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Preparation of mouse and human α-synuclein fibrils

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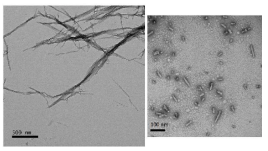

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Abstract

This protocol describes the preparation of homogeneous a-synuclein fibrils. The primary objective is to generate alpha-synuclein fibrils with high homogeneity in size, devoid of other protein species such as monomers and oligomers. The protocol employs shaking to generate the fibrils and sonication to reduce the fibrils to their terminal length. The expected outcome is the production of highly homogeneous fibrils, with a consistent size range of 25±5 nm in radius, and at high concentrations without the presence of monomers and oligomers. This protocol provides a method for producing stable, homogeneous sonicated fibrils that can be stored in batches for in vitro and in vivo experiments. By following this protocol, the fibrils produced will consistently maintain the same size and concentration, thereby ensuring the reliability and reproducibility of subsequent experimental results.

Protocol materials

250g Guanidine hydrochloride **G-Biosciences Catalog #**BC85 Step 13

Thermo Scientific™ Low Protein Binding Collection Tubes (1.5 mL) **Catalog #**PI90411 Step 3

Safety warnings

Hazard Identification and Risk of Exposure to the Hazards:

Inhalation or spread through food or drink that contain fibrils aerosols or fibrils.

Protective gloves, safety glasses and lab coat must always be used when handling anything that possibly could contain α-synuclein fibrils. Food or drink is strictly prohibited in any environment where α-syn fibrils are used.

Routes of Transmission: *Prior to assigning containment requirements, it is imperative to understand the routes of transmission.*

Some issues to address:

1. What are the exposure routes/risks of most concern:

Inhalation or spread through food or drink that contain fibril aerosols or fibirls accordingly. Fibrils possibly might reach the brain regions through the olfactory epithelium; Risk of accidental needlestick/droplet splash while handling fibrils for *in vitro* or *in vivo* work.

1. *What are the consequences of exposure (potential illness, etc)*

Fibrils may be considered as infectious material. Minimum to no hazard is expected from α-syn protein. *There is no evidence that transmission of fibrils can lead to development Parkinson's disease.* However, taking into account prion-like properties of α-syn fibrils should therefore be handled cautiously and wisely. Strictly recommended using disposable materials and Personal Protective Equipment (PPE) such as gloves, face mask, etc.

PRECAUTIONS:

Laboratory work where high concentration of fibrils (more than 300 uM) is needed must comply with biosafety level 2 (BSL2) containment as described in the current edition of the CDC/NIH's Biosafety in the Microbiological and Biomedical Laboratories: *http://www.cdc.gov/od/ohs/biosfty/bmbl5/bmbl5toc.htm*

Sharps safety precautions:

The use of sharps (glass pipettes, glass slides and cover slips, scalpels and lancets) should be eliminated, when possible. Appropriate precautions should be taken to avoid percutaneous injuries. These items should be disposed of immediately in a puncture-resistant sharps container. Bending, recapping or clipping of needles is prohibited. As described in CDC's sharps safety website: https://www.cdc.gov/sharpssafety/index.html

Procedural Methods and Materials:

- Laboratory work where high concentration of fibrils (more than 300 uM) is needed must comply with biosafety level 2 (BSL2) containment. This means all aerosol generating procedures must be performed

within the biosafety cabinet.

- *All the fibrils work involves using PPE, aerosol-tight centrifuges, water bath sonicator in a closed cabinet, homogenization of frozen brain samples using probe-tip sonicator under the hood (collection of protein fractions in BSL2 cabinets), chromatography equipment in a closed-door fridge, sealed plates, safe lock microfuge tubes (or tubes wrapped/sealed with parafilm), and use of filtered tips for pipettes. All personnel must strictly adhere to these procedures.*
- Use of proper PPE as stated in the section below. Use of available N95 respirators is voluntary (same for the use of available sleeve protectors). Follow safety precautions for sharps (for e.g., to avoid accidental needle sticks) while working with PFFs in the lab and for doing *in vivo* work.

Personal Protective Equipment (PPE): Appropriate PPE includes gloves, lab coat and safety glasses, face mask (voluntary N95 respirator use and sleeve protectors), face / bench top splash shield for specific procedures as stated above.

Methods to minimize personal exposure: Strictly adhere to sharps safety precautions using needles or any material that can potentially cause wounds. Use disposable supplies where possible. Use the minimal amounts of α-fibrils needed for an experiment. Keep fibrils in closed tubes. 10% of SDS solution in water must be used for decontaminating work areas. Do not use NaOH or Sodium Hypochlorite or ethanol. Do not leave samples containing fibrils unattended at the bench.

Methods to prevent the release of fibrils/protect workers from aerosols, splashes, splatters: protective gloves and clothing always be always be worn when handling frozen vials. High concentration of fibrils(>1mg/mL) always be handled under Biosafety cabinet and containment caps will be used while centrifugation. Centrifuge cups will be opened inside a biosafety cabinet. Face shield or benchtop splash shield will be used when working at the open bench.

Specimen transport and removal of material(s) from the laboratory: Transported in secondary container (plastic/Styrofoam) in a closed box. The closed box is carried in a bag.

Standard microbiological methods: hand washing after removal of gloves and before leaving the work area, no mouth pipetting, strictly no food or drink in refrigerators where material is stored, no eating in work area.

Cleaning & Disinfection: Work area must be cleaned with *10% SDS in water.* Wipes used must be immediately disposed into biohazard waste container. Any piece of equipment or supplies that possibly have been exposed to fibrils must be wiped with 10% of SDS.

Waste Generation and Disposal Methods: The solutions that contain α-syn fibrils must be decontaminated with 10% of SDS in water for 30 minutes and be thrown as a biohazard waste in a sealed container/bag (use a minimal volume of fibrils needed for an experiment, do not generate large volumes of fibril-containing liquids). Use small biohazard bags to collect tips and consumables of experiment performed, appropriately tie neck of bag in single knot and place in into secondary biohazard waste container.

Spill and Accident Response Procedure: *Describe all emergency procedures including spill clean-up. Describedisinfectants and environmental decontamination.* (ex., Outside of a BSC: If spill is a respiratory hazard, evacuate 30 minutes to allow aerosols to settle. Place absorbent towels over the spill, apply freshly prepared 10% SDS solution to entire area of spill starting on the outer edges and working inward, pick up sharp items with mechanical device (not hands), place all clean-up materials in a biohazard bag)

Generation of α-synuclein fibrils via shaking cycles

5d 0h 30m

1 Thaw down an aliquot of α-synuclein monomer stock (track down a batch number with identified EU number, concentration, A260/280 ratio) on ice On ice /quick water bath incubation at 42 °C with shaking

2 Spin down an aliquot of α-synuclein monomer stock solution (20000 x g , 00:10:00 4 °C) and measure the concentration using nanodrop

10m

Add 3 µL of 10x diluted aliquot in PBS onto nanodrop pedestal;
Parameters:
- other proteins; coefficient extinction: 5.98; MW: 14.4 kDA **(for wild-type human α-synuclein)**
- other proteins; coefficient extinction: 7.45; MW: 14.4 kDA **(for wild-type mouse α-synuclein)**

Perform two measurements and confirm <10% standard error between two measurements
If necessary, prepare 20X and 30X dilutions to confirm findings.

Equipment

NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer	NAME
UV-Vis Spectrophotometer	TYPE
Thermo Scientific	BRAND
ND-ONE-W	SKU

3 Calculate the reaction mix: [M] 5 mg/mL concentration of α-synuclein in 200 µL in PBS
Use protein low-binding tubes:

Thermo Scientific™ Low Protein Binding Collection Tubes (1.5 mL) **VWR International Catalog #**PI90411

4 Incubate the reaction at 1000 rpm, 37°C for 120:00:00 using program settings: 1 min ON; 1 min OFF

5d

Equipment

Eppendorf Thermomixer C Model 5382	NAME
Thermomixer C	TYPE
Eppendorf	BRAND
5382000023	SKU

Equipment

ThermoTop®	NAME
Smart block	TYPE
Eppendorf	BRAND
5308000003	SKU

5 Spin down the insoluble fraction at 15000 rpm, 10°C, 00:10:00 ; remove 100 µL of supernatant and add 1 mL of fresh PBS to the pellet 10m

Note

Example of reaction mix after 5 days of incubation

6 Gently resuspend and spin down the fibrils at 15000 rpm, 10°C, 00:10:00 ; remove around 900 µL of supernatant (be careful, avoiding disturbing insoluble fraction) and add 1 mL of fresh PBS to the pellet (repeat 3 times) 10m

Equipment

Centrifuge 5425/5425 R - Microcentrifuge	NAME
Centrifuge	TYPE
Eppendorf	BRAND
5406000240	SKU

STEP CASE

Conjugation of full-length fibrils 7 steps

Conjugation of full-length fibrils with pHrodo/Alexa dyes/BODIPY-FL **NHS ester**
7 steps
1. Dissolve 100 ug of dye in 50 ul of sterile DMSO (2 mg/mL)
2. Dilute fibrils to 1mg/mL concentration in PBS containing 0.1M bicarbonate (total volume: 0.95 mL)
3. Add 50 ug of dissolved dye to each reaction tube
4. Incubate overnight in eppendorf tube (foil-wrapped) with continuous shaking
5. In the morning, spin down the dye-fibril solution at 10,000 g for 10 min at 10C
6. Transfer the supernatant and dissolve the pellet with 1 mL of PBS (repeat 5 times)

7 Take out 900 ul of PBS leaving about 100 ul of fibril pellet in the tubes. Transfer the fibrils into 0.2 mL PCR tubes (thick wall). *** spin down the PCR tubes using bench-top centrifuge

Equipment

Mini-centrifuge

NAME

Centrifuge

TYPE

Fisher

BRAND

S67601B

SKU

https://www.fishersci.com/shop/products/fisherbrand-standard-mini-centrifuge-standard-mini-centrifuge/s67601b

LINK

Any standard mini centrifuge with adapters for different tube sizes will suffice

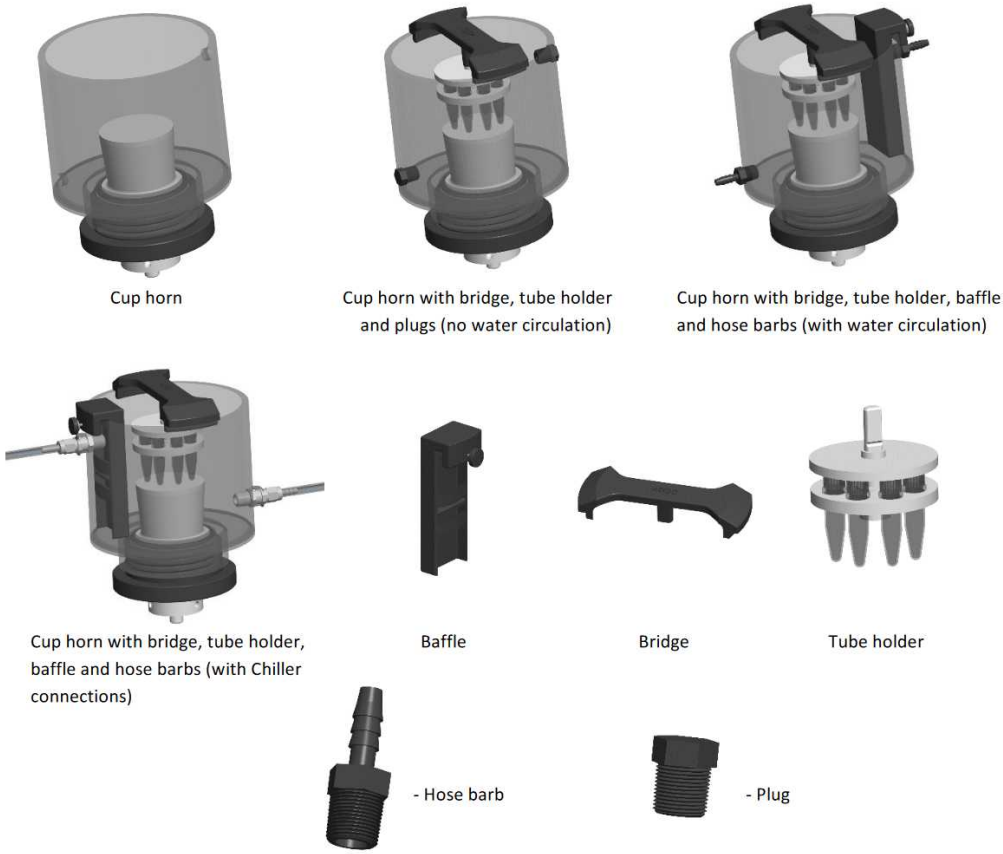
SPECIFICATIONS

8 Sonicate the generated fibrils using water bath sonicator at 10C, 30% amplitude and for 1 hour (no OFF ON cycles). Check the level of water in the water-bath is in a line with the tube content.

Sonication

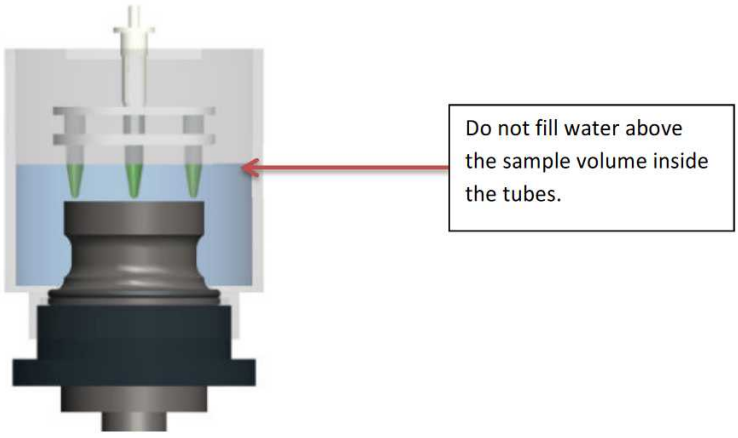
5d 0h 30m

9



General components of cup horn for water-bath sonicator (adopted from Qsonica manual)

10



Note: DI water is recommened.

Schematics indicating the optimal water level required for successful sonication

Size range and concentration measurements

11 Measure size of sonicated particles using DLS. Indication that α-synuclein fibrils are sonicated to the terminal size can be extrapolated from the distribution of size populations after 10 aqs. 5 sec apart DLS measurement.
1. There is one single population of size in a range of 10-100 nm
2. Average radius is 20-30 nm for human fibrils and 15-25 for mouse fibrils
3. %Mass is no less 95% for the size population from 10-100 nm
See examples below

Protocol

NAME

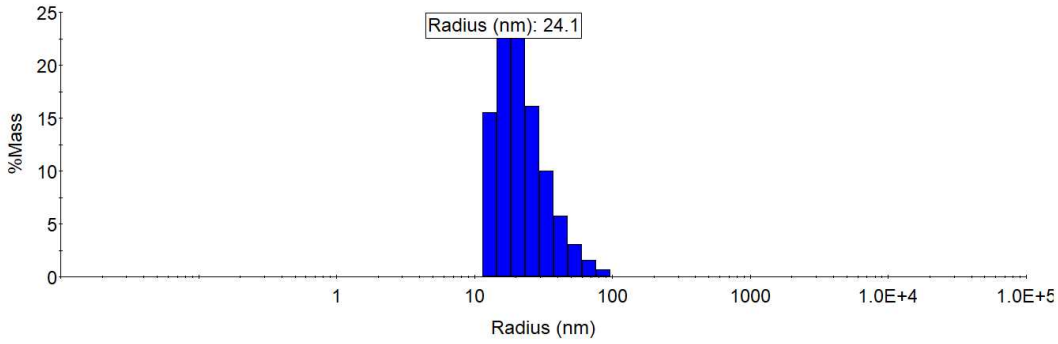
Dynamic Light Scattering measurements

CREATED BY

andrew.west west

PREVIEW

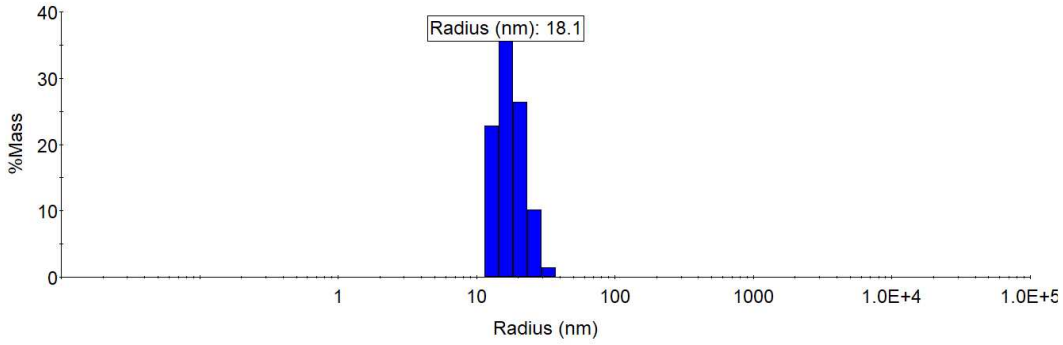
12



Example of the human α-synuclein fibrils size range after 1 hour of sonication

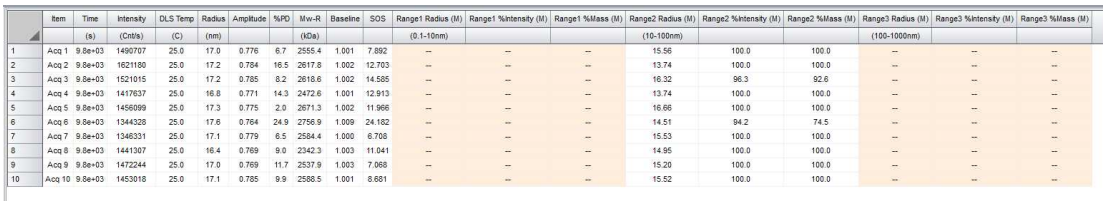
	Peak	Radius	Mw-R	%Pd	%Intensity	%Mass	%Number
		(nm)	(kDa)				
1	Peak 1	22.4	485...	12.9	95.4	84.4	100.0

Item	Time	Intensity	DLS Temp	Radius	Amplitude	%PD	Mw-R	Baseline	SOS	Range1 Radius (M)	Range1 %Intensity (M)	Range1 %Mass (M)	Range2 Radius (M)	Range2 %Intensity (M)	Range2 %Mass (M)	Range3 Radius (M)	Range3 %Intensity (M)	Range3 %Mass (M)
	(s)	(Cnt/s)	(C)	(nm)			(kDa)			(0.1-10nm)			(10-100nm)			(100-1000nm)		
1 Acq 1	9.1e+03	1409636	25.0	27.3	0.775	19.0	7749.4	1.005	18.861	--	--	--	24.20	100.0	100.0	--	--	--
2 Acq 2	9.1e+03	1321535	25.0	28.9	0.786	24.7	8806.9	1.017	34.656	--	--	--	24.88	94.2	28.6	--	--	--
3 Acq 3	9.2e+03	1559858	25.0	27.5	0.772	20.3	7853.1	1.007	30.838	--	--	--	23.55	98.5	84.4	--	--	--
4 Acq 4	9.2e+03	1356850	25.0	27.0	0.764	34.4	7550.7	1.007	18.730	--	--	--	19.35	98.6	91.6	--	--	--
5 Acq 5	9.2e+03	1579752	25.0	26.8	0.767	22.3	7419.1	1.000	14.683	--	--	--	19.96	100.0	100.0	--	--	--
6 Acq 6	9.2e+03	1495781	25.0	27.6	0.758	27.8	7888.8	1.009	35.540	--	--	--	23.29	92.9	49.0	--	--	--
7 Acq 7	9.2e+03	1463890	25.0	27.0	0.776	48.6	7518.3	1.010	79.107	--	--	--	22.11	85.4	50.7	616.48	10.9	12.6
8 Acq 8	9.2e+03	1648846	25.0	27.3	0.779	21.1	7696.7	1.003	17.656	--	--	--	23.31	100.0	100.0	--	--	--
9 Acq 9	9.2e+03	1407852	25.0	25.7	0.763	18.9	6680.8	1.003	8.982	--	--	--	21.38	100.0	100.0	--	--	--
10 Acq 10	9.2e+03	1514130	25.0	26.2	0.775	17.5	7019.5	1.002	22.231	--	--	--	21.03	100.0	100.0	--	--	--



Example of the mouse α-synuclein fibrils size range after 1 hour of sonication

	Peak	Radius	Mw-R	%Pd	%Intensity	%Mass	%Number
		(nm)	(kDa)				
1	Peak 1	16.4	232...	2.6	98.9	98.0	100.0



	Item	Time	Intensity	DLS Temp	Radius	Amplitude	%PD	Mw-R	Baseline	SOS	Range1 Radius (M)	Range1 %Intensity (M)	Range1 %Mass (M)	Range2 Radius (M)	Range2 %Intensity (M)	Range2 %Mass (M)	Range3 Radius (M)	Range3 %Intensity (M)	Range3 %Mass (M)
		(s)	(Cnt/s)	(C)	(nm)			(kDa)			(0.1-10nm)			(10-100nm)			(100-1000nm)		
1	Acq 1	9.8e+03	1490707	25.0	17.0	0.776	6.7	2555.4	1.001	7.892	--	--	--	15.56	100.0	100.0	--	--	--
2	Acq 2	9.8e+03	1621180	25.0	17.2	0.784	16.5	2617.8	1.002	12.703	--	--	--	13.74	100.0	100.0	--	--	--
3	Acq 3	9.8e+03	1521015	25.0	17.2	0.785	8.2	2618.6	1.002	14.585	--	--	--	16.32	96.3	92.6	--	--	--
4	Acq 4	9.8e+03	1417637	25.0	16.8	0.771	14.3	2472.6	1.001	12.913	--	--	--	13.74	100.0	100.0	--	--	--
5	Acq 5	9.8e+03	1456099	25.0	17.3	0.775	2.0	2671.3	1.002	11.966	--	--	--	16.66	100.0	100.0	--	--	--
6	Acq 6	9.8e+03	1344328	25.0	17.6	0.764	24.9	2756.9	1.009	24.182	--	--	--	14.51	94.2	74.5	--	--	--
7	Acq 7	9.8e+03	1346331	25.0	17.1	0.779	6.5	2584.4	1.000	6.708	--	--	--	15.53	100.0	100.0	--	--	--
8	Acq 8	9.8e+03	1441307	25.0	16.4	0.769	9.0	2342.3	1.003	11.041	--	--	--	14.95	100.0	100.0	--	--	--
9	Acq 9	9.8e+03	1472244	25.0	17.0	0.769	11.7	2537.9	1.003	7.068	--	--	--	15.20	100.0	100.0	--	--	--
10	Acq 10	9.8e+03	1453018	25.0	17.1	0.785	9.9	2588.5	1.001	8.681	--	--	--	15.52	100.0	100.0	--	--	--

13 Measure the concentration of sonicated fibrils:
prepare serial dilutions: 2x; 4x; 8x in 3M Guanidine HCL

250g Guanidine hydrochloride **G-Biosciences Catalog #**BC85

Start with preparation of 2x dilution: 4 uL of sonicated fibrils + 4 ul of 6M GuHCL
4x: add 4 uL of 2x diluted sample + 4 uL of 3M GuHCL
8x: add 4 uL of 4x diluted sample + 4 uL of 3M GuHCL
Incubate for 5 min at RT

Add 3 uL of diluted sample onto nanodrop piedestal;
Parameters: other proteins; coefficient extinction: 5.98; MW: 14.4 kDA for human fibrils
Parameters: other proteins; coefficient extinction: 7.45; MW: 14.4 kDA for mouse fibrils

- Confirm that the standard error between the two measurements is less than 10%.
- Calculate the stock concentration by multiplying the measured concentration by the dilution factor

Equipment

NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer	NAME
UV-Vis Spectrophotometer	TYPE
Thermo Scientific	BRAND
ND-ONE-W	SKU