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Differential extraction of detergent-insoluble protein aggregates from cultured mammalian cells - a benchtop centrifuge method

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Protein aggregation is strongly associated with accelerated ageing and (neuro)degeneration, and considerable effort is directed towards studying this process in vivo. Reliable, unbiased quantification and analysis of endogenous, insoluble protein aggregates requires their specific isolation from the total proteome. Here, we describe a user-friendly differential detergent fractionation method to sequentially extract increasingly insoluble proteins from cultured cells. We also discuss options for downstream quantification and analysis. The use of a benchtop centrifuge instead of ultracentrifugation makes this method simple and cost-effective.

S. BERGINK, W. HUITING 2022. Differential extraction of detergent-insoluble protein aggregates from cultured mammalian cells - a benchtop centrifuge method. **protocols.io** https://protocols.io/view/differential-extraction-of-detergent-insoluble-pro-b4hrqt56

Protein fractionation, protein aggregation, insoluble proteins, detergent insolubility, subcellular fractionation

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Key Resources

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals		
Igepal CA-630	N/A	N/A
cOmplete EDTA free protease inhibitor cocktail	Roche	Cat#11836170001
Benzonase nuclease, Purity > 90%	Merck Millipore	Cat#70746
Critical Commercial Assays		
Detergent compatible protein quantification assay: e.g. DC protein microassay	Bio-Rad	Cat#5000116
Software and Algorithms		
ImageJ	[27]	https://imagej.nih.gov/ij/
Other		
Vortex	N/A	N/A
Refrigerated benchtop microcentrifuge	N/A	N/A
Dry block thermoshaker	N/A	N/A
Bright mini-LED	N/A	N/A
30G needles (e.g. B Braun Sterican 30G)	N/A	N/A
1 ml syringes (e.g. B Braun Omnifix-F Solo)	N/A	N/A

Buffer A

Reagent	Final concentration	Stock concentration	Volume (per ml)
HEPES, pH = 7.4	125 mM	0.5 M	250 ml
NaCl	500 mM	2 M	250 ml
MgCl2	5 mM	0.5 M	10 ml
MilliQ			490 ml
Total			**1000** ml

Buffer A is the 5X primary buffer stock solution. Make in advance and store at 4°C for up to 4 weeks.

Wash buffer EB

Reagent	Final concentration	Stock concentration	Volume (per ml)
Buffer A	1X	5X	200ml
MilliQ			800 ml
Total			1000 ml

Buffer I

Reagent	Final concentration	Stock concentration	Volume (per ml)
Buffer A	1X	5X	200ml
Igepal CA-630	1% (v/v)	20 % (v/v)	50 ml
cOmplete EDTA free protease inhibitor cocktail	1X	50X	20 ml
Benzonase nuclease	~50 U/ml	25-29 U/ml	2 ml
MilliQ			728 ml
Total			1000 ml

Buffer S

Reagent	Final concentration	Stock concentration	Volume (per ml)
Buffer A	1X	5X	200ml
SDS	1% (v/v)	20 % (v/v)	50 ml
MilliQ			750 ml
Total			1000 ml

We recommend that buffers EB, I and S are prepared fresh from stock solutions on the day of the experiment.

Buffer U

Reagent	Final concentration	Stock concentration	Volume (per ml)
Urea	8 M	-	-
SDS	2% (v/v)	20 % (v/v)	100 ml
DTT	50 mM	1 M	50 ml
Tris pH 7.4	100 mM	2 M	50 ml
MilliQ			728 ml
Total			**1000** ml

We recommend that buffer U is prepared in advance, and stored as one-time-use aliquots. In solution, urea is more stable at lower temperatures, so store aliquots at-80°C for up to 3 months.

Note: Buffer U can be challenging to make, because 8 M is close to the solubility limit of urea. In addition, the urea usually constitutes a large volume by itself. We recommend preparing at least 50 ml buffer U in advance. Add SDS, DTT and Tris solutions to urea and carefully add MilliQ to reach the desired volume. Adding moderate heat (20-25°C)while stirring will accelerate the endothermic dissolution of urea in water. However, don't increase heat further, as it will lead to the decomposition of urea into reactive cyanate ions.

4X DTT SB

Reagent	Final concentration	Stock concentration	Volume (per ml)
SDS	8 % (w/v)	20% (w/v)	400 ml
Tris pH 6.8	250 mM	2 M	125 ml
DTT	200 mM	1 M	200 ml
Bromophenol blue	0.025% (w/v)	0.5% (w/v)	50 ml
Glycerol	20% (v/v)	100%	200 ml
MilliQ			25ml
Total			**1000** ml

4X DTT sample buffer (4XSB) can be prepared in advance, aliquoted for one-time-use and stored at -20°C for up to a year.

Other solutions

Name	Instructions
HEPES, NaCl, MgCl2 stocks	Make in advance using milliQ, and store at 4 °C for up to 6 months.
cOmplete EDTA free protease inhibitor stock	Dissolve one tablet in 1 ml milliQ to make a 50X stock solution. Aliquot per 20 ml (i.e. one-time-use for 1 ml buffer I), and store at -20 °C for at least 12 weeks.
Benzonase nuclease	Aliquot per 50 units (i.e. one-time-use for 1 ml buffer I), and store at -20 °C for up to 3 years.
DTT	Make fresh when needed using milliQ. Alternatively, make in advance, aliquot for one-time-use, and store at -20 °C for up to a year.
Bromophenol blue (0.5% w/v)	Make in advance by adding 5 mg of bromophenol blue into 1 ml milliQ and dissolve. Store at -20 °C for up to a year.

Alternatives:

- HEPES is preferred as buffering agent in buffer A because of its pH range (6.8-8.2) and a more stable pKa at lower temperatures. Tris can be used in buffer A as well (preferably in a 250 mM final concentration), but make sure to check the pH of your buffer at 4°C before use.
- cOmplete EDTA free protease inhibitor can be replaced with any other commercially available protease inhibitor, as long as it does not contain EDTA in a concentration greater than 1 mM,

as this will inhibit benzonase activity. It can also be replaced by adding a combination of pepstatin A (1.5mM), leupeptin (2mM) and PMSF (2 mM).

- Benzonase nuclease can be replaced with other commercially available nucleases like DENARASE. Concentrations may need to be optimized when using a different nuclease.
- In buffer U, 8 M urea can be replaced with 6 M urea + 2 M thiourea when stronger chaotropic conditions are needed to increase final protein solubilization even further.

Fractionation

1 **Prepare cell culture starting material according to your research question.**

a. Harvest cells via scraping in ice-cold PBS.

b. Pellet cells in 1.5 microcentrifuge tubes via low-speed centrifugation in a pre-cooled benchtop centrifuge (5 minutes, 500 x g, 4 °C).

c. Discard supernatant.

2 **Prepare assay buffers as needed.**

a. Prepare buffer A.

i. Prepare a minimum of 120 ml + (100 ml per 500 mg expected protein yield from lysis) buffer A per sample. Example: for one well of a 6-well culture plate, with HEK293t cells at 90% confluency, the expected total protein yield after lysis would be 400-500 mg. If three samples will be generated from one well each, prepare 3 x (120 + 100) = 660 ml of buffer A.

b. From buffer A, prepare buffers EB, I and S.

i. Prepare 550 ml wash buffer EB per sample.

ii. Prepare 200 ml of both buffer I and buffer S per sample.

c. Prepare buffer U.

3 **Lyse pelleted cells in 1.5 ml microcentrifuge tubes in 150 ml ice-cold buffer I.**

a. Vortex cell pellets in buffer I until dissolved completely, then put tubes on ice.
b. Vortex cell pellets 5-10 seconds every 10 minutes for 30 minutes.
c. Homogenize cell lysates further with a 1 ml syringe and 30G needle (5 strokes).
d. Vortex homogenates 5-10 seconds, leave on ice for another 10 minutes.
e. Visually verify that cell lysis is complete (no cell clumps left).

4 Perform protein measurement, preferably using a detergent compatible protein quantification method (e.g. DC protein assay, Bio-Rad) and performing measurements in triplicate.

5 Equalize protein concentration between samples using remaining buffer I. Equalize to ~250 mg of total protein per sample.

6 **Take desired volume from the equalized homogenate to serve as whole cell lysate (fraction WC, i.e. loading control)**

a. Take 15-25 mg for simple assay verification (e.g. staining, Western blotting), take more for multiple downstream analyses.
b. Add 4XSB into homogenate. Mix by brief vortexing.
c. Boil (5 min, 95 °C).
d. Store at -20 °C.

7 Centrifuge remaining homogenate in a pre-cooled (4 °C) benchtop centrifuge at 14,000 x g for 45 minutes.

8 **Take desired volume from supernatant to serve as Igepal soluble fraction (fraction I)**

1. Add 4XSB into supernatant. Mix by brief vortexing.
2. Boil (5 min, 95°C).
3. Store samples at -20°C.

9 Carefully take off the rest of the supernatant, discard.

10 **Wash the protein pellet.**

a. Add 250 ml ice-cold wash buffer EB without disturbing the protein pellet.
b. Centrifuge in a pre-cooled (4 °C) benchtop centrifuge at 14,000 x g for 15 minutes.
c. Take off the supernatant, discard.

11 **Dissolve the 1% igepal-CA630 protein pellet in buffer S.**

a. Add appropriate volume of buffer S (volumeS = volumestart - volumeWC - volumeI) at room temperature.

b. Carefully break up the protein pellet using a p200 pipettip.
c. Put the tube in a dry block heating shaker at room temperature, shaking at 1000-1200 rpm for 1 hour.
d. Check if protein pellets are completely dissolved. If not, extend shaking time.

12 Centrifuge the protein suspension in a benchtop centrifuge at 14,000 x g for 45 minutes at room temperature.

13 **Take desired volume from supernatant to serve as SDS soluble fraction (fraction S)**.
a. Add 4XSB into supernatant.
b. Mix by brief vortexing.
c. Boil (5 min, 95 °C).
d. Store samples at -20 °C.

14 Carefully take off the rest of the supernatant, discard.

15 **Wash pellet.**
a. Add 250 ml wash buffer EB (room temperature) without disturbing the protein pellet.
b. Centrifuge in a benchtop centrifuge at room temperature, 14,000 x g for 15 minutes.
c. Take off supernatant, discard.

16 **Dissolve the 1% SDS insoluble protein pellet in buffer U**
a. Add 35-50 ml buffer U to the protein pellets to ensure proper dissolution.
b. Put the tube in a dry block heating shaker at room temperature, shaking at 1000-1200 rpm for 3 hours to overnight.
c. Check if pellet has dissolved properly. If not, extend shaking time.
d. Freeze samples in liquid nitrogen and store at -80 °C.

Note: Avoid heat greater than 25°C when dissolving proteins in buffer U.

Downstream quantification and analysis

17 **Run WC, I and S fractions in a polyacrylamide gel.**
a. Load 3-5 ug of protein per sample. This is generally sufficient to obtain a good signal/noise ratio in staining or stain-free quantification.

18 **Run U fractions (SDS insoluble proteins) separately in a polyacrylamide gel**
a. Add 1 ml of 0.5% bromophenol blue to each sample to make loading easier
b. Vortex, and briefly centrifuge
c. Load 25-50% of fraction U.

After separation, stain protein fractions using a method of your choice.

19 a. For simple analysis, we recommend colloidal Coomassie (G-250) staining for the WC, S and I fractions. This offers user-friendliness, good sensitivity and reproducibility.
b. Fraction U generally contains very few proteins, so we recommend a highly sensitive Silver-staining

20 **Quantify stained protein fractions**
a. Use imaging software to plot and measure whole lane intensities (e.g. with the freely available package ImageJ) to obtain a relative quantification between fractions.
b. When unstained ladder was included, normalize lane intensities to ladder intensities to get an approximate absolute quantification.