ProteoExtract®
Subcellular Proteome Extraction Kit

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1. Introduction

One major challenge in functional proteomics is the separation of complex protein mixtures to allow detection of low abundance proteins and provide for quantitative and qualitative analysis of proteins impacted by environmental parameters. Prerequisites for the success of such analysis are standardized and reproducible procedures for sample preparation prior to 1 or 2 DGE and/or liquid chromatography (LC). Due to the complexity of total proteomes and the divergence of protein properties it is required to prepare standardized partial proteomes of a given organism in order to maximize the coverage of the proteome and to increase the chance to visualize low-abundance proteins.

The unique ProteoExtract® Subcellular Proteome Extraction Kit (**S-PEK**) enables the differential extraction of proteins according to their subcellular localization. The special mild S-PEK procedure yields proteins in their native state making it highly suited for demanding proteomics applications including enzyme activity assays e.g. reporter gene assays and subcellular redistribution assays to monitor protein shuttling e.g. singnaling proteins.
Optimized protocols are available for freshly prepared tissue culture cells, frozen cell pellets and fragmented tissues. The kit contains four Extraction Buffers prepared with ultra-pure chemicals to ensure high reproducibility, protease inhibitor cocktail to prevent protein degradation and Benzonase® nuclease to remove contaminating nucleic acids.

S-PEK takes advantage of the differential solubility of certain subcellular compartments in special reagent mixtures preserving the structural integrity of the subcellular structures before and during the extraction.
For suspension-grown cells the S-PEK extraction starts with gentle sedimentation and washing of the cells. For frozen cells and fragmented tissues, extraction starts with resuspension in the first extraction buffer. In case of adherent cells, the S-PEK procedure is performed directly in the tissue culture dish. The cells or the parts of the cells remain attached to the plate during the entire extraction procedure until the appropriate extraction reagent is used. Thus, the early destruction of the cellular structure by enzymatic or mechanical detachment of cells from the tissue culture plate and any mixing of different subcellular compartments is prevented. A schematic overview of the S-PEK extraction procedure applied to adherent tissue culture cells is shown in Fig 1 A and morphological changes of the cells are documented.

ProteoExtract®Subcellular Proteome Extraction Kit yields the total proteome fractionated into four sub proteomes of decreased complexity. With Extraction Buffer I cytosolic proteins are released (fraction 1). Subsequently, membranes and membrane organelles are solubilized with Extraction Buffer II, without impairing the integrity of nucleus and cytoskeleton (fraction 2). Next, nucleic proteins are enriched with Extraction Buffer III (fraction 3). Components of the cytoskeleton are finally solubilized with Extraction Buffer IV (fraction 4).

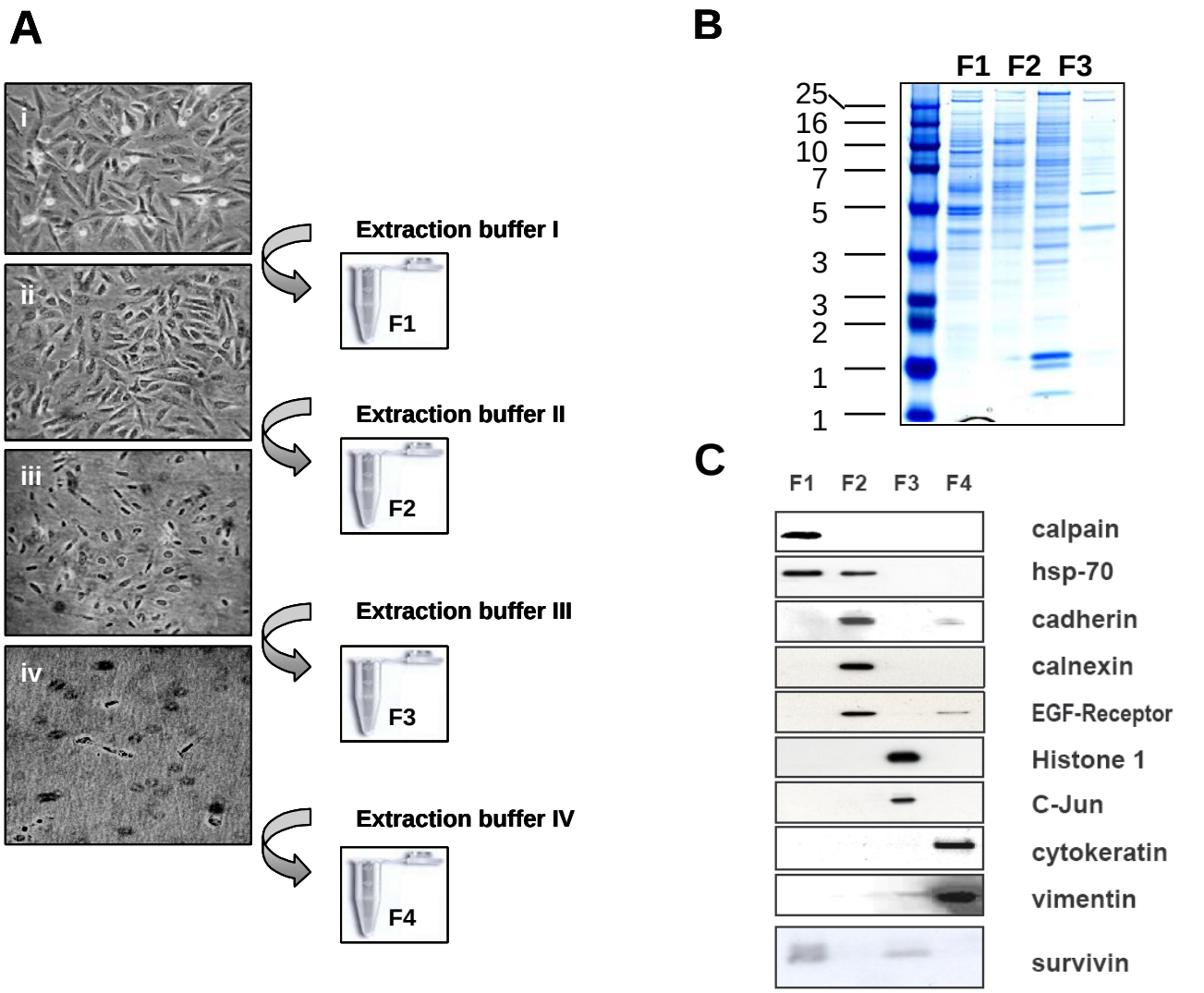

Fig.1: Schematic representation of S-PEK extraction of adherent tissue culture cells.

A: Adherent tissue culture cells (SAOS-cells) were extracted with the ProteoExtract® Subcellular Proteome Exraction Kit. Images i-iv show cells before and after the extraction with the respective extraction buffer (200-fold enlarged). During the entire extraction procedure the SAOS cells keep attached to the tissue culture plate.

B: SDS-PAGE of subcellular fractions after S-PEK extraction of adherent tissue culture cells demonstrating that protein patterns of the respective fractions are clearly distinct. (F1-4: Fraction 1-4).

C: Documentation of selectivity of subcellular extraction using S-PEK by immunoblotting against marker proteins. Adherent tissue culture cells were extracted using the S-PEK procedure. The proteins were separated by SDS-PAGE and blotted onto PVD. Immunoblotting (IB) with antibodies directed against the indicated marker proteins show the separation of the cell components according to their subcellular localization. For c-fos, the protein was immunoprecipitated (IP) prior to detection by IB. (F1-4: Fraction 1-4)

The efficiency of subcellular fractionation by S-PEK has been shown by 1 DE and immunoblotting of selected marker proteins (Fig 1B). More than 90 % of marker proteins are assigned to their expected subcellular fractions (Fig. 1C). Please note that HSP70 is present in both the cytoplasm and the mitochondria of cells and thus is detected in both the cytoplasmic and membrane/organelle fraction after subcellular extraction.

The S-PEK procedure is ideally suited to investigate changes of subcellular localization of regulatory proteins depending on experimental or disease parameters. To demonstrate this application the well-described translocation of NFkappaB from the cytosol to the nucleus upon stimulation of cells with tumor necrosis factor alpha (TNFalpha) (Mejdoubi et al., 1999; Butcher et al., 2001) was chosen. NFkappaB translocation was studied in TNFalpha-stimulated A431 cells that were subsequently extracted with the S-PEK kit. Nuclear translocation of NFkappaB could be easily demonstrated by immunoblotting of fractions and densitometric analysis of filters (Fig. 2) while the control protein calpain did not undergo any translocation between fractions upon TNFalpha-stimulation of cells (data not shown). Thus, using the S-PEK kit, translocations of regulatory proteins can be investigated.

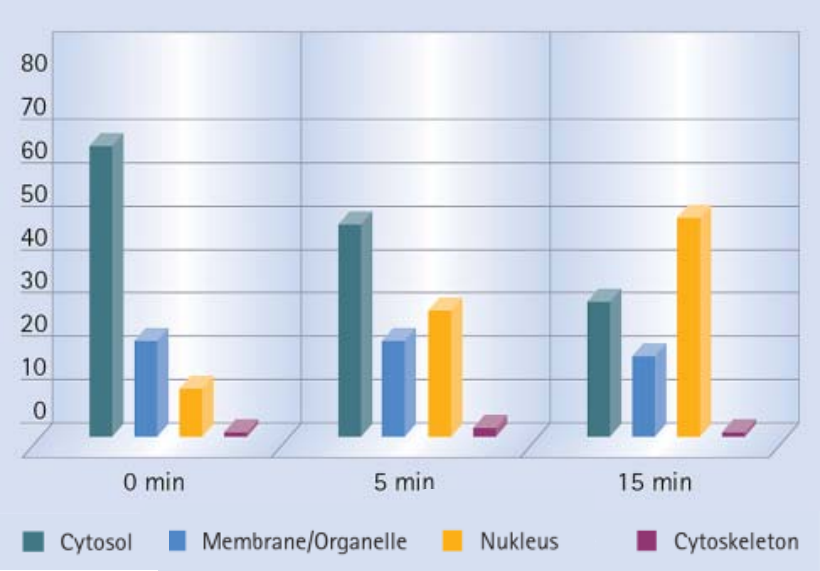

Fig.2: Analysis of protein distribution profiles to characterize cellular changes, exemplified by a time-course analysis of NFkappaB redistribution in stimulated cells.

A431 cells were stimulated with 0.2 µg/ml, and stepwise extraction of cytosolic fraction (F1), organelle/membrane fraction (F2) nucleic fraction (F3) and cytoskeleton proteins (F4) was performed using the subcellular described extraction procedure. Cell fractions were submitted to SDS-PAGE and separated proteins blotted onto nitrocellulose membrane. The membrane was probed with anti-NFkappaB. The time-course analysis demonstrates a measurable translocation of NFkappaB from the cytoplasm to the nucleus as early as 5 min and a stronger response at 15 min.

S-PEK was sucessuflly used with a wide variety of human tissue culture cells. The cells are grown in T25-culture flasks with about 1 x 10^6 cells/flask at approximately 80 % confluence. Please note that the actual number of cells at 80 % confluence of the monolayer may change considerably amongst different cell types. The buffer volumes given in the extraction protocols (chapter 7.) have been scaled up for the use of T25-culture flasks.

Table 1: Protein concentrations of particular fractions obtained by S-PEK extraction of adherent tissue culture cells.

Protein amounts were obtained after the extraction of 80% confluent cell monolayer of the respective cells in T25-flasks. Please use the values as guideline to estimate the amount of starting material needed to achieve a certain protein yield when using S-PEK extraction. Please note that different tissue culture cells may yield considerably different protein amounts.

Cell type (human)	Fraction	Protein concentration [mg/ml]	Protein amount [mg]	Proportion to total protein [mg]	Deviation [%]
Epithelial carcinoma A431	Cytosol Membrane/organelle Nucleus Cytoskeleton	0.55 0.75 0.2 0.15	0.55 0.75 0.1 0.075	33% 45% 13% 9%	< 10 < 10 < 10 < 5
Mamma carcinoma MCF7	Cytosol Membrane/organelle Nucleus Cytoskeleton	0.64 0.47 0.24 0.1	0.64 0.47 0.12 0.05	44% 32% 17% 7%	< 10 < 10 < 10 < 5
Liver carcinoma HEPG2	Cytosol Membrane/organelle Nucleus Cytoskeleton	3.1 1.8 0.6 0.35	3.1 1.8 0.3 0.175	53% 31% 10% 6%	< 10 < 10 < 10 < 5
Osteosarcom SAOS2	Cytosol Membrane/organelle Nucleus Cytoskeleton	0.44 0.5 0.2 0.1	0.44 0.5 0.1 0.05	36% 41% 17% 6%	< 10 < 10 < 10 < 5

& The values represented in table 1 are mean values of independent experiments. The grand total may differ from 100 % because of this.

Furthermore a protocol to use the S-PEK procedure with cells grown in suspension culture is provided. Finally a protocol than can be applied to forzen cell pellets ot to fragmented tissue (tissue clusters) previously dissected and isloated accodring to user specific protocols.

2. Kit Contents

- Wash Buffer: 1 x 100 ml
- Extraction Buffer I: 1 x 22 ml
- Extraction Buffer II: 1 x 22 ml
- Extraction Buffer III: 1 x 11 ml
- Extraction Buffer IV: 1 x 10 ml
- Protease Inhibitor Cocktail: 1 x 500 µl
- Benzonase®(≥ 250 U/µl) 1 x 45 µl

3. Storage Conditions

- Wash Buffer: Store at + 4°C.
- Extraction Buffers I -III: The Buffers can be stored at + 4 °C up to 6 month.
  For prolonged storage, freeze the buffers in convenient aliquots at - 20°C. Before extraction, buffers must be thawed at RT. A water bath at +25 °C will speed up the process. After thawing, mix components by gently shaking or vortexing. Avoid repeated freezing and thawing cycles
- Extraction Buffer IV: Buffer IV can be stored at room temperature.
- Protease Inhibitor Cocktail
  The Protease Inhibitor Cocktail is supplied in DMSO and can be stored at 4°C up to 6 month. For prolonged storage, freeze the cocktail in convenient aliquots at - 20°C. During the sample preparation procedure it must be kept at RT to prevent freezing of DMSO.
- Benzonase®: The enzyme should be stored at 4°C. During extraction keep on ice

4. Samples

The ProteoExtract™ Subcellular Proteome Extraction Kit is designed for mammalian samples:

- Adherent tissue culture cells
- Suspension grown tissue culture cells
- Frozen cell pellets
- Fragmented tissues

5. Reagents and Equipment Not Provided

- Platform mixer e.g. IKA Vibramax (when extracting adherent tissue culture cells)
- Cell culture equipment, media etc. for cell growth (e. g., RPMI, DMEM).
- Micropipettes and tips, 10 µl, 200 µl and 1 ml size (e.g., Eppendorf, Gilson or equivalent)
- Cooled centrifuge and rotor for 50 ml tube size (Eppendorf, Heraeus, Nalgen, etc.)
- Cooled micro centrifuge and rotor up to 10,000 x g for 2 ml tube size (e.g. Eppendorf)
- Thermo mixer or rolling facility (e.g., Eppendorf

6. Kit Components Needed for One Extraction

The buffer amounts required for one subcellular extraction depend on the amount of starting cell material is shown in Table 2a . Table 2b lists the sample to buffer ratio needed for commonly used cell culture sizes (flasks & dishes).
As guideline to estimate the amount of starting material please refer to Table 1 listing protein yields from selected cell types revealed after S-PEK extraction.

Table 2a: Buffer volumes necessary for one S-PEK extraction based on cell numbers

Cell type	Adherent tissue culture cells	Suspension grown cells/ frozen cell pellet	Fragmented tissue*
Cell amount	**3 - 5 x 10^6 cells**	**3 - 5 x 10^6 cells**	**25 - 50 mg**
Wash Buffer	2 x 2 ml	2 x 2 ml	2 x 2 ml
Extraction Buffer I	1 ml	1 ml	1 ml
Extraction Buffer II	1 ml	1 ml	1 ml
Extraction Buffer III	0.5 ml	0.5 ml	0.5 ml
Extraction Buffer IV	0.5 ml	0.5 ml	0.5 ml
Protease Inhibitor Cocktail	5 µl per fraction	5 µl per fraction	5 µl per fraction
Benzonase	1.5 µl	1.5 µl	1.5 µl

*** Tested for rat liver & bovine liver**

Table 2b: Buffer volumes necessary for one S-PEK extraction using confluent adherent cells

Cell type	Adherent tissue culture cells Flasks			Adherent tissue culture cells Petri dishes		
Size	**T_{25} flask**	**T_{75} flask**	**T_{175} flask**	**35 mm**	**60 mm**	**100 mm**
Wash Buffer	2 x 2 ml	2 x 5 ml	2 x 10 ml	2 x 0.8 ml	2 x 2 ml	2 x 6 ml
Extraction Buffer I	1 ml	3 ml	7 ml	0.4 ml	1 ml	3 ml
Extraction Buffer II	1 ml	3 ml	7 ml	0.4 ml	1 ml	3 ml
Extraction Buffer III	0.5 ml	1.5 ml	4 ml	0.2 ml	0.5 ml	1.5 ml
Extraction Buffer IV	0.5 ml	1.5 ml	4 ml	0.2 ml	0.5 ml	1.5 ml
Protease Inhibitor Cockta	5 µl per fraction	15 µl per fraction	30 µl per fraction	2 µl per fraction	5 µl per fraction	15 µl per fraction
Benzonase	1.5 µl	4.5 µl	10.5 µl	0.5 µl	1.5 µl	4.5 µl

7. Protocols

7.1. Subcellular extraction of adherent tissue culture cells

The S-PEK protocol for adherent tissue culture cells is scaled for the extraction of cell monolayer from T_{25}-culture flasks (3-5 x 10^6 cells). Please refer to Table 2 to calculate the sample to buffer ratio needed for your actual cell amount used.
For optimal adherence of cells vital cells that are in their logarhytmic growth phase at approximately 80 % confluence should be used. Nevertheless certain cell types might detach from the support during the extraction. In case the cells detach at some stage proceed with the respective step of the protocol 7.2. "Subcellular fractionation of suspension-grown tissue culture cells".

To monitor the extraction procedure morphological changes of the cells can be examined by phase contrast microscopy (compare Fig. 1A)

1. Before extraction mix buffers well by vortexing. During the extraction procedure keep Buffers I-III and Benzonase® on ice and Buffer IV and Protease Inhibitor Cocktail at room temperature. Make sure that buffers are thawed before starting the extraction.
2. Carefully remove the growth medium without disturbing the cell monolayer.
3. Wash cells by careful overlaying the cell monolayer with 2 ml ice cold Wash Buffer. Gentle agitate the cell culture flask(s) at +4 °C for 5 min (if cells detach, transfer the cell suspension in a appropriate centrifuge tube and continue with step 7.2.4)
4. Aspirate Wash Buffer completely without disturbing the cell monolayer.
5. Repeat washing steps 3 and 4 to remove contaminating media components. (If cells detach, transfer the cell suspension in an appropriate centrifuge tube and continue with step 7.2.5).
6. Mix 1 ml ice cold Extraction Buffer I and 5 µl Protease Inhibitor Cocktail. Immediately add the mixture into the flask without disturbing the monolayer. Carefully move flask until all cells are covered with buffer. Incubate for 10 min at +4 °C under gentle agitation (if cells detach, transfer the cell suspension after incubation in a appropriate centrifuge tube and continue with step 7.2.7).
7. Transfer the supernatant (**fraction 1**) into a sample tube using a pipette without disturbing the cell layer. Make sure that all liquid is removed. Keep the fraction 1 on ice.
8. Mix 1 ml ice cold Extraction buffer II and 5 µl Protease Inhibitor Cocktail. Immediately add the mixture into the flask without disturbing the monolayer. Carefully move flask to cover all cells with buffer. Incubate for 30 min at +4 °C under gentle agitation (if cells detach, transfer the cell suspension after incubation in a appropriate centrifuge tube and continue with step 7.2.10).
9. Transfer the supernatant (**fraction 2**) into a sample tube using a pipette without disturbing the cell layer. Make sure that all liquid is removed. Keep the fraction 2 on ice.

10. Mix 500 µl ice-cold Extraction buffer III with 5 µl Protease Inhibitor Cocktail and 1.5 µl (≥ 375 U) Benzonase®. Immediately add the mixture into the flask without disturbing the monolayer. Carefully move flask until all cells are covered with buffer. Incubate under gentle agitation for 10 min at 4°C. (when cells detach, transfer the cell suspension after incubation in a appropriate centrifuge tube and continue with step 7.2.13)
11. Transfer the supernatant (fraction 3) into a sample tube using a pipette. Be careful not to disturb the cell layer and to completely remove all the liquid. Place fraction III on ice.
12. Mix 500 µl room temperature Extraction Buffer IV and 5 µl Protease Inhibitor Cocktail. Add the mixture into the flask. Carefully move flask until all residual material is covered with buffer. The remaining cell structures will detach upon treatment with buffer 4. After complete solubilization of the residual materials, transfer extract (**fraction 4**) in a tube and store on ice. (See also Technical Appendix, point 4).

Note: If used the same day, place the extracts on cold for downstream applications and analysis. For long-term storage, prepare aliquots (e. g. 100 µl) and store at –20° C or colder until use.

For use in 1 or 2 DE refer to Chapter 8, Technical Appendix.

7.2. Suspension grown tissue culture cells

The protocol for subcellular fractionation of suspension grown tissue culture cells is scaled for the extraction of 3-5 x 10^6 cells. Please refer to Table 2 to calculate the sample to buffer ratio needed for the actual cell amount used.

1. Before extraction mix buffers well by vortexing. During the extraction procedure keep buffers I-III and Benzonase® on ice and Buffer IV and Protease Inhibitor Cocktail at room temperature. Make sure that buffers are thawed before starting the extraction.
2. Transfer the cell culture into an appropriate centrifuge tubes and sediment at 100-300 x g and 4° C for 10 min. Remove and discard supernatant by aspiration.
3. Wash the pellet with 2 ml ice cold Wash Buffer. Release the cell pellet by gently flicking the tube. Incubate for 5 min at 4°C under gentle agitation. A rotary shaker is recommended to avoid formation of cell clumps.
4. Sediment cells at 100-300 x g and +4 °C for 10 min. Carefully remove the supernatant without disturbing the pellet and discard.
5. Repeat washing steps 3 and 4.
6. Mix 1 ml ice cold Extraction Buffer I and 5 µl Protease Inhibitor Cocktail. Immediately add the mixture to the cell pellet. Resuspend the cell pellet by gently flicking the tube. Incubate for 10 min at +4 °C under gentle agitation. A rotary shaker is recommended to avoid formation of cell clumps.
7. Sediment insoluble material at 500-1000 x g and +4 °C for 10 min.

8. Transfer the supernatant (**fraction 1**) into a tube and keep it on ice.

9. Mix 1 ml ice cold Extraction Buffer II and 5 µl Protease Inhibitor Cocktail. Immediately add the mixture to the cell pellet. Resuspend the cell pellet by gently flicking the tube. Incubate for 30 min at +4 °C under gentle agitation. A rotary shaker is recommended to avoid formation of cell clumps.

10. Sediment insoluble material at 5000-6000 x g and +4 °C for 10 min.

11. Transfer the supernatant (**fraction 2**) into a tube and keep it on ice.

12. Mix 500 µl Extraction buffer III with 5 µl Inhibitor Cocktail and 1.5 µl (≥ 375 U) Benzonase®. Immediately add the mixture to the cell pellet by pipetting up and down. Incubate for 10 min at +4 °C under gentle agitation. A rotary shaker is recommended to avoid formation of cell clumps.

13. Sediment insoluble material at 6800 x g and +4 °C for 10 min.

14. Transfer the supernatant (**fraction 3**) into a tube and keep it on ice.

15. Mix 500 µl room temperature Extraction Buffer IV and 5 µl Protease Inhibitor Cocktail. Immediately add the mixture to the cell pellet. Carefully suspend residual particles by pipetting up and down (**fraction 4**).

Note: If used the same day, place the extracts on cold for downstream applications and analysis. For long-term storage, prepare aliquots (e. g. 100 µl) and store at –20° C or colder until use.

For use in 1 or 2 DE refer to Chapter 8, Technical Appendix.

7.3. Subcellular extraction of fragmented tissue and frozen cell pellet

The protocol applies to 25 - 50 mg of fresh & flash frozen fragmented tissue cells or 3-5 x 10^6 flash frozen cells. For preparation of fragmented tissue, please refer to the literature such as e.g. Reymond et al.
Important: For frozen cell pellets make sure that cells are washed with a suitable buffer before freezing. Use only flash frozen (liquid nitrogen) cell pellets or tissues.

1. Mix 1 ml ice cold Extraction Buffer I and 5 µl Protease Inhibitor Cocktail. Immediately add the mixture to the fragmented tissue/frozen cell pellet. Gently homogenize the fragmented tissue/frozen cell pellet by gently flicking the tube. Incubate for 10 min at +4 °C under gentle agitation. A rotary shaker is recommended to avoid formation of cell clumps.

2. Sediment insoluble material at 500-1000 x g and +4 °C for 10 min.

3. Carefully transfer the supernatant (**fraction 1**) into a fresh tube and keep it on ice.

4. Mix 1 ml ice cold Extraction Buffer II and 5 µl Protease Inhibitor Cocktail. Immediately add the mixture to the pellet. Resuspend the pellet by gently flicking the tube. Incubate for 30 min at +4 °C under gentle agitation. A rotary shaker is recommended to avoid formation of cell clumps.
5. Sediment insoluble material at 5000-6000 x g and +4 °C for 10 min.
6. Carefully transfer the supernatant (**fraction 2**) into a tube and keep it on ice.
7. Mix 500 µl Extraction Buffer III with 5 µl Inhibitor Cocktail and 1.5 µl (≥ 375 U) Benzonase®. Immediately add the mixture to the pellet. Resuspend the pellet by gently flicking the tube. Incubate for 10 min at +4 °C under gentle agitation. A rotary shaker is recommended to avoid formation of cell clumps.
8. Sediment insoluble material at 7000 x g and +4 °C for 10 min.
9. Carefully transfer the supernatant (**fraction 3**) into a tube and keep it on ice.
10. Mix 500 µl room temperature Extraction Buffer IV and 5 µl Protease Inhibitor Cocktail. Immediately add the mixture to the pellet and suspend the residual particles by pipetting up and down (**fraction 4**).

Note: Keep extracts on ice if used at the same day. For long-term storage, prepare convenient sized aliquots (e. g. 100 µl) and store at –20° C or colder.

For use in 1 or 2 DE refer to Chapter 8, Technical Appendix.

8. Technical Appendix

Problem	Answer
1. Upon centrifugation of suspension-grown cells, no compact pellet is formed.	It cannot be ruled out that certain types of cells do not form compact pellets at 100 x g. In these cases increase acceleration of the centrifuge to e.g. 300 x g.
2. Upon washing or extraction of adherent tissue culture cells, detachment of the cell layer from the support occurs.	When cells detach early while extracting adherent cell monolayer, transfer the resulting suspension into a micro centrifuge tube and continue with the respective step of the protocol given in chapter 7 for suspension-grown cells. This does normally not affect the quality of your result.
3. How do I determine the protein concentration of the subcellular protein extracts?	As the extraction buffers contain components that might interfere with protein quantification assays specific protein assays such as "Dc-Kit"; BioRad or "Non-interfering Protein Assay Kit"; Calbiochem, Cat. -No. 488250 are required to determine the protein concentration. Alternatively you may precipitate an aliquot of the extract prior quantification
4. When storing extraction buffer 4 or fraction 4 on ice, a precipitate occurs.	This does normally not affect the result of your experiment. In case of precipitation of buffer components from extraction buffer 4 or fraction 4 gently warm the sample to room temperature, mix well and use immediately for analysis.
5. How do I prepare subcellular fractions generated with S-PEK for one-dimensional SDS-PAGE?	S-PEK fractions can directly be analyzed by one-dimensional SDS-PAGE: Dilute the sample with an equal volume of 2 x SDS-PAGE sample buffer (not provided: e. g. 125 mM Tris/HCl, pH 6.8; 10 % (w/v) SDS; 30 % (v/v) Glycerol; 100 mM DTT; 0.002 % (w/v) bromophenol blue) and heat to + 95 °C for 5 min.
6. How can I concentrate S-PEK fractions?	In case the protein concentration of fractions generated by S-PEK is not sufficient for your purpose, we recommend to use the Protein Precipitation Kit (Cat. No. 539180) to concentrate the proteins. Dissolve pellet in a buffer suitable for your further downstream applications.
7. How do I prepare S-PEK fractions for two-dimensional SDS-PAGE?	When protein concentrations are high, S-PEK fractions **1,2 and 3** might be used directly for 2 DGE. Samples must be diluted 1: 4 with common loading buffer for IEF (not provided, e.g. 5 M Urea, 2 M Thiourea, 4 % CHAPS, Ampholytes, 100 mM DTT) and incubated for 60 min at room temperature prior to loading on IEF. Fraction **4** must include a clean-up step (e.g. as described in 6.), prior to IEF. For improved results in 2DGE we strongly recommend precipitation (clean-up) of all four S-PEK fractions using the Protein Precipitation Kit (Cat. No. 539180) prior to IEF.

9. Literature

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10. Application References

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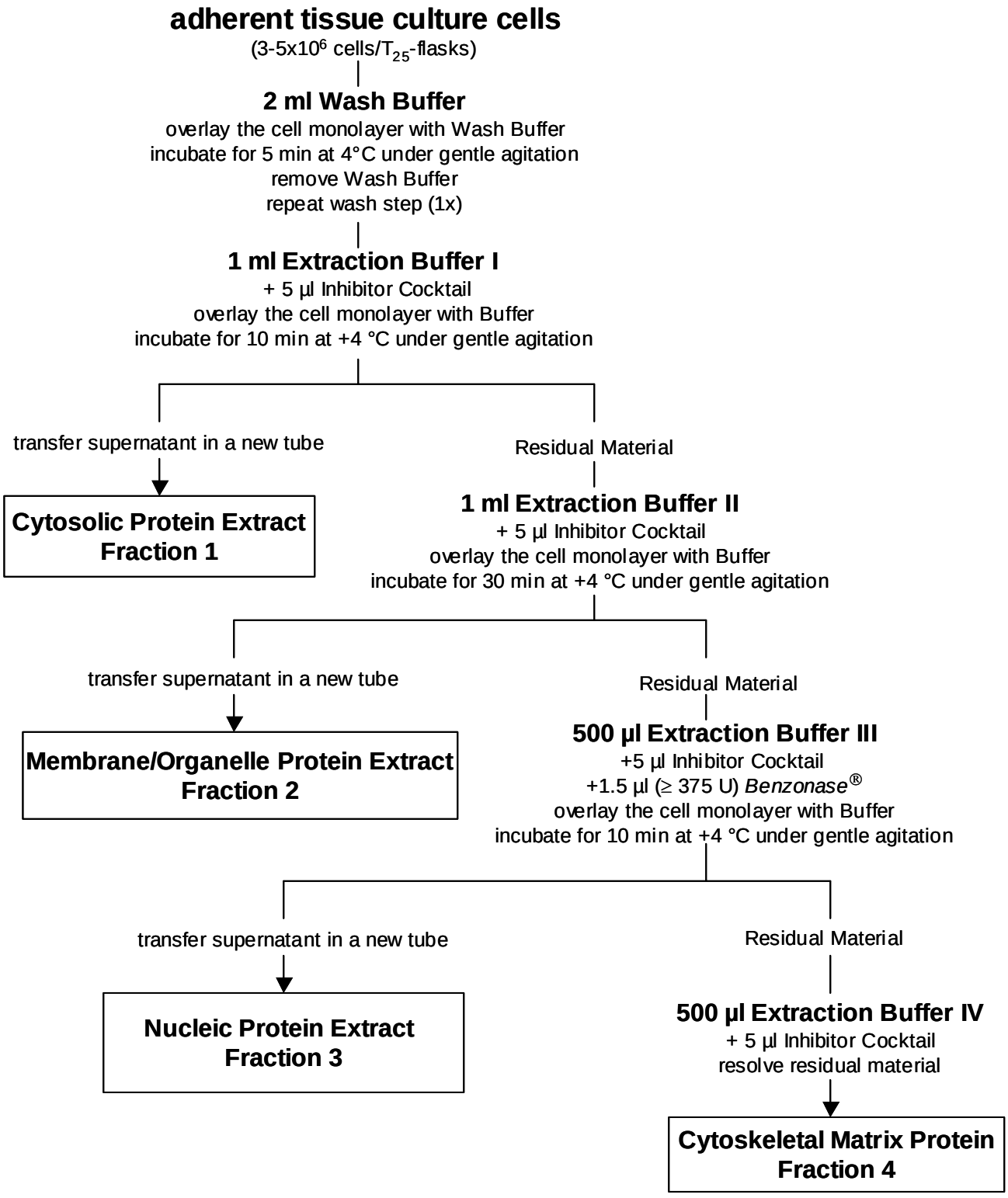
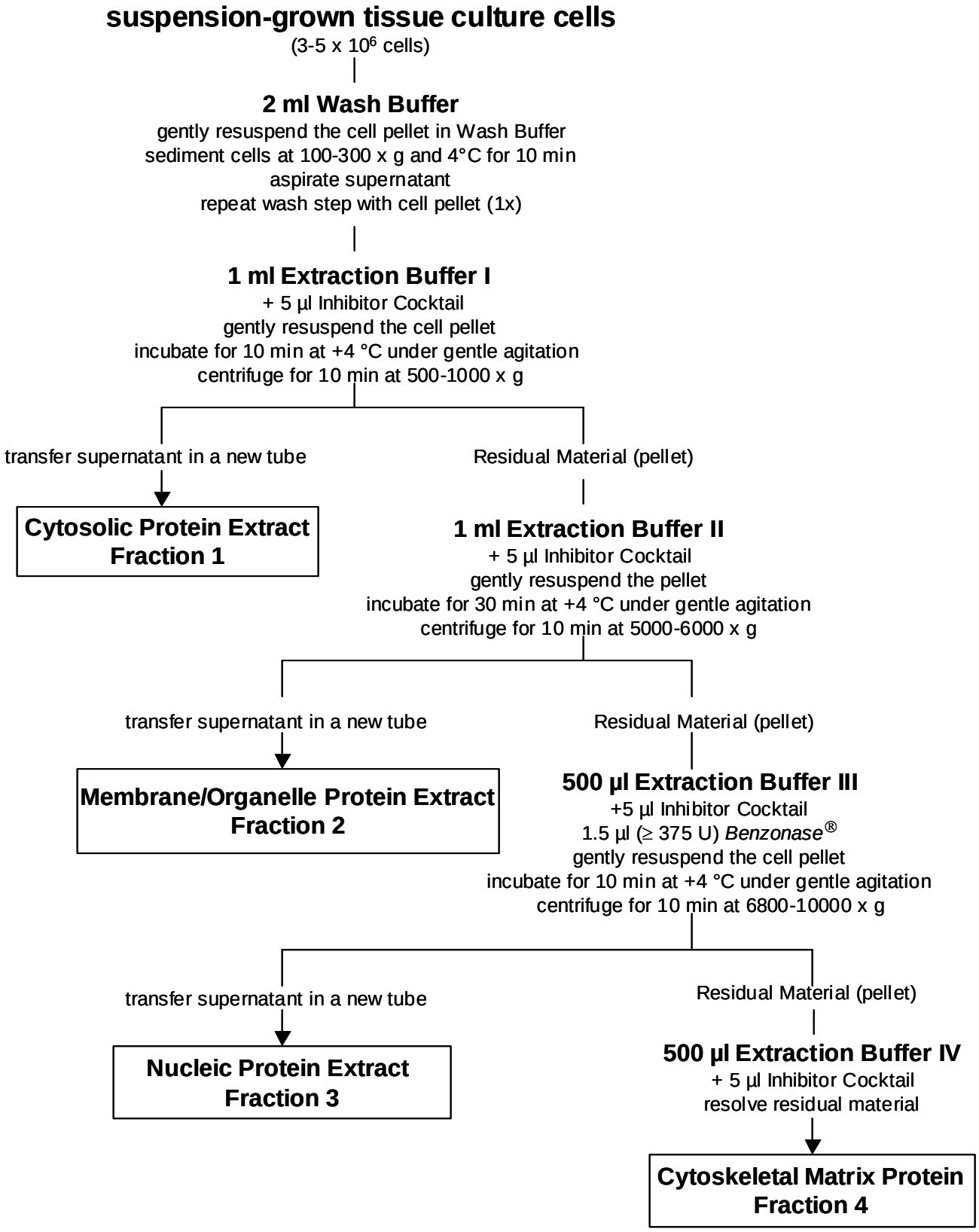
10. Flow Charts For Subcellular Extraction with S-PEK





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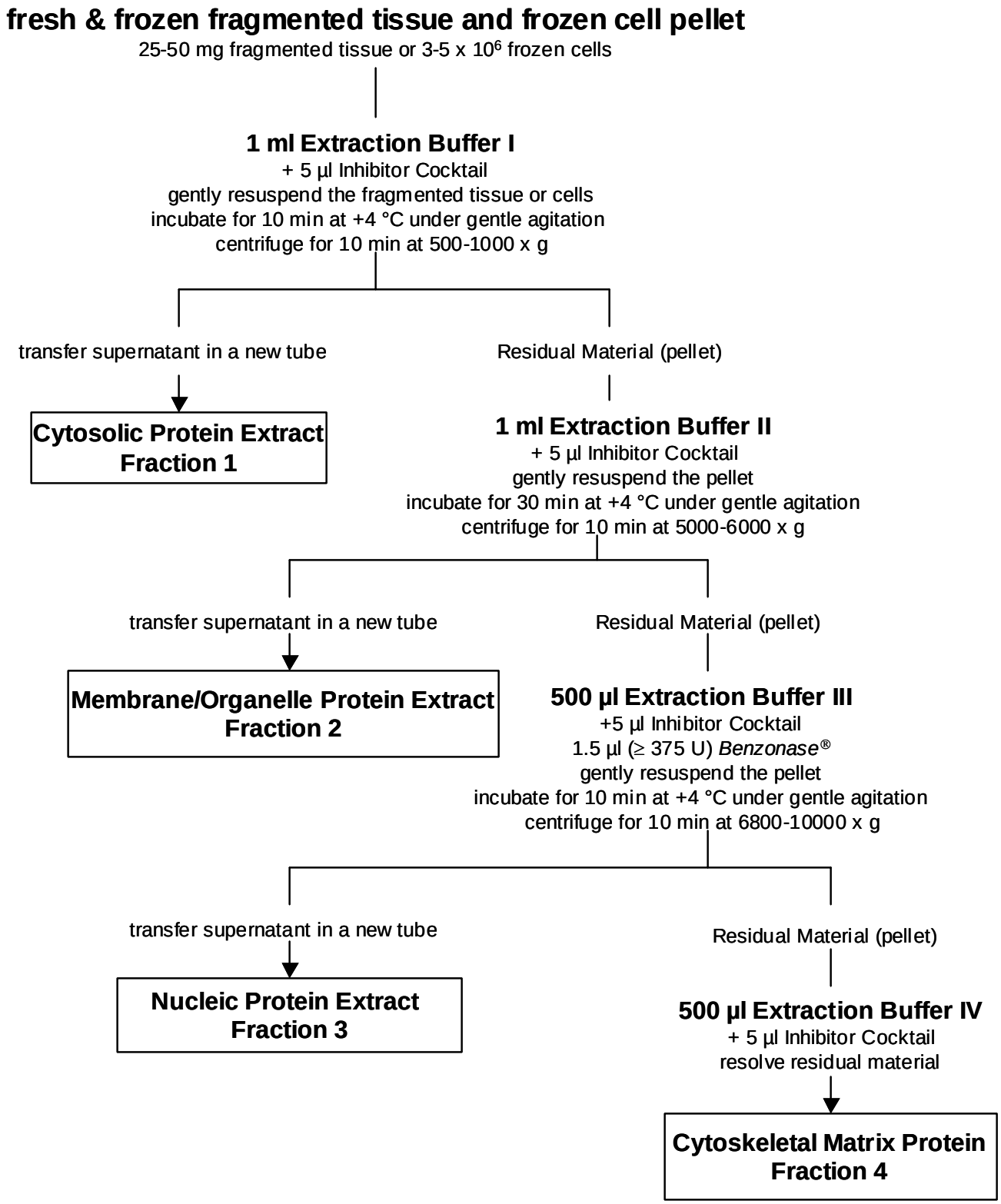

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