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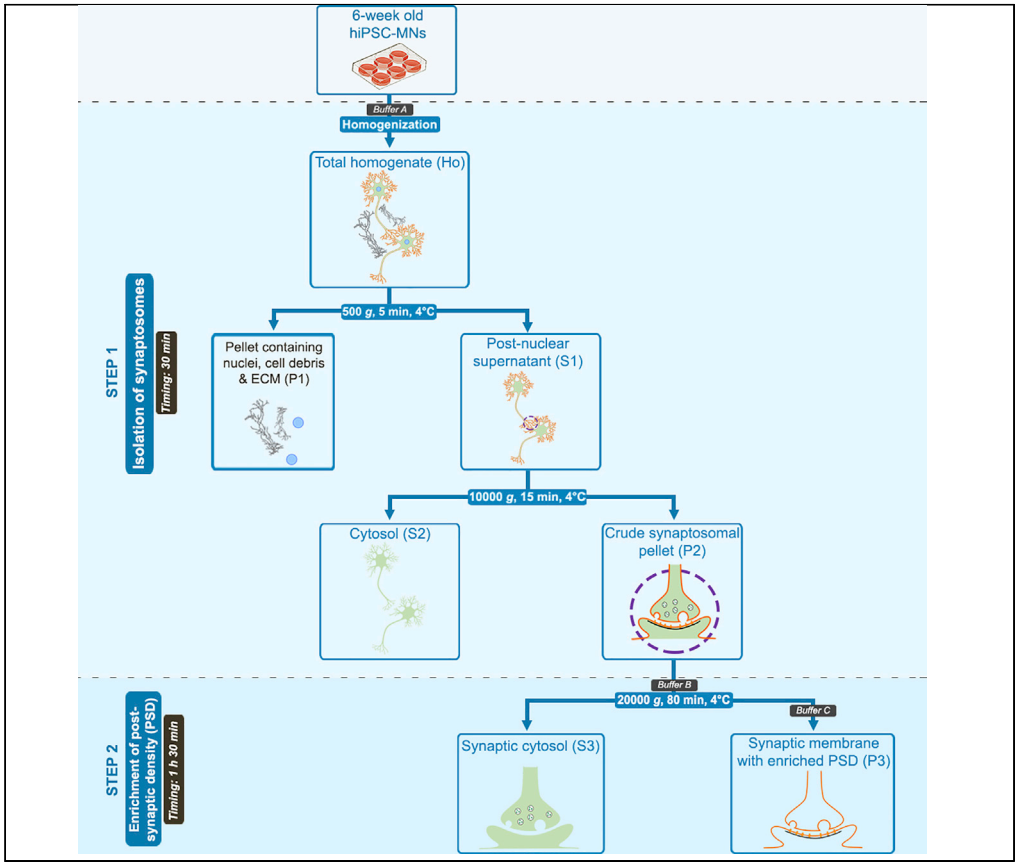
Fast and efficient synaptosome isolation and post-synaptic density enrichment from hiPSC-motor neurons by biochemical sub-cellular fractionation



We describe here a time-efficient, in-house protocol for synaptosome isolation and enrichment of the post-synaptic density (PSD) from hiPSC-derived motor neurons. By using biochemical sub-cellular fractionation, the crude synaptosome is first isolated from the cytosol and is then further separated into the synaptic cytosol and the enriched PSD fraction. The protocol can also potentially be adapted to other hiPSC-derived neuronal types, with necessary changes made to cell seeding density and buffer volumes.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Fast and efficient synaptosome isolation from hiPSC-derived motor neurons

Subsequent fractionation into synaptic cytosol and highly enriched PSD

Confirmation of percentage enrichment by standard Western blotting

Substantial protein quality and concentration for high-throughput proteomics

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Fast and efficient synaptosome isolation and post-synaptic density enrichment from hiPSC-motor neurons by biochemical sub-cellular fractionation

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SUMMARY

We describe here a time-efficient, in-house protocol for synaptosome isolation and enrichment of the post-synaptic density (PSD) from hiPSC-derived motor neurons. By using biochemical sub-cellular fractionation, the crude synaptosome is first isolated from the cytosol and is then further separated into the synaptic cytosol and the enriched PSD fraction. The protocol can also potentially be adapted to other hiPSC-derived neuronal types, with necessary changes made to cell seeding density and buffer volumes.

BEFORE YOU BEGIN

Most neurological diseases reflect strongly on their synaptic integrity and function either as a cause or consequence,[1–3] and therefore a means to evaluate the localization and expression of proteins in synaptic compartments would prove a vital resource to understand disease pathology and search for potential drug targets.

Extraction of synaptosomes and the PSD has been a long-established method[4,5] useful in studying relative expression levels of synaptic proteins including neurotransmitters and ion channels, and for qualitatively estimating the localization of proteins of interest in different synaptic compartments.

The majority of these methods use a sucrose density gradient-based, ultracentrifugation approach by which the various fractions are serially separated.[6,7] However, this is an intensive, time-consuming process that lasts more than 4 h, and it requires a large amount of tissue, to the order of 5 mouse brains per group per experiment, to ensure a significant yield downstream.[8]

Thus, the optimization of faster but still efficient protocols represents a great advantage for the field of synaptic research. Accordingly, our group has previously established a faster protocol to quickly (approx. 2 h) isolate synaptosomes from brain tissue. This method requires only a single mouse brain per experimental group[9] and has already been widely used by other research labs.[10]

Based on these considerations, we aimed at providing a fast and reliable protocol for the isolation of synaptic sub-fractions from hiPSC-derived neurons.[11] Neuronal models derived from hiPSCs have become indispensable to neuroscientific research over the past decade, from modeling various diseases to screening for therapeutics. A customary search of academic publications revealed a stark absence of synaptosome extraction methods for hiPSC-neurons and therefore a quick, streamlined protocol would prove to be extremely helpful in elucidating synaptic pathomechanisms.

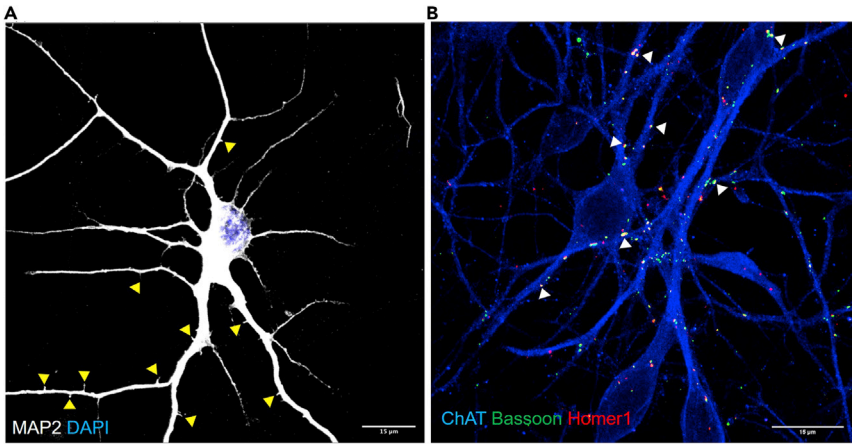

Figure 1. DIV42 hiPSC-MNs show synaptic maturity

(A) Representative confocal image at 63× magnification of a MN immunostained for the nuclear marker DAPI (blue) and the pan-neuronal dendritic marker MAP2 (white) showing dendritic spines (highlighted by yellow arrowheads) as a readout for neuronal maturation. Scale bar: 15 μm.

(B) Representative confocal image at 63× magnification of a ChAT^{+} (blue) MN showing pre-synaptic marker Bassoon (green) and post-synaptic marker Homer1 (red) forming synapses (colocalization highlighted by white arrowheads). Scale bar: 15 μm.

In this article, we show a detailed step-by-step synaptosome isolation and PSD enrichment method for hiPSC-derived motor neuron (MN) cultures, which yields a high enrichment of proteins in their respective sub-cellular fractions. This method is faster and requires a much lower quantity of starting material than the traditional methods (which usually rely on density gradients and ultracentrifugation), and it is more cost-effective than commercially available reagents, thereby ensuring rapid yet sensitive evaluation of synaptic modulations in various neurological disorders.

Notably, besides being suitable for standard western blot analysis, it can even be applied to produce samples of required quality and concentrations for the investigation of the total synaptic proteome by mass spectrometry.

Institutional permissions (if applicable)

Experiments with human iPSCs have been performed in compliance with the guidelines of the Federal Government of Germany and approved by the Ethical Committee of Ulm University (approval Nr. 19/12). The use of human material was approved by the Declaration of Helsinki concerning Ethical Principles for Medical Research Involving Human Subjects, and experiments were performed according to the principles set out in the Department of Health and Human Services Belmont Report.

Experimenters are advised to acquire necessary permissions from the relevant institutions.

Culture of hiPSC-derived motor neurons

Timing: >50 days

hiPSC-MNs were cultured as previously established in our lab.[12] The cells were seeded on 6-well tissue culture plates coated with growth factor reduced Matrigel, at a seeding density of 300,000 cells/well.

Based on our previous findings on time-dependent synaptic maturation and electrophysiological activity,[13] we isolated synaptosomes and PSDs from motor neurons that were cultured for 6 weeks (DIV42), when they display a well-established synaptic phenotype (Figure 1).

Note: Other types of neuronal cultures, or even motor neurons differentiated from hiPSCs using protocols different to ours, may require a proper evaluation of the synaptic maturation occurring *in vitro* in order to identify the most suitable timepoint for the experiments.

Buffers and necessary equipment

Timing: 30 min

1. All buffers are to be prepared beforehand as indicated in the materials and equipment section and stored accordingly.
   a. On the day of the experiment, allow the required buffer aliquots to thaw on ice.
2. For each cell line and/or plate to be used, label the following set of 1.5 mL centrifuge tubes: P, Ho, S1, S2, P2, S3 and P3, and place them on ice.
3. Place the glass douncers on ice.
4. Make sure that the centrifuges are cooled down to 4°C.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit anti-Choline acetyltransferase (1:1000)	Abcam	Cat#ab181023; RRID: AB_2687983
Rabbit anti-Homer1 (1:1000)	Abcam	Cat#ab184955; RRID: AB_2744679
Rabbit anti-Synaptophysin (1:1000)	Abcam	Cat# ab14692; RRID: AB_301417
Mouse anti-β-Actin (1:25000)	Sigma-Aldrich	Cat#A5316; RRID: AB_476743
Rabbit anti-Mouse HRP	Agilent	Cat#P0260, RRID: AB_2636929
Goat anti-Rabbit HRP	Agilent	Cat#P0448, RRID: AB_2617138
Chemicals, peptides, and recombinant proteins		
DPBS, no calcium, no magnesium	Gibco	Cat#14190094
EDTA	Applichem	Cat#131669.1209
EGTA	Sigma-Aldrich	Cat#E4378-10G
Growth factor reduced Matrigel	Corning	Cat#356231
HEPES	Carl Roth	Cat#9105.4
Protease Inhibitor Cocktail	Roche	Cat#4693132001
Sodium deoxycholate	Merck	Cat#1065040250
Sodium fluoride	Merck	Cat#1064490250
Sodium orthovanadate	Sigma-Aldrich	Cat#S6508-10G
Triton X-100	Sigma-Aldrich	Cat#T8787-100ML
Trizma base (Tris)	Sigma-Aldrich	Cat#93362-250G
β-Glycerol phosphate	Applichem	Cat#A2253,0100
Experimental models: Cell lines		
Human: hiPSC line	Systems Bioscience	Cat#SC600A-WT
Other		
1.5 mL centrifuge tubes	Eppendorf	Cat#0030120086
6-well tissue culture plate	Falcon	Cat#353046
Cell scraper	Sarstedt	Cat#83.3951
Dounce tissue grinder 2 mL pestle	Kimble	Cat#885302-0002
Dounce tissue grinder 2 mL tube	Kimble	Cat#885303-0002
High-speed centrifuge	Eppendorf	Cat#5430R

MATERIALS AND EQUIPMENT

Buffer A		
Reagent	Final concentration	Amount
HEPES, pH 7.4	10 mM	0.119 g
EDTA	2 mM	0.037 g
Sodium orthovanadate	5 mM	0.046 g
Sodium fluoride	30 mM	0.063 g
β-glycerol phosphate	20 mM	0.216 g
Protease inhibitor cocktail	N/A	1 tablet
ddH_2O	N/A	50 mL
Total	**N/A**	**50 mL**
Make 1 mL aliquots and store at –20°C up to 6 months.		

Buffer B		
Reagent	Final concentration	Amount
HEPES, pH 7.4	10 mM	0.119 g
EDTA	2 mM	0.037 g
EGTA	2 mM	0.038 g
Sodium orthovanadate	5 mM	0.046 g
Sodium fluoride	30 mM	0.063 g
β-glycerol phosphate	20 mM	0.216 g
Triton X-100	1%	0.5 mL
Protease inhibitor cocktail	N/A	1 tablet
ddH_2O	N/A	49.5 mL
Total	**N/A**	**50 mL**
Make 200 µL aliquots and store at –20°C up to 6 months.		

Buffer C		
Reagent	Final concentration	Amount
Tris, pH 9.0	50 mM	0.302 g
Sodium orthovanadate	5 mM	0.046 g
Sodium fluoride	30 mM	0.063 g
β-glycerol phosphate	20 mM	0.216 g
Sodium deoxycholate	1%	0.5 g
Protease inhibitor cocktail	N/A	1 tablet
ddH_2O	N/A	50 mL
Total	**N/A**	**50 mL**
Make 70 µL aliquots and store at –20°C up to 6 months.		

STEP-BY-STEP METHOD DETAILS

The protocol provided below is described for one hiPSC-MN cell line plated on one full 6-well plate (for fewer number of wells per 6-well plate, see troubleshooting 1). A schematic representation of the protocol is provided in Figure 2.

> ***Note:*** Take into consideration that the timing for the various steps will be delayed incrementally depending on the number of cell lines and/or plates simultaneously used (See troubleshooting 2).

⚠ CRITICAL: All steps are to be performed on ice.

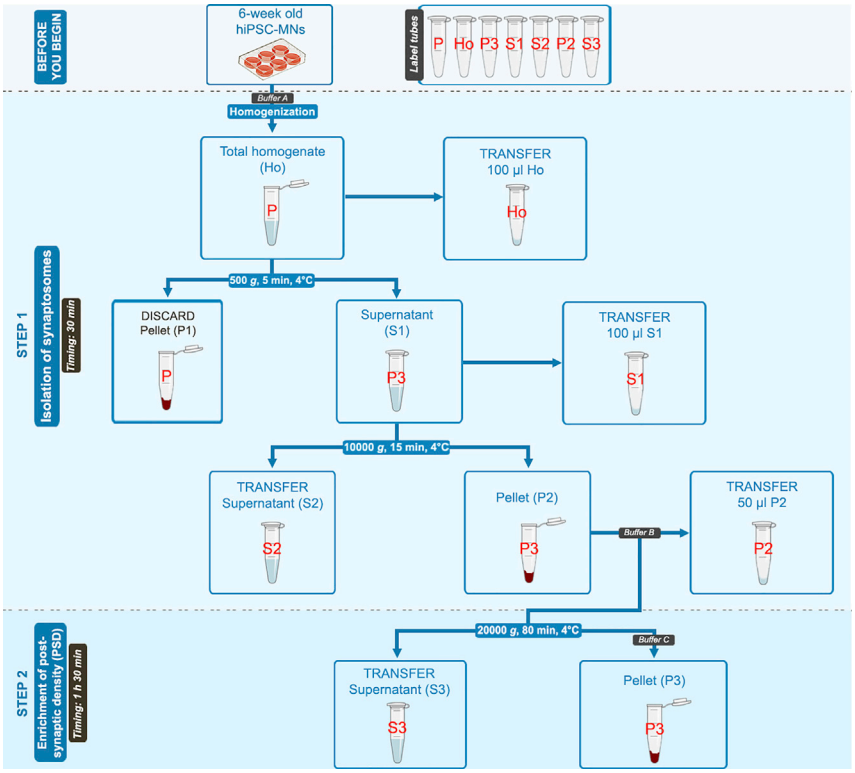

Figure 2. Step-by-step scheme of the protocol

The labels for the 1.5 mL centrifuge tubes are shown in red font, whereas the notations of the different sub-cellular fractions and their respective instructions are shown in either blue or black font.

Isolation of synaptosomes

Timing: 30 min

The first part of the protocol involves the preparation of the total homogenate of the MNs by mechanical disruption. Then the total lysate is purified from the cell debris, nuclei and the extracellular matrix by centrifugation. Upon further centrifugation, the cytosol is obtained in the supernatant, and the crude synaptosomal fraction is extracted from the pellet.

Note: Always start with one 6-well plate.

1. Washing the cells.
   a. Completely aspirate the cell culture medium from all wells.
   b. Quickly wash twice with 500 µL of DPBS per well.
2. Homogenate (Ho) preparation.
   a. Distribute a total of 1 mL of Buffer A to all wells of the 6-well plate.
   b. Use a cell scraper to collect the cells from all the wells and transfer them to a glass douncer.
   c. Homogenize manually for 30 strokes and transfer the homogenate (Ho) to the 1.5 mL centrifuge tube labeled "P."
   d. Collect 100 µL separately in the 1.5 mL centrifuge tube labeled "Ho."
3. Lysate (S1) preparation.
   a. Centrifuge the remaining Ho in tube "P" at 500 × *g* for 5 min at 4°C.
   b. Transfer the supernatant containing the lysate (S1) to the 1.5 mL centrifuge tube labeled "P3."

⚠ CRITICAL: Due to the softness of the pellet, please proceed cautiously without disturbing it when collecting the supernatant (see troubleshooting 3).

Note: The supernatant is transferred to the P3 tube because after each subsequent centrifugation the pellet remains in this same tube until the last step when the actual P3 fraction is obtained.

c. Collect 100 µL separately in the 1.5 mL centrifuge tube labeled "S1."

Note: The pellet (P1) remaining in tube "P" (containing the cell debris, nuclei and extracellular matrix) can be discarded.

4. Synaptosome (P2) isolation.
   a. Centrifuge the P3 tube containing the remaining lysate at 10,000 × *g* for 15 min at 4°C.
   b. Transfer the supernatant containing the cytosol (S2) to the 1.5 mL centrifuge tube labeled "S2."
   c. Resuspend the pellet completely in 200 µL of Buffer B to obtain the crude synaptosomal fraction (P2).
   d. Transfer 50 µL separately to the 1.5 mL centrifuge tube labeled "P2."

Enrichment of post-synaptic density (PSD)

Timing: 1 h 30 min

The second part of the protocol involves a further centrifugation step that separates the synaptic cytosol in the supernatant from the PSD fraction in the pellet.

5. Separation of synaptic cytosol fraction (S3).
   a. Centrifuge the remaining P2 at 20,000 × *g* for 80 min at 4°C.
   b. Transfer the supernatant containing the synaptic cytosol fraction (S3) to the 1.5 mL centrifuge tube labeled "S3."
6. Enrichment of PSD fraction (P3).
   a. Completely resuspend the pellet (remaining in the 1.5 mL centrifuge tube labeled "P3") in 70 µL of Buffer C to obtain the enriched PSD fraction (P3) (see troubleshooting 4).
7. Estimation of protein concentration and storage.
   a. Estimate the protein concentrations of the Ho, S1, S2, P2, S3 and P3 fractions (see troubleshooting 5).
   b. Snap freeze the samples in liquid nitrogen and store them at –80°C up to 1 year.

Note: The fractions can also be stored as smaller aliquots in order to avoid repeated freeze-thaw cycles.

EXPECTED OUTCOMES

Protein concentration estimation by BCA method (Table 1) shows consistently high yields of proteins sufficient for downstream applications such as Western blotting as well as high-throughput experiments such as proteomics.

Confirmation for the subcellular fractionation can be performed by Western blotting (Figure 3). The S1 fraction should contain all non-nuclear proteins, the S2 fraction should be enriched with only cytosolic proteins, the S3 fraction with synaptic cytosol proteins like vesicular proteins and neurotransmitters, and the P3 should be enriched with PSD proteins such as ion channels, scaffolding proteins and the actin cytoskeleton.

Table 1. Estimated protein concentrations for Western blotting of DIV42 hiPSC-MN sub-cellular fractions by BCA method

	Protein concentration (μg/μL)			
Fractions	*Experiment #1*	*Experiment #2*	Standard deviation	Collected volume (μL)
S1	3.9818	4.5651	0.4124	100
S2	3.2979	4.2120	0.6463	700–800
S3	1.4174	1.6160	0.1404	150–200
P3	1.1633	1.2580	0.0669	70

LIMITATIONS

One limitation of this protocol is that the separated fractions are not always completely pure. However, it is to be kept in mind that the aim of this technique is not purification of the subcellular fractions, but rather the enrichment of specific proteins within their respective compartments. This limitation is not a major factor as long as the percentage enrichment of a protein is greater than 100% in its respective fraction and lower than 100% in the others. If not, the buffer volumes have to be adjusted until this is the case.

In line with this, the protocol does not allow a further fractionation of synaptic sub-compartments such as the Triton X-100-insoluble fraction within the PSD, which requires sucrose gradients and longer ultracentrifugation steps.[14]

Though this stepwise procedure is specific for hiPSC-derived neuronal cultures, in order to apply it to other models (both *in vitro* and *in vivo* such as rodent primary neurons or brain areas), extensive internal and application-specific standardization is required. This includes adjustments to the amount of starting material, buffer volumes and concentrations, homogenization method and centrifugation times, among others.

TROUBLESHOOTING

Problem 1

Cells from a full 6-well plate are not available (see step-by-step method details).

Potential solution

Depending on the number of wells available, the buffer volumes can be adjusted as shown in the table below to yield similar enrichment levels:

No. of wells of a 6-well plate	Buffer A	Buffer B	Buffer C
5 wells	900 μL	180 μL	65 μL
4 wells	800 μL	165 μL	60 μL
3 wells	700 μL	150 μL	55 μL

Problem 2

Multiple 6-well plates are to be processed (see step-by-step method details).

Potential solution

If there are more than one 6-well plates, it is advised to perform steps 1 and 2 for each of them separately and place the respective "P" tubes on ice, until ready to proceed with step 3. Make sure to clean the glass douncer with running water and allow it to dry before proceeding to the next plate in order to avoid cross-contamination between different samples.

Problem 3

There is no clear separation of the pellet (P1) from the supernatant (S1) or S1 is turbid or P1 has been disturbed (see step 3b).

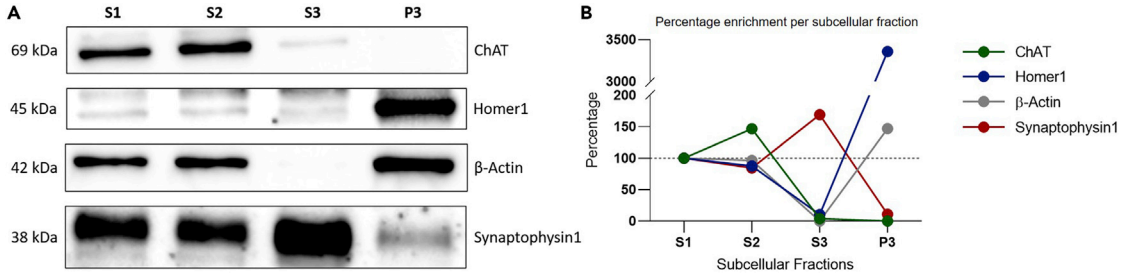

Figure 3. Confirmation of subcellular enrichment of synaptic proteins by Western blot

(A) Representative blot shows expected subcellular localization of respective proteins. 4 μg of protein was loaded for each fraction.

(B) Cytosolic protein choline acetyltransferase (ChAT) is quantitatively confirmed to be enriched in the S2 fraction (146.69%), synaptic vesicular protein Synaptophysin1 in the S3 fraction (169.21%), and the PSD scaffolding protein Homer1 (3358.33%) and cytoskeletal β-Actin (147.1%) in the P3 fraction. Percentage enrichment was calculated as the percentage of the ratio of the protein level in the respective fraction to its amount in the S1 fraction.

Potential solution

If the supernatant is not completely clear or its purity is questionable, repeat step 3. If it is difficult to avoid disturbing the pellet, then leave behind around 100–150 μL of the supernatant when pipetting (applies equally for all experimental groups used). The remaining S1 should be sufficient for all downstream steps.

Problem 4

Pellet P3 is difficult to resuspend in Buffer C (see step 6a).

Potential solution

Resuspending P3 requires rapid pipetting and may take longer than anticipated. Be careful not to introduce bubbles or froth, and avoid using a vortex mixer. If necessary, centrifuge the tube briefly and resume pipetting until the pellet is completely dissolved.

Problem 5

P3 samples precipitate during protein estimation by Bradford method (see step 7a).

Potential solution

The high detergent concentration in Buffer C leads to precipitate formation in the P3 samples. Therefore, it is recommended to use the BCA method (refer to the manufacturer's protocol) which has a higher tolerance for detergents, and is in general more sensitive than the Bradford method.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Alberto Catanese (alberto.catanese@uni-ulm.de).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate or analyze datasets or code.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

Conceptualization, S.R., A.C.; methodology, investigation and writing – original draft, S.R.; writing – review and editing, S.R., A.C.; resources, supervision, and funding acquisition, T.M.B., A.C.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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