CALBIOCHEM®

Partial Mammalian Proteome Extraction Kit

Cat. No. 539789

Product Information

FOR RESEARCH USE ONLY. NOT FOR HUMAN OR DRUG USE. MSDS AVAILABLE UPON REQUEST.

Germany Tel 0800 6931 000

USA & Canada Tel (800) 628-8470

United Kingdom Tel 0115 9430 840

email address for technical inquiries: technical@calbiochem.com

An Affiliate of Merck KGaA, Darmstadt, Germany

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1. Introduction

Proteome analysis implies the ability to separate proteins with high resolution and reproducibility prior to characterization by mass spectrometry, microsequencing or equivalent means. Currently, two-dimensional-electrophoresis (2DE) remains the highest resolution technique for protein separation when complex samples need to be arrayed for analysis.

The sample preparation of any protein mixture for subsequent 2DE is of major importance, as it will affect the overall performance of the technique. In general, pretreatment of samples involves solubilization, denaturation and reduction of proteins in the presence of special reagents. The prerequisites for an efficient sample preparation method are reproducible solubilization of all types of proteins, prevention of protein degradation as well as a thorough removal of contaminating nucleic acids e.g. by enzymatic digestion. Due to their very specific characteristics, some proteins may be well denatured and solubilized by a given detergent or chaotrope, whereas other proteins will require another reagent. Consequently, the future of solubilization is to find mixtures of detergents and chaotropes able to cope with the diversity of proteins encountered in the complex samples to be separated by 2DE. Additionally each biological sample requires special procedures e.g. for cell disruption. For this reason, we have developed the Mammalian Partial Proteome Extraction Kit (P-PEK) as a tool to extract proteins of variable solubility from both tissue culture cells and dissected mammalian tissues in the presence of different detergents and chaotropes.

P-PEK is designed for serial sample preparation of complex protein mixtures using reagent mixtures with increasing solubilization strength. Each of the provided Extraction Reagents solubilizes a different subset of cellular proteins. The most soluble proteins of mammalian cells are released by mechanical disruption of the cells in Extraction Reagent 1 (Fraction 1). Proteins of intermediate solubility are subsequently extracted with Extraction Reagent 2 (Fraction 2). For solubilization of proteins otherwise insoluble in Extraction Reagent 2 the kit provides Extraction Reagent 3 with a special formulation for efficient membrane protein extraction (Fraction 3). The resulting fractions of proteins subsequently extracted with Extraction Reagent 1, 2 and 3 can be directly analyzed on separate 2DE-gels leading to an increased number of spots to be visualized in total. Finally the proteins still insoluble in Extraction Reagents 3 can be solubilized using the provided SDS-Buffer that can be analyzed by one-dimensional SDS-PAGE (Fraction 4). A schematic representation of P-PEK as well as corresponding 2D-gels using human *Hep G2* tissue culture cells are shown in Figure 1.

To get optimal resolution and reproducibility in 2DE-protein patterns, the kit contains reagents composed of ultrapure chemicals. In order to preserve the protein profile, a ready-to-use Protease Inhibitor Cocktail is added. To reduce sample viscosity and increase spot resolution, Benzonase®, a proprietary non-specific nuclease, is included for efficient nucleic acid degradation. Reduction of extracted proteins is performed with DTT. Despite that DTT is negatively charged at alkaline pH, it was observed to be superior over TBP in the P-PEK procedure. Furthermore, besides being more difficult to handle due to spontaneous decomposition, TBP was found to be unstable in concentrated urea solutions as used in sample preparation for 2DE [1].

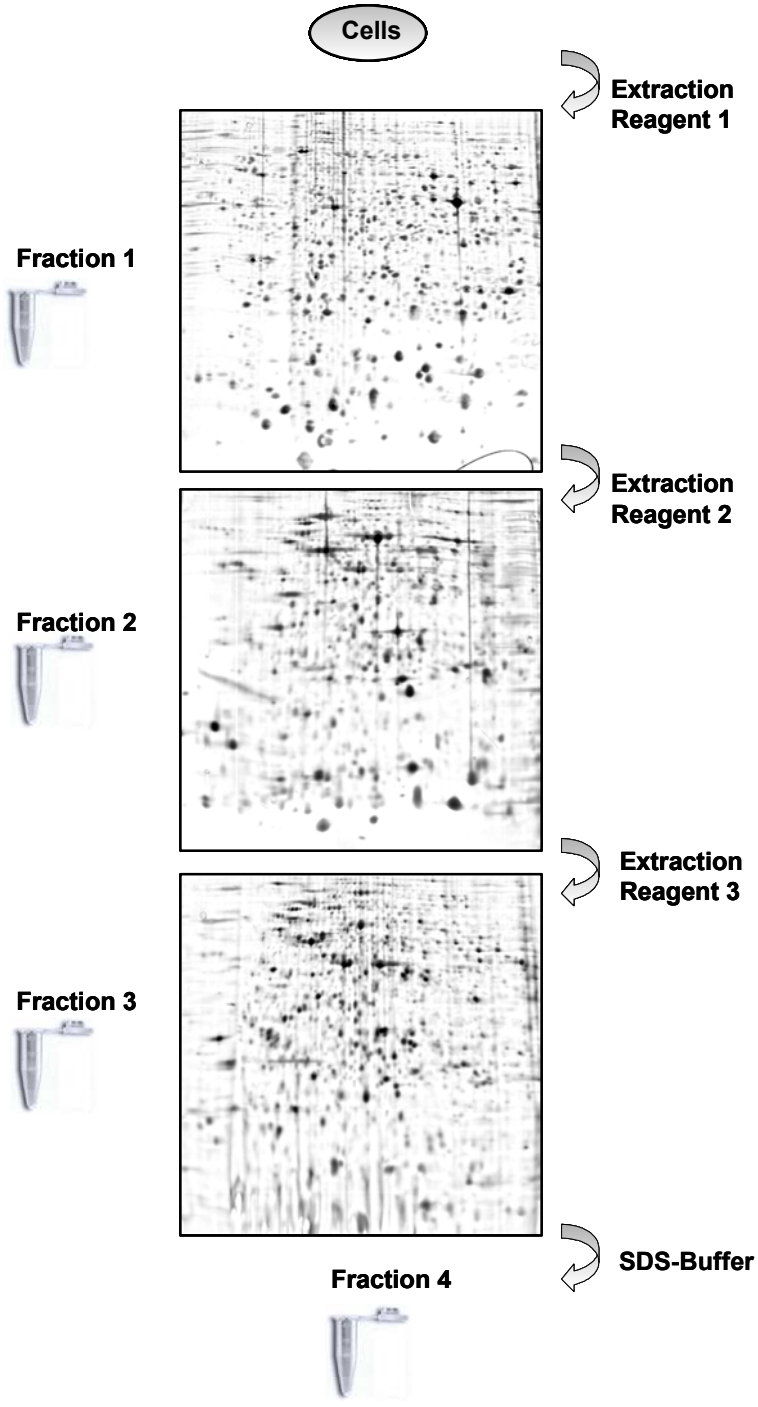

Figure 1: Schematic view of the Partial Mammalian Proteome Extraction Procedure (P-PEK). Sample preparation from human Hep G2 tissue culture cells was performed as described in the manual. 200 µg protein extract of fractions 1 to 3 were separated by 2DE. First dimension was done by isoelectric focusing in immobilized pH gradient gels (pI 4-7). The second dimension was carried out by SDS-PAGE in 12% polyacrylamide gels. The Proteins were visualized by silver staining.

Prior to sequential extraction, lysis conditions are very important for the success of the sample preparation and they depend strongly on the cell type used. To allow for an efficient release of easily soluble proteins, tissue culture cells as well as mammalian tissues are sufficiently disrupted by homogenization in Extraction Reagent 1 using a combination of a glass homogenizer and the application of shearing force by passing the cell suspension through a narrow needle. Special extraction procedures have been developed and are described below.

Independent of the sample preparation method chosen, it is most important to minimize protein modifications that might result in artefactual spots on 2DE-maps. Reagents and samples contain urea. For this reason, **heating over +30 °C must be avoided** as this may introduce considerable charge heterogeneity due to carbamoylation of the proteins by isocyanate formed from the decomposition of urea. Due to the importance of temperature control during sample preparation, the P-PEK-protocols for mammalian samples work without any sonication step to avoid impairment of the poor temperature control when sonicating small volumes. Use the information given in table 1 as a guideline to score for the success of your extraction procedure.

Table 1: Typical protein amounts in fractions obtained by the P-PEK procedure for selected tissue culture cells and tissues.

Material	Fraction	Protein concentration [mg/ml][$]
***Hep G2* Liver Carcinoma Cells**[#]	Extraction Reagent 1 Extraction Reagent 2 Extraction Reagent 3 SDS-Buffer	4.6 3.7 0.7 2.4
Rat Liver[#]	Extraction Reagent 1 Extraction Reagent 2 Extraction Reagent 3 SDS-Buffer	45 10.5 3.8 11
Rat Brain[#]	Extraction Reagent 1 Extraction Reagent 2 Extraction Reagent 3 SDS-Buffer	12.5 6 4 4

[$] Protein concentrations are rounded mean values from independent experiments.
[#] Please note that different types of cells may yield considerably different amounts of protein per unit.

2. Kit Components (for 20 reactions)*

* Please note that the Extraction Reagents 2 and 3 must be used to dilute the protein extract in case analytical gels are performed. This may decrease the total number of experiments that can be performed with the reagents provided. The components of the kit require different storage conditions. Please refer to chapter 3 for more information.

- **Wash Buffer**: 2 vials
  - 100 ml / vial
  - Supplied ready-to-use
- **Extraction Reagent 1** (Imidazole/Sucrose): 2 vials
  - 110 ml / vial
  - Supplied ready-to-use
- **Extraction Reagent 2** (Urea/Detergent/DTT): 1 vial
  - 40 ml / vial
  - Supplied as dry powder
  - **To be reconstituted by the addition of 24 ml high-quality water**
- **Extraction Reagent 3 A** (Urea/Thiourea/Sulfobetain/Non-ionic Detergent/DTT): 1 vial
  - 15 ml / vial
  - Supplied as dry powder
  - **To be reconstituted by the addition of 9 ml of the content from Extraction Reagent 3 B**
- **Extraction Reagent 3 B**: 1 vial
  - 10 ml / vial
  - Supplied ready for reconstitution of Extraction Reagent 3 A
- **SDS-Buffer A**: 1 vial
  - 0.04 g / vial
  - Supplied as dry powder
  - **To be reconstituted by the addition of 5 ml of the content of SDS-Buffer B**
- **SDS-Buffer B**: 1 vial:
  - 6 ml / vial
  - Supplied ready for reconstitution of SDS-Buffer A
- **Protease Inhibitor Cocktail**: 1 vial
  - 0.45 ml / vial
  - Supplied ready-to-use
- **Benzonase®**: 1 vial
  - ≥ 250 U / µl
  - 45 µl / vial
- **Glass Beads**: 1 vial
  - 19 gr / vial

12 items

3. Storage Conditions for Reagents

- **Wash Buffer**
  The Wash Buffer is provided ready-to-use. Upon arrival, the buffer should be stored at +4 °C.
- **Extraction Reagent 1**
  Extraction Reagent 1 is provided ready-to-use. Upon arrival, the Extraction Reagent 1 must be stored at +4 °C. After the first use, store frozen at –20 °C in aliquots of convenient volume to avoid repeated freezing and thawing (see Chapter 5).
- **Extraction Reagent 2 and 3 Vial A**
  Extraction Reagent 2 and 3 Vial A are supplied as dry powder and should be stored unopened at +4 °C. These reagents should be reconstituted just prior to use as described below. After reconstitution, the reagents must be stored frozen at –20 °C, preferably in aliquots of convenient volume to avoid repeated freezing and thawing (see Chapter 5).
- **SDS-Buffer A**
  SDS-Buffer A is supplied as dry powder and should be stored unopened at +4 °C. The reagent should be reconstituted just prior to use as described below. After reconstitution, the reagent must be stored frozen at –20 °C, preferably in aliquots of convenient volume to avoid repeated freezing and thawing (see Chapter 5).
- **Extraction Reagent 3 Vial B and SDS-Buffer Vial B**
  Extraction reagent 3 Vial B and SDS-Buffer Vial B are provided ready-to-use. Upon arrival, the solutions must be stored at +4 °C.
- **Protease Inhibitor Cocktail:**
  The Protease Inhibitor Cocktail is supplied in DMSO at ambient temperature. The vial must be transferred to –20 °C immediately upon arrival for storage. During the sample preparation procedure the vial must be kept at RT to prevent freezing of DMSO. It is not required the store the Protease Inhibitor Cocktail in aliquots.
- **Benzonase®**
  Benzonase® (≥ 250 U/µl) should be transferred to –20 °C immediately upon arrival.

4. Preparation Instructions for Reagents

Extraction Reagent 1 (Two Vials)

Aliquot the unused material in volumes of convenient size and store frozen at –20 °C until further use (see chapter 5). Thaw the frozen aliquots prior to at room temperature. The use of a water bath at +25 °C will facilitate thawing of the Extraction Reagent. Discard unused portions of each aliquot.

Extraction Reagent 2 (One Vial)

Add 24 ml of high quality water to the contents of the vial and mix by gently swirling the vial. The solution will become cold to the touch and needs to be warmed to +25 °C for complete solubilization. **Do not allow the temperature of the material to raise above 30°C** since the urea inside may begin to form cyanates that will carbamoylate proteins during ongoing sample preparation. Aliquot the unused material in volumes of convenient size and store frozen at –20 °C until further use (see chapter 5). Thaw the frozen aliquots prior to use and discard unused portions of each aliquot.

Extraction Reagent 3 (Two Vials, Vial A and B)

Add 9 ml of the content of vial B to the contents of vial A and mix by gently swirling the vial. The solution will become cold to the touch and needs to be warmed to +25 °C for complete solubilization. **Discard Extraction Reagent Vial B with the rest of its content after reconstitution of Extraction Reagent Vial A.**
Do not allow the temperature of the material to raise above 30°C since the urea inside may begin to form cyanates that will carbamoylate proteins during ongoing sample preparation. Aliquot the unused material in volumes of convenient size (see Chapter 5) and store frozen at –20 °C until further use. Thaw the frozen aliquots prior to use and discard unused portions of each aliquot.

SDS-Buffer (Two Vials, Vial A and B)

Add 5 ml of the content of vial B to the contents of vial A and mix by gently swirling the vial. **Discard SDS-Buffer Vial B with the rest of its content after reconstitution of SDS-Buffer Vial A.** Aliquot the unused material in volumes of convenient size and store frozen at –20 °C until further use (see chapter 5). Thaw the frozen aliquots prior to at room temperature. The use of a water bath at +25 °C will facilitate thawing of the SDS-Buffer. Discard unused portions of each aliquot.

Protease Inhibitor Cocktail (One Vial)

The Protease Inhibitor Cocktail is supplied in DMSO and has to be stored at -20°C. Prior to use the solution is thawed at RT. During sample preparation keep the cocktail at RT and not on ice since +4 °C is sufficient to freeze DMSO again. After usage store again at – 20 °C.

Benzonase® (One Vial)

Benzonase® (≥ 250 U/µl) is supplied in glycerol and has to be stored at – 20°C until use.

5. Reagents Needed for One Partial Proteome Extraction

For one extraction the following volumes of reagents are needed:

- 4 - 8 ml Wash Buffer (depending on the sample)
- 5 - 10 ml Extraction Reagent 1 (depending on the sample)
- 1000 µl Extraction Reagent 2
- 500 µl Extraction Reagent 3 (reconstituted from both vials A and B)
- 200 µl SDS-Buffer (reconstituted from both vials A and B)
- 20 µl Protease Inhibitor Cocktail
- 1.6 µl (≥ 400 Units) Benzonase®
- 0.5 ml Glass Beads (optional)

Supplied reagents are sufficient for 20 sample preparations.

6. Reagents and Equipment not provided

- High purity water, conductivity < 5 µS (e.g., Merck)
- 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 microcentrifuge and rotor up to 25,000 x g for 2 ml tube size (Eppendorf, Heraeus, Nalgen etc.)
- Thermomixer or rolling facility (e.g., Eppendorf)
- *Optional:* Ultracentrifuge and rotor up to 75,000 x g for 2 ml tube size (e.g. Beckman, Sorvall etc.); UV-Photometer
- *For tissue extraction*: Mortar and pestle made from porcelain, small spatula. These instruments must be cooled to –70 °C or colder prior to be used in the P-PEK procedure! Precooled glass homogenizer.

7. Extraction from tissue culture cells

Please use the following protocol for the extraction of proteins from tissue culture cells. Instructions for preparation of instruments and reagents are given in chapters 4 and 6 of this manual and should be read carefully prior to protein extraction. Guidelines for sample handling, determination of protein concentration and preparation of the extracts for IPG-2DE are described in the **Technical Appendix** below.

Special procedures for preparation of washed cell pellets from both adherent and suspension-grown tissue culture cells are described below.
Follow protocol A1 for adherent cells
Follow protocol A2 for suspension grown cells.

If your starting sample is an already prepared frozen cell pellet you can directly proceed with step 1.1 of the protocol described below.

Abbreviations used: RT: Room temperature

Part A: Tissue culture cell pellet preparation

A1 Preparation of a cell pellet from adherent tissue culture cells

- Grow cells in appropriate media until 1 - 2 x 10^8 of sub-confluent cells are reached.
- Aspirate the growth medium without disturbing the cell monolayer. Be careful to remove the entire medium from the cell monolayer.
- Add Wash Buffer (1 ml/75 cm^2 flask) to the cell monolayer to remove residual medium components. Wash cell monolayer by gently agitating the culture flask for 5 times. Aspirate the Wash Buffer completely without disturbing the cell layer.
- Carefully suspend the attached cells in fresh Wash Buffer (1 ml/75 cm^2 flask) using a rubber policeman.
- Transfer the cell suspension into a 2 ml-microcentrifuge tube and pellet cells at 150 x g for 10 min at +4 °C.
- Discard the supernatant. Be careful to remove all liquid from the cell pellet after centrifugation.
- Freeze the washed cell pellet at –20 °C or colder for 10 min. The cell pellet may be stored under these conditions.
- Continue with step 1.1 of the protocol for tissue culture cell extraction

A2 Preparation of a cell pellet from suspension-grown tissue culture cells

- Grow cells in appropriate media until 1 - 2 x 10^8 cells in logarithmic growth phase (viability by e.g. trypan blue exclusion should not be below 90%) are reached.
- Isolate the cells by centrifugation at 150 x g for 10 min at +4 °C.
- Carefully aspirate the medium and resuspend cells in Wash Buffer (2 ml/10^8 cells).
- Isolate the cells by centrifugation at 150 x g for 10 min at 4°C

- Aspirate the supernatant without disturbing the cell pellet. Discard the supernatant.
- Resuspend the cell pellet in Wash Buffer (2 ml/10^8 cells) by pipetting up and down with a 1 ml tip.
- Transfer the cell suspension into a 2 ml-microcentrifuge tube and isolate cells by centrifugation at 150 x g for 10 min at +4 °C.
- Discard the supernatant. Be careful to remove all liquid from the cell pellet after centrifugation.
- Freeze the washed cell pellet at –20 °C or colder for 10 min. The cell pellet may be stored under these conditions.
- Continue with step 1.1 of the protocol for tissue culture cell extraction

Part B: **Protein Extraction from tissue culture cells**

1. Tissue culture cell disruption and extraction of most soluble proteins with Extraction Reagent 1

1.1 To the frozen cell pellet, add first 15 µl Protease Inhibitor Cocktail and then 1000 µl of ice-cold Extraction Reagent 1. Vortex thoroughly for 1 min to resuspend the cells.

1.2 Transfer cell suspension into a precooled glass homogenizer. Homogenize material by a minimum of 10 strokes on ice until a homogeneous suspension without visible cell clumps is produced.

1.3 Pass the suspension produced in 1.2 through a G22-needle for 10 times. Transfer suspension into a precooled microcentrifuge tube.

1.4 Add 0.8 µl (200 U) Benzonase® and mix gently by inverting the tube 5 times.

1.5 Incubate cell suspension at +4 °C for 15 min under gentle agitation. If available the use of a thermomixer at 1000 rpm and +4 °C for 15 min would be advantageous.

1.6 Clarify the sample by centrifugation at 20.000 x g and +4 °C for 30 min. If available, the use of an ultracentrifuge for 30 min at 75.000 x g and +4 °C would be advantageous.

1.7 Transfer the supernatant completely to a fresh microcentrifuge tube and recover as **fraction 1**.

1.8 To wash away remaining proteins soluble in Extraction Reagent 1, add to the pellet 1000 µl of fresh ice-cold Extraction Reagent 1. Transfer pellet in a glass homogenizer. And homogenize material by a minimum of 10 strokes on ice until a homogeneous suspension is produced.

1.9 Centrifuge at 20,000 x g and +4 °C for 15 min. If available, the use of an ultracentrifuge for 15 min at 75,000 x g and +4 °C would be advantageous.

1.10 Save the supernatant as wash fraction without disturbing the pellet. Be careful to remove all the liquid from the disrupted cell pellet before proceeding.

1.11 Repeat steps 1.8 to 1.10 four times. Combine supernatants as wash fraction with the previously saved wash fraction. *Optional:* Measure the absorption at 280 nm in the wash fractions. You may continue the washing procedure for more than four times to ensure that in your cell sample of interest no significant changes in absorption can be observed anymore in between washing steps.

2. Extraction of tissue culture cell proteins of intermediate solubility with Extraction Reagent 2

2.1 To the washed pellet prepared according to 1.11 add in the order indicated: 5 µl Inhibitor Cocktail, 500 µl room temperature Extraction Reagent 2 and 0.8 µl (200 U) Benzonase®.

2.2 Resuspend the pellet by pipetting up and down with a 1 ml pipette tip for a minimum of 10 times until the pellet is completely resuspended.
2.3 Incubate at RT for 15 min under gentle agitation to achieve maximum solubilization of proteins. If available the use of a thermomixer at 1000 rpm and RT for 15 min would be advantageous.
2.4 Clarify the sample by centrifugation at 20,000 x g and +10 °C for 10 min. If available the use of an ultracentrifuge at 75,000 x g for 30 min at +10 °C would be advantageous.
2.5 Transfer the supernatant completely to a fresh microcentrifuge tube and recover as **fraction 2**. Be careful to remove all the liquid from the pellet before proceeding.

3. Extraction of tissue culture cell proteins of low solubility with Extraction Reagent 3

3.1 To the pellet add 500 µl of room temperature Extraction Reagent 3.
3.2 Resuspend pellet by pipetting up and down with a 1 ml pipette tip for a minimum of 10 times until the pellet is completely resuspended.
3.3 Incubate at RT for 15 min under gentle agitation. If available the use of a thermomixer at 1000 rpm and RT for 15 min would be advantageous.
3.4 Clarify the sample by centrifugation at 20,000 x g for 10 min at +10 °C. If available the use of an ultracentrifuge at 75,000 x g for 30 min at +10 °C would be advantageous.
3.5 Transfer the supernatant completely to a fresh microcentrifuge tube and recover as **fraction 3**. Be careful to remove all the liquid from the pellet before proceeding.

4. Extraction of otherwise insoluble tissue culture cell proteins with SDS-Buffer

4.1 The final pellet is resuspended in 200 µl SDS-Buffer by thorough vortexing and pipetting up and down with a 200 µl pipette tip for a minimum of 10 times until the pellet is completely resuspended.
4.2 Heat the sample to +95 °C for 5 min.
4.3 Quickly cool sample to RT on ice. Avoid precipitation of SDS by cooling to +4 °C.
4.4 Clarify the sample by centrifugation at 20,000 x g for 30 min at +10 °C.
4.5 Transfer the supernatant completely to a fresh microcentrifuge tube and recover as **fraction 4**.

8. Extraction from mammalian tissues

Please use the following protocol for the extraction of proteins from tissues. Instructions for preparation of instruments and reagents are given in chapters 4 and 6 of this manual and should be read carefully prior to protein extraction. Guidelines for sample handling, determination of protein concentration and preparation of the extracts for IPG-2DE are described in the **Technical Appendix** below.

Abbreviations used: RT: Room temperature

Part A: Tissue preparation

- For cooling down, a mortar, a pestle and a spatula are placed into a styrofoam box that contains liquid nitrogen or dry ice. The mortar and pestle should be manufactured from agate or from glass. Glass was found to be more stable in liquid nitrogen.
- Mince tissue to pieces of approximately 2 mm in size directly after dissection and freeze tissue pieces at –70 °C or colder. The tissue may be stored under these conditions.
- Place frozen tissue in the cooled mortar and grind with the pestle to powder. Care should be taken that small pieces of the material do not jump out of the mortar when starting to break up the hard, frozen material.
- Transfer 500 mg of the tissue powder into a cold 2 ml microcentrifuge tube by using the cooled spatula.
- Compress the powder collected by knocking on the tube. This sample can be immediately subjected to extraction or stored at -20°C or colder

Part B: Protein Extraction from tissue samples

1. Tissue disruption and extraction of most soluble proteins with Extraction Reagent 1

1.1 To the frozen tissue powder add first 10 µl Protease Inhibitor Cocktail and then 2000 µl of ice-cold Extraction Reagent 1.
1.2 Vortex thoroughly for 1 min to resuspend the tissue.
1.3 Transfer tissue suspension into a precooled glass homogenizer. Homogenize material by a minimum of 10 strokes on ice until a homogeneous suspension without visible tissue pieces is produced. *Important:* If with your tissue sample no homogeneous suspension can be obtained, please refer to step 11 of the **Technical Appendix** below.
1.4 Pass the suspension produced in 1.3 through a G22-needle for 10 times. Transfer suspension into a precooled microcentrifuge tube.
1.5 Add 0.8 µl (200 U) Benzonase® and mix gently by inverting the tube 5 times.
1.6 Incubate tissue suspension at +4 °C for 15 min under gentle agitation. If available the use of a thermomixer at 1000 rpm and +4 °C for 15 min would be advantageous.
1.7 Clarify the sample by centrifugation at 20,000 x g and +4 °C for 30 min. If available, the use of an ultracentrifuge for 30 min at 75,000 x g and +4 °C would be advantageous.
1.8 Transfer the supernatant completely to a fresh microcentrifuge tube and recover as **fraction 1**.

1.9 To wash away remaining proteins soluble in Extraction Reagent 1, add to the pellet 2000 µl of fresh ice-cold Extraction Reagent 1 and resuspend pellet in a glass homogenizer by a minimum of 10 strokes on ice until a homogeneous suspension is produced.

1.10 Centrifuge at 20,000 x g and +4 °C for 15 min. If available, the use of an ultracentrifuge for 15 min at 75,000 x g and +4 °C would be advantageous.

1.11 Save the supernatant as wash fraction without disturbing the pellet. Be careful to remove all the liquid from the disrupted cell pellet before proceeding.

1.12 Repeat steps 1.9 to 1.11 four times. Combine supernatants as wash fraction with the previously saved wash fraction. *Optional:* Measure the absorption at 280 nm in the wash fractions. You may continue the washing procedure for more than four times to ensure that in your tissue sample of interest no significant changes in absorption can be observed anymore in between washing steps.

2. Extraction of tissue proteins of intermediate solubility with Extraction Reagent 2

2.1 To the washed pellet prepared according to 1.12 add in the order indicated 10 µl Inhibitor Cocktail, 1000 µl room temperature Extraction Reagent 2 and 0.8 µl (200 U) Benzonase®.

2.2 Resuspend the pellet by pipetting up and down for a minimum of 30 times until a homogeneous suspension is produced.

2.3 Incubate the cell suspension at RT for 15 min under gentle agitation. If available the use of a thermomixer at 1000 rpm and +25 °C for 15 min would be advantageous.

2.4 Clarify the sample by centrifugation at 20,000 x g for 30 min at +10 °C. If available, the use of an ultracentrifuge at 75,000 x g for 15 min at +10 °C would be advantageous.

2.5 Transfer the supernatant completely to a fresh microcentrifuge tube and recover as **fraction 2**.

3. Extraction of tissue proteins of low solubility with Extraction Reagent 3

3.1 To the pellet add 500 µl of either room temperature Extraction Reagent 3.

3.2 The pellet is resuspended by pipetting up and down for a minimum of 30 times until a homogeneous suspension is produced.

3.3 Incubate cell suspension at RT for 15 min under gentle agitation. If available the use of a thermomixer at 1000 rpm and +25 °C for 15 min would be advantageous.

3.4 Clarify the sample by centrifugation at 20,000 x g for 30 min at +10 °C. If available, the use of an ultracentrifuge at 75,000 x g for 15 min at +10 °C would be advantageous.

3.5 Transfer the supernatant completely to a fresh microcentrifuge tube and recover as **fraction 3**. Be careful to remove all the liquid from the pellet before proceeding.

4. Extraction of otherwise insoluble tissue proteins with SDS-Buffer

4.1 The final pellet is resuspended in 200 µl SDS-Buffer by thorough vortexing and pipetting up and down with a 200 µl pipette tip for a minimum of 20 times until a homogeneous suspension is produced.

4.2 Heat the sample to +95 °C for 5 min.

4.3 Quickly cool sample to RT on ice.

4.4 Clarify the sample by centrifugation at 20,000 x g for 30 min at +10 °C.

4.5 Transfer the supernatant completely to a fresh microcentrifuge tube and recover as **fraction 4**.

9. Technical Appendix

Important: Please understand the following information as guidelines for specific technical questions encountered when preparing cell extracts generated with the P-PEK procedures for subsequent one or two-dimensional gel electrophoresis. For technical problems regarding the gel electrophoresis process please refer to the instruction manual of your electrophoresis equipment.

Question	Answer
1. How do I handle Reagents and fractions during protein extraction with P-PEK?	**Do not warm reagents and samples above 30 °C to avoid protein carbamoylation by urea.** Do not cool Extraction Reagents 2 and 3 as well as corresponding fractions below +10 °C since otherwise components may precipitate. Generally, samples should be subjected to as minimum handling as possible and kept cold at all times. For a longer time, storage in a freezer at -20°C or colder is mandatory. Avoid repeated freezing and thawing of the samples. It is recommended to freeze in aliquots and thaw only once. ***Important*:** During sample preparation precipitation of reagent or extract components may occur at low ambient temperatures or when the temperature of the sample is not properly controlled. This does normally not affect the quality of your result. If precipitation of reagent or extract components occurs, warm up the sample immediately to +25 °C under gentle agitation until content is completely dissolved.
2. How do I prepare the fractions generated with P-PEK for one-dimensional SDS-PAGE?	To analyze the cell extracts by 1D-SDS-PAGE, dilute the desired portion 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 boil samples at +95 °C for 5 min prior to loading on SDS-PAGE. ***Important*:** Please note that for of fraction 2 and 3 the boiling step must be omitted when subsequent protein identification by means of mass spectrometry or equivalent is desired (see above). In this case, incubate samples for 60 min at RT before loading onto SDS-PAGE.
3. How do I load equal amounts of protein from fractions prepared with P-PEK on IPG-strips for comparative experiments?	You may generate fractions from equal starting numbers of cells and load equal portions of the fractions on IPG-strips. This requires a precise counting of cells and precise liquid handling during sample preparation. Errors in cell counting or liquid handling will cause misleading results. We recommend determination of the protein concentration in the fractions (see below) prior to 2DE.

4. How much protein should I load for 2DE using IPG strips?	100 to 200 µg protein is suitable for analytical gels (17-18 cm size) when silver or fluorescent dye staining is used for protein visualization. For preparative gels and Coomassie Blue staining, 1-3 mg protein can be loaded (17-18 cm gel size). If other gel sizes will be used, please refer to the instruction manual of your equipment to adjust the amount of material to your problem.
5. How do I measure the protein concentration in the fractions?	Due to the detergents, reducing agents and chaotropes in **fractions 2 and 3** a protein assay including a precipitation step to remove interfering substances (e.g. "Rc Dc-Kit"; BioRad or "Non-interfering Protein Assay Kit"; Calbiochem, Cat.-No 488250) is required to determine the protein concentration. Please follow information given by the manufacturers. Alternatively you may precipitate a desired portion of the extract using TCA/Acetone (Yuan *et al.*, 2002) and dissolve the protein pellet in a suitable reagent for your protein assay of choice. ***Important*:** Do not dilute fractions 2 and 3 with water prior to protein assay is not recommended since it may lead to precipitation of proteins that need high chaotrope and detergent concentrations for solubility.
6. How can I concentrate fractions in case the protein concentration is not sufficient for my purpose?	In case the protein concentration of a fraction generated by P-PEK is too low for your needs as e.g. preparative loading on IPG-strips, we recommend to precipitate the desired amount of protein using TCA/Acetone (Yuan *et al.*, 2002) and to dissolve the pellet directly in a volume of the extraction reagent suitable for rehydration of the IPG-strip of choice. For fractions 1 and 2, Extraction Reagent 2 should be used, for fraction 3, Extraction Reagent 3 should be used to dissolve the precipitate. Incubation in a sonication bath may be required to dissolve the pellet. Please be sure to check for proper temperature control (see above).
7. Storage and preparation of **fraction 1** obtained by P-PEK for IPG-2DE	***Important*:** Fraction 1 is prepared in a hypotonic buffer and requires the addition of detergents and chaotropes for 2DE. The fraction should be kept on ice until usage for 2DE. It is recommended to use fraction 1 the same day as prepared. For longer storage store at -20 °C or colder. However, due to the low ionic strength of the buffer and the high protein concentration, freezing may lead to precipitation of some proteins. If longer storage is necessary, it is recommended to prepare fraction 1 for 2DE by the addition of Extraction Reagent 2 prior to freezing. Fraction 1 needs to be diluted with Extraction Reagent 2 in a ratio of

at least 1:4 prior to be loaded on IPG-strips to provide appropriate detergents and chaotropes required for 2DE. To ensure proper reduction and denaturation of proteins prior to 2DE, incubate diluted sample for 60 min at RT before loading.
***Important*:** We recommend the addition of carrier ampholytes in a concentration between 0.2 and 0.5 % (w/v). Table 2 below will give a guideline for preparation of fractions for IPG using reswelling of IPG strips with the protein solution.

The wash fraction must be treated equal to fraction 1 in case analysis is desired.

8. Storage and preparation of **fraction 2** for IPG-2DE

Fraction 2 can be kept at +10 °C or RT until usage for 2DE the same day. Do not cool below +10 °C to avoid precipitation of reagent contents. **Do not warm above 30 °C** (see above). For longer storage freezing at -20 °C or colder is recommended.

Fraction 2 is ready to be loaded on IPG strips without further preparations. Please refer to the manufacturers instructions for your 2DE equipment used for sample loading procedures.

Fraction 2 may need to be diluted with Extraction Reagent 2 to allow for equal protein loads.
***Important*:** We recommend the addition of carrier ampholytes in a concentration between 0.2 and 0.5 % (w/v). Table 2 below will give a guideline for preparation of fractions for IPG using reswelling of IPG strips with the protein solution.

9. Storage and preparation of **fraction 3** for IPG-2DE

Fraction 3 should be kept at +10 °C or RT until usage for 2DE the same day. Do not cool below +10 °C to avoid precipitation of reagent contents. **Do not warm above 30 °C** (see above). For longer storage freezing at -20 °C or colder is recommended.

Fraction 3 is ready to be loaded on IPG strips without further preparations. Please refer to the manufacturers instructions for your 2DE equipment used for sample loading procedures.

Fraction 3 may need to be diluted with Extraction Reagent 3 to allow for equal protein loads.
***Important*:** We recommend the addition of carrier ampholytes in a concentration between 0.2 and 0.5 % (w/v). Table 2 below will give a guideline for preparation of fractions for IPG using reswelling of IPG strips with the protein solution.

10. Can I analyze **fraction 4** obtained with SDS-Buffer on IPG-2DE?

Fraction 4 should be kept at +10 °C or RT until usage for 2DE the same day. For longer storage freezing at -20 °C or colder is recommended.

***Important*:** SDS-Buffer contains SDS that is able to disrupt most non-covalent protein interactions but precludes its inclusion in IEF gels because of its anionic character. We recommend analysis of fraction 4 by one-dimensional SDS-PAGE (see above).

However, fraction 4 may be diluted 1:10 with Extraction Reagent 2 prior to loading on IPG-2DE to quench the effect of SDS.

Alternatively the desired amount of protein from fraction 4 can be precipitated using TCA/Acetone (Yuan *et al.*, 2002). A minimum of two washing steps with the precipitating agent should be performed before dissolving the pellet. Dissolve the pellet directly in a volume of the extraction reagent suitable for rehydration of the IPG-strip of choice. In this case use Extraction Reagent 3 for dissolving the precipitated pellet.

11. I cannot produce a homogeneous tissue suspension in step 1.3 of the protocol for mammalian tissues. How can I improve the result?

Some tissues may be especially hard to disrupt. If you experience your tissue not being sufficiently homogenized by the given protocol, add 1 ml glass beads to the suspension produced in step 1.3 of the given protocol. Please note that you must use a larger container than mentioned in the protocol due to the additional volume of the glass beads. Vortex thoroughly for 1 min, then incubate 1 min on ice to avoid overheating of the sample. Repeat vortexing-cooling cycle for four more times. Finally allow glass beads to settle for 1 min and aspirate tissue suspension. Be careful to not transfer any glass beads since the glass beads will be harmful to your glass homogenizer. Continue with step 2.4 of the protocol.

Table 2:Scheme for preparation of fractions obtained by P-PEK for IPG-2DE. The following example is calculated for 400 µl sample solution for isoelectric focusing, suitable to reswell a 17-18 cm-IPG strip or equivalent size. We recommend to reswell the IPG-strip directly in the extract containing the desired protein amount.

		Notes
Sample (e.g. 200 µg protein)	x µl	-
Extraction Reagent	Fill volume to 400 µl	-
Carrier Ampholytes*	Max 20 µl	A concentration of 0.2-0.5 % (w/v) carrier ampholytes is recommended
400 - 420 µl		

Serva Ampholytes (Servalyt®) are recommended.

Example: If your protein concentration is 2.5 mg/ml in fraction 1 obtained with P-PEK and you wish to run an analytical gel with 200 µg of protein load and 0.5 % (w/v) carrier ampholytes, the following pipetting scheme must be applied:

Sample (2.5 mg/ml)	80 µl
Extraction Reagent 2	320 µl
Carrier Ampholytes (Serva ampholytes (Servalyt®) are recommended)	5 µl stock solution (40 % w/v)
	405 µl

10. Literature

[1] Amersham Pharmacia Biotech web discussion list: http://www.apbiotech.com/discussion/ postings.asp?subjectid=1&areaid=3

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