Aug 19, 2021

Sequential extraction and immunoblotting

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Isabel Lam: Protocol from VolpicelliDaley et al Nat Protocols 2014.

dx.doi.org/10.17504/protocols.io.bu2wnyfe

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ABSTRACT

This protocol examines the fraction of alpha-synuclein (as assessed by alpha-synuclein and/or PS129 western blot) that is present in the triton-soluble or SDS-soluble fraction. Addition of alpha-synuclein pre-formed fibrils (PFFs) to neuron cultures seeds the recruitment of endogenous or transgenic alpha-synuclein into aggregates characterized by detergent insolubility. Monomeric alpha-synuclein will be present in the triton-soluble fraction, whereas PFF-induced alpha-synuclein oligomers will be present in the triton-insoluble, SDS-soluble fraction. This protocol encompasses preparation of cell extracts with Triton X-100, followed by sequential extraction of the Triton-insoluble material with SDS. The protein in the different detergent fractions are quantified by BCA, followed by SDS-PAGE and WB for alpha-synuclein, PS129, TUJ1, and loading control such as GAPDH.

ATTACHMENTS

dh35biqa7.pdf

DOI

dx.doi.org/10.17504/protocols.io.bu2wnyfe

PROTOCOL CITATION

Isabel Lam 2021. Sequential extraction and immunoblotting. **protocols.io** https://dx.doi.org/10.17504/protocols.io.bu2wnyfe

KEYWORDS

Immunoblotting, Alpha-synuclein, Sequential extraction

CREATED

May 15, 2021

LAST MODIFIED

Aug 19, 2021

OWNERSHIP HISTORY

May 15, 2021 Urmilas

Jun 23, 2021 hendersa

PROTOCOL INTEGER ID

49974

MATERIALS TEXT

Materials:

- **50 mL** Falcon tube
- Bucket with ice
- Cell scraper
- Sonicator (I used Misonix Sonicator S-4000 from Wolfe Lab, next to ultracentrifuge)
- Ultracentrifuge (I used Optima MAX-TL Ultracentrifuge TLA-120.2 S/N 12U1811)
- Polyallomar tube

Reagents:

- 1X PBS
- 1% (vol/vol) TX-100/TBS with protease and phosphatase inhibitors
- 2% SDS/TBS with protease and phosphatase inhibitors
- 4X Laemmli buffer
- TBS/5% (wt/vol) milk
- 10X TBS
- BCA assay reagents
- SDS-PAGE reagents

Sequential extraction and immunoblotting 7h 50m

1 

Rinse the neurons twice with PBS.

2 

Place the dish **On ice** . By working one well at a time, completely aspirate the PBS and add the following volumes of ice-cold 1% (vol/vol) TX-100/TBS with protease and phosphatase inhibitors: **250 µl** per well for a six-well plate, and **500 µl** for a 6-cm dish.

3 Use a cell scraper to thoroughly scrape all neurons from each well.

4 Place the neurons in a polyallomar tube for a table top ultracentrifuge; keep the tube **On ice** .

5 

Sonicate the tube ten times at a 0.5-s pulse and at 10% power.

5.1 Use Misonix Sonicator S-4000, with Program Settings: Amplitude 10, Process Time **00:00:10** , 12s Pulse-ON time **00:00:01** , Pulse-OFF time **00:00:01** .

5.2 Wipe sonication tip with 1% SDS after coming into contact with PFFs, then 70% ethanol.

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6 30m

Incubate the tube **On ice** for **00:30:00** .

7 30m

Centrifuge the tube at **100000 x g** at **4 °C** for **00:30:00** .

7.1 30m Optima MAX-TL Ultracentrifuge TLA-120.2 S/N 12U1811 **100000 x g** **00:30:00** **4 °C** .

8

Add 4× Laemmli buffer to ~ **150 µl** – **200 µl** of TX-100 supernatant . Save ~ **20 µl** of supernatant for protein assay. Retain the supernatant **On ice** or in a **-20 °C** freezer.

Some of the supernatant is usually lost during Step 3–8; we recommend measuring the remaining supernatant before determining how much to remove for Laemmli buffer.

9

To the pellet, add the same volume of ice-cold 1% (vol/vol) TX-100/TBS with protease and phosphatase inhibitors: **250 µl** per well for a six-well plate, and **500 µl** for a **6 cm** dish.

10 Sonicate ten times at a 0.5-s pulse and at 10% power. Keep the tip of the probe toward the bottom of the tube to prevent frothing. Make sure that the pellet is completely dispersed.

a. Sonicator: Fisherbrand™ Model 120 Sonic Dismembrator (120V, 50/60Hz)
b. I used Misonix Sonicator S-4000 with Program Settings: Amplitude 10, Process Time **00:00:10** , Pulse-ON time: **00:00:01** , Pulse-OFF time: **00:00:01**

11 30m

Centrifuge the mixture at **100000 x g** at **4 °C** for **00:30:00** .

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12 Discard the supernatant.

13 

Add 2% (vol/vol) SDS/TBS to the pellet with protease and phosphatases inhibitors. To a six-well plate, add **125 µl** of 2% (wt/vol) SDS/TBS per well, and to a **6 cm** dish add **250 µl** of 2% (wt/vol) SDS/TBS.

14 Sonicate 15 times, at a 0.5-s pulse and at 10% power. Keep the tip of the probe toward the bottom of the tube. Make sure that the pellet is completely dispersed.

a. I used Misonix Sonicator S-4000 with Program Settings: Amplitude 10, Process Time 00:00:15, Pulse-ON time: 00:00:01, Pulse-OFF time: 00:00:01

15 Remove the supernatant and place it into a new microcentrifuge tube.

16 Perform a BCA/protein assay on TX-100 supernatant and SDS extract. Typically, a 1:5 dilution for the BCA assay is sufficient.

17 Dilute 2% (wt/vol) SDS extract from Step 15 into Laemmli buffer to 2× volume for the corresponding TX-100 fraction (regardless of the protein concentration of the SDS fraction).

For example, if you have **180 µl** of TX-100 extract (from Step 8) at **[M]1 mg/ml** and **90 µl** of SDS extract, add **60 µl** of 4× Laemmli buffer to the TX-100 extract and **30 µl** of 4× Laemmli buffer to the SDS extract. Load **12.5 µl** of both the TX-100 extract (**10 g**) and SDS extract. We suggest using a 2× volume because it makes the insoluble a-syn species more abundant and thus easier to visualize and quantify by immunoblotting.

18 Load the samples on a 4–20% (wt/vol) gel and run them according to the manufacturer's directions.

We use 85 V constant voltage until the dye front runs off the gel. Be sure not to let the 10-kDa marker to run off the gel.

2h 30m

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19

Transfer the proteins from the gel to a nitrocellulose membrane according to the manufacturer's instructions at 100 V for **01:15:00** or **Overnight** at 40 V.

20 Block the membrane for **01:00:00** with TBS/5% (wt/vol) milk. 1h

21 1h

Dilute the primary antibodies in TBS/5% (wt/vol) milk and incubate them **Overnight** at **4 °C** with shaking.

22 10m

Rinse the membrane three times with TBS/T, **00:10:00** each rinse.

23 1h

Incubate the membrane with HRP-conjugated secondary antibodies for **01:00:00** at **Room temperature** .

24 10m

Rinse the membrane three times with TBS/T, **00:10:00** each rinse.

25 Develop with enhanced chemiluminescence.

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