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PURIFICATION OF PROTEINS FROM PFA FIXED SAMPLES BAK_WITH BIOTIN PULLDOWN FOR LC-MS/MS_2023

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Choi SG, Tittle T, Garcia-Prada D, Kordower JH, Melki R, Killinger BA. Alpha-synuclein aggregates are phosphatase resistant. bioRxiv [Preprint]. 2024 Apr 9:2023.11.20.567854. doi: 10.1101/2023.11.20.567854. PMID: 38645137; PMCID: PMC11030248.

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

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Abstract

This protocol details the purification of proteins from PFA fixed samples and extraction of proteins from formalin-fixed tissues. Also included, biotin pulldown prior to LC-MS/MS. Samples generated for this protocol have been used for mass spectrometry, immunoblotting, and pulldowns.

Attachments

PDF

PURIFICATION OF PROT...

221KB

Image Attribution

Killinger BA, Mercado G, Choi S, Tittle T, Chu Y, Brundin P, Kordower JH. Distribution of phosphorylated alpha-synuclein in non-diseased brain implicates olfactory bulb mitral cells in synucleinopathy pathogenesis. NPJ Parkinsons Dis. 2023 Mar 25;9(1):43. doi: 10.1038/s41531-023-00491-3. PMID: 36966145; PMCID: PMC10039879.

Materials

Wash Buffer (1L):

	A	B
	10X TBS pH 7.6	100 mL
	Triton X-100	10 mL
	SDS	1 g
	0.5M EDTA pH 8.0	2 mL

Dissolve in milliq water. Up to final volume 1L. Store at room temperature for 6 months.

Reversal Buffer (RB) (500ML):

	A	B
	SDS	25g
	0.5M EDTA pH 8.0	2 mL
	Tris-Base	30.35 g
	NaCl	4.38 g

Dissolve in milliq water. pH to 7.6. Up to final volume 500mL. Store at room temperature for up to 1 year.

PMSF solution (100 mM). Acute toxicity, handle powder carefully.

1. Place 1.5mL tube onto scale, tare.
2. Add small amount of PMSF to tube, record weight.
3. Add calculated volume of isopropanol to PMSF. Mix well. To calculate: PMSF mass (mg) / 17.4 = volume of isopropanol.

Stable in isopropanol at RT for at least 6 months. Stable for ~15 min once added to aqueous solution.

Fixation solution:

	A	B
	Ethanol	50%
	Acetic acid	10%

Troubleshooting

Procedure Day 1 (Extract proteins and test concentration) 1h 35m

1 Wash sections in DM 3 X 10 min.

1.1 Wash sections in DM for 00:10:00 (1/3) 10m

1.2 Wash sections in DM for 00:10:00 (2/3) 10m

1.3 Wash sections in DM for 00:10:00 (3/3) 10m

2 Place sections in 1.5mL Eppendorf tube.

3 Add 0.5 mL of reversal buffer.

4 Briefly sonicate on low power to disperse tissue.

5 Add 5 µL of [M] 100 millimolar (mM) PMSF. Mix well. Quick spin.

6 Heat on block for 98 °C for 00:30:00 with cap locks. 30m

7 Remove from heat block carefully (caps will pop if not careful).

8 After 00:05:00 of cooling, vortex well. 5m

9 Centrifuge at 22000 x g for 00:30:00 Room temperature . 30m

10 Collect S1 (extracted proteins).

Western blot (Optional)

11 Perform methanol/chloroform cleanup on 100 µL of S1.

12 Resuspend resulting pellet in 100 µL 5% SDS.

13 Perform BCA assay on 100 µL. Each BCA test takes 20 µL. The remaining volume can be used for western blot. Alternatively, more protein can be prepared from S1 if needed.

14 Calculate volume required for 20 µg protein.

15 20ug / concentration of S1 ug/ul = ul of sample required

Procedure Day 2 (Capture biotinylated proteins and wash) 10h 3m

16 Add S1 to 10 mL TBST in 15mL conical tube. Mix well.

17 Add 40 µL of prepared magnetic streptavidin beads to each tube.

18 Nutation for 01:00:00 Room temperature. 1h

19 Place tube on magnetic stand for 00:01:00 (until all beads have been drawn out of solution). 1m

20 Carefully remove supernatant no to disturb beads. Add 10 mL wash buffer.

21 Nutation for 00:30:00. 30m

22 Place tube on magnetic stand for 00:01:00 (until all beads have been drawn out of solution). 1m

23 Add 10 mL wash buffer.

24 Nutation for 00:30:00 . 30m

25 Place tube on magnetic stand for 00:01:00 (until all beads have been drawn out of solution). 1m

26 Add 10 mL wash buffer

27 Nutation Overnight at 4 °C . 8h

Day 3 (Elute captured proteins) 12m

28 Place tube on magnetic stand for 00:01:00 (until all beads have been drawn out of solution). 1m

29 Carefully remove supernatant no to disturb beads.

30 Add 1 mL of wash buffer.

31 Mix samples until beads are suspended in wash buffer.

32 Using a pipette with tip cut off, transfer suspended beads to a 1.5mL protein low bind tube.

33 Place tube on magnetic stand for 00:01:00 (until all beads have been drawn out of solution). 1m

34 Remove buffer and surface wash with milliq 2X.

35 Quick spin and remove liquid from tube (should only have beads left).

36 Add 80 µL 1X SDS-page sample buffer containing reducing agent.

37 Mix well and quick spin.

38 Place on heat block set to 98 °C for 00:10:00. 10m

39 Mix well and quick spin.

40 Place on magnet.

41 Transfer eluent to two separate lo-bind tube and discard used beads. 35 µL in 1 tube (used for premass spec QC), the remaining 45 µL in another (used for mass spec).

42 Immediately place in -80 °C for long-term storage.

Day 4 (Prepare proteins for Mass Spec) 3h 8m

43 Prepare Bis-Tris 4-12% wedge gel 15 well.

44 Make 800 mL 1X MOPS running buffer.

45 Load 5 µL of MW standard in one side of gel.

46 For MS load exactly 40 µL of the samples into wells. Careful not to spill into opposing wells. Only load samples into every other well to prevent cross contamination.

47 Ensure all wells have a buffer. For empty wells fill with 40 µL 1X loading buffer.

48 Run the gel at 150V for a few minutes. Watch carefully until the sample has completely entered gel.

49 Stop running, remove gel with clean gloves, and clean equipment.

50 Fix gel in 100mL fixation solution (refer materials section) for 01:00:00 Room temperature . 1h

51 Wash gel in several changes of milliq, until gel has swollen to original size.

52 Place in 100 mL colloidal Coomassie blue stain solution. Cover loosely, and heat in microwave for few minutes until solution just starts to boil.

53 Incubate gel in heated colloidal Coomassie blue stain solution for 00:08:00 . 8m

54 Remove gel from stain solution and wash several times with milliq water. Clear background is achieved with ~ 02:00:00 of washing. 2h

55 Using a clean razor excise the entire sample from the gel.

56 Place each sample into a clean 1.5mL tube. Add 500 µL of milliq water.

57 Samples can be stored at 4 °C until submitted to mass spec core.

Optional: Pre mass spec QC

45m

58 **Estimating capture concentration** - **Purpose:** To provide mass spec core with a rough estimate of the amount of protein in the capture sample.

> Note
>
> This prevents potential overloading, or digestion problems in the mass spec core.

58.1 Prepare gel as described above (Day 4, Steps 43-45)

59 Load 20 µL of QC aliquot into wells.

60 Load 2 µg BSA into 1 well.

61 Run the gel at 150V for approximately 00:45:00 or until the die has reached end of gel.

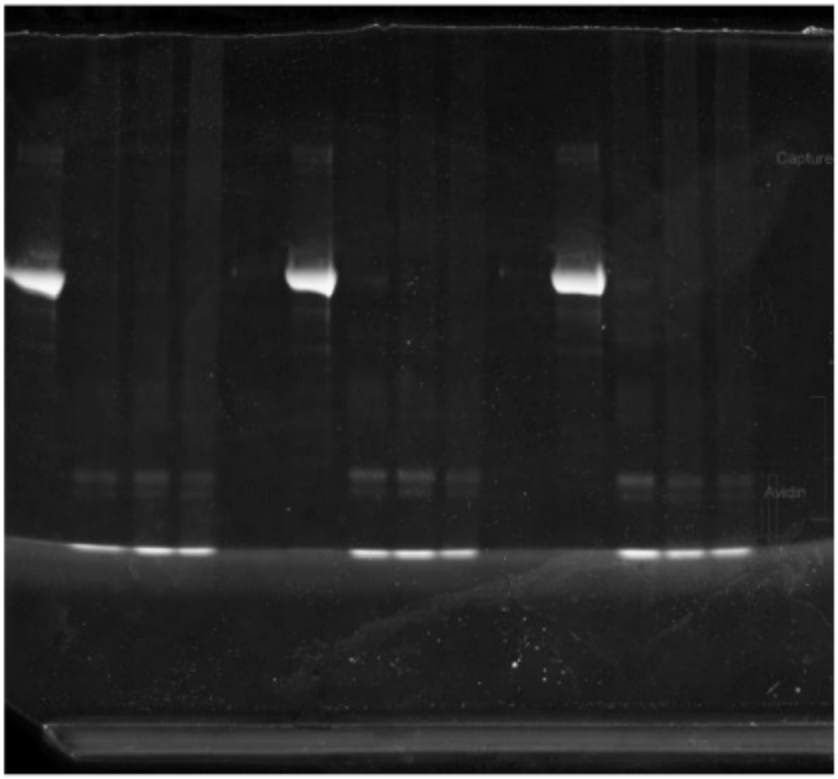
45m

62 Stain gel with Coomassie as described above (Day 4, Steps 50-54)

63 Image on Odyssey using NIR.

64 Forward image to NW mass spectrometry core.

Example of results; order = 2ug BSA, 3 captures, 2ug BSA, 3 captures, 2ug BSA, 3 captures



Dot blot to estimate target enrichment

8h 0m 30s

65 Cut PVDF membrane into ~1X3 inch piece.

66 Activate PVDF in 100% methanol for 00:00:30 30s

67 Equilibrate activated PVDF in diH20.

68 Just prior to blotting samples, place hydrated PVDF on wypall and quickly pat dry.

69 Place PVDF on new dry wypall.

70 Spot 1 µL of each sample onto PVDF.

71 Allow PVDF to dry completely. (~1h or Overnight) 8h

72 Reactivate spotted PVDF as described above and equilibrate in dH20.

73 Blot can now be probed with antibodies using standard protocols.