 mg/mL.

EXAMPLE: If the protein concentration is 25 mg/mL, add 100 μL protein to 400 μL PBS in a 1.5 mL tube. If the protein concentration is 6mg/mL (in 10 mM Tris, 50 mM NaCl, pH 7.6), add 44 μL of a 40 mM phosphate, 230 mM NaCl solution to 156 μL of the monomeric aSyn sample. This will result in a final buffer formulation of ~100 mM NaCl, ~7.5 mM Tris, and ~10 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. Vortex tubes at high speed for 3 seconds 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 37°C incubator since most shakers do not heat the top of the tube, resulting in condensation.

8. Shake for 7 days at 1,000 RPM. *Solution should turn turbid during this period.*
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 temp.

Step 2. Quality control to verify fibril formation

11. Thaw an aliquot at room temperature and perform Thioflavin T assay to confirm presence of amyloid fibrils:

- Dilute 1mM Thioflavin T stock in PBS to 25 μ M final concentration (1:40 dilution)
- Pipet 95 μ L of the 25 μ M Thioflavin T per well of 384 well plate.
- Pipet PFFs up and down to mix, add 2.5 μ L to wells with Thioflavin T
- For controls, include 2.5 μ LPBS alone and 2.5 μ L monomeric aSyn.
- Incubate at room temperature for 2 minutes to 1 hour.
- 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. The presence of fibrils can also be assessed by sedimentation.

- Dilute 2 μ L of 5mg/mL PFFs in 20 μ LPBS
- Spin in ultracentrifuge (e.g. TLA-100) at 100,000 xg for 30 min at 25 $^{\circ}$ C.
- Remove supernatant, dilute in 5X Laemlli buffer
- Add 20 μ LPBS to pellet, pipet up and down several times until resuspended, dilute in 5X Laemlli buffer
- Boil samples at 95 $^{\circ}$ C for 5 min.
- Run equal volumes of supernatant and pellet fractions on 15% polyacrylamide gel
- 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.

Step 3. Preparation of fibrils for intracerebral injection.
Perform ~1-2 hours prior to surgery. This takes about 30 min.^{3,5,7,8,9}

NOTE: Prior to *in vivo* or *in vitro* use, it is highly recommended that sonication parameters be established to result in fibrils of $\leq 50\text{nm}$ in length (as longer fibrils result in limited or lack of toxicity). Fibril length can be verified using electron microscopy or dynamic light scattering. Once appropriate sonication parameters are identified to result in fibrils of $\leq 50\text{nm}$ in length, these parameters should be strictly followed. A change in PFF sample volume or concentration may require modified sonication parameters.

Reagents:

- We have successfully elicited synuclein pathology following injection with fibrils in C57Bl/6, CD1, C57Bl6/Sv129, and C57B/C3H mice.
- Sterile dPBS
- **5 mg/mL aSyn PFFs** (as prepared in Step 1). Thaw at room temperature immediately before use.

Equipment:

- Fume hood (BSL2)
- Sonicator with 1/8" tip (Qsonica XL-2000)
- Stereotaxic surgery setup

Protocol:

1. Perform all sonication steps in a fume hood or biosafety cabinet. *Ensure that hood is externally ducted and does not re-circulate exhaust into the laboratory space.*
2. Thaw sufficient aliquots of 5 mg/mL PFFs at room temperature immediately before use. It is recommended to measure protein concentration again (See Step 1, Protocol Step 3) as freeze-thaw may change protein concentration.
3. Dilute PFFs to required concentration by adding PFFs to a sterile microcentrifuge tube containing the appropriate volume of sterile dPBS. *Note that pffs are assembled in dPBS. For mouse injections, we typically use 2-2.5 mg/mL PFFs.*
4. Using a probe sonicator, sonicate at power level 2 for a total of 60 pulses (~0.5 seconds each). Pause briefly between every 10-12 pulses to prevent solution from heating up excessively and to avoid frothing.

NOTE: We have also tested this protocol using select high-energy bath sonicators such as the Covaris and Bioruptor systems. Results so far indicate that they are also suitable for preparation of PFFs prior to addition/injection. These systems can sonicate closed tubes and are preferable where aerosol generation is a concern. However, be cautious when using bath sonication in place of probe sonication and be sure to verify fibrils are $\leq 50\text{nm}$ in length for proper toxicity.

5. Close cap and tap side of tube so that any liquid on the side of the tube is now at the bottom. It should appear clear and colorless, although small fragments that scatter light may still be visible.
6. Allow sonicated PFF solution to settle for 1 min. *PFF suspension is now ready for stereotaxic injection.*
7. Gently flick tube to mix contents prior to use and pipette up and down between surgical injections. It is recommended to use an aliquot for up to 4 hours during a surgical session. If an 8 hour surgical session is planned, use one aliquot in the morning and a new aliquot in the afternoon.

NOTE: If aSyn monomers are being used as the control, be sure endotoxin units (EUs) are near or below 0.5 EU/mL. The Pierce High Capacity Endotoxin Removal Kit is a reliable method for removing endotoxins. Please note you may lose a good portion of your sample in the process and should re-measure protein levels after endotoxin cleanup.

Expected results

The amount of time required for detectable levels of aSyn pathology to develop following injection is dependent on the strain of animal used. For example, PFF-injection into homozygous aSyn Tg mice (M83 line) results in visible pathology in as little as 3-4 weeks near the injection site. In heterozygous mice, the timeframe is typically 2-3 months when using the same human aSyn PFFs, suggesting that pathology accumulation is dependent on expression levels. Pathologic aSyn should be visible as phospho-aSyn (i.e. at Ser129) positive intraneuronal and neuritic inclusions in neurons. In lines that express sufficient amounts of aSyn in astrocytes, glial pathology may also be seen. PBS-treated and centrifuged aSyn monomer-control brains should show minimal p-aSyn-staining. Additional antibodies such as ones recognizing aSyn, or misfolded conformations of aSyn, should also detect inclusions, as will those against ubiquitin.

References

1. Volpicelli-Daley, L.A. et al. Addition of exogenous α -synuclein pre-formed fibrils to primary neuronal cultures to seed recruitment of endogenous α -synuclein to Lewy body and Lewy neurite-like aggregates. *Nat. Protoc.* **9(9)**:2135-46 (2014).
2. Martinez, T.N. et al. [Characterization, comparison, and cross-validation of *in vivo* alpha-synuclein models of parkinsonism](#). *The Society for Neuroscience meeting* (2014).
3. Luk, K.C. et al. Intracerebral inoculation of pathological alpha-synuclein initiates a rapidly progressive neurodegenerative alpha-synucleinopathy in mice. *J Exp Med* **209**, 975-86 (2012).
4. Giasson, B.I., Murray, I.V., Trojanowski, J.Q. & Lee, V.M. A hydrophobic stretch of 12 amino acid residues in the middle of alpha-synuclein is essential for filament assembly. *J Biol Chem* **276**, 2380-6 (2001).
5. Luk, K.C. et al. Exogenous alpha-synuclein fibrils seed the formation of Lewy body-like intracellular inclusions in cultured cells. *Proc.Natl.Acad.Sci.U.S.A.* **106**, 20051-20056 (2009).
6. Murray, I.V.J. et al. Role of alpha-synuclein carboxy-terminus on fibril formation in vitro. *Biochemistry* **42**, 8530-8540 (2003).
7. Volpicelli-Daley, L.A. et al. Exogenous alpha-synuclein fibrils induce Lewy body pathology leading to synaptic dysfunction and neuron death. *Neuron* **72**, 57-71 (2011).
8. Tarutani, A., et al. The effect of fragmented pathogenic alpha-synuclein seeds on prion-like propagation. *J Biol Chem*, **291(36)**: 18675-18688.
9. Bousset, L., et al. An efficient procedure of removal and inactivation of alpha-synuclein assemblies from laboratory materials. *J Parkinsons Dis*, **6(1)**: 143-151.