CALBIOCHEM®

Complete Mammalian Proteome Extraction Kit

Cat. No. 539779

Product Information

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1. Introduction

Two-dimensional-electrophoresis (2DE) remains the highest resolution technique for protein separation when complex samples need to be arrayed for characterization, as in proteomics. Pretreatment of samples for isoelectric focusing (IEF) followed by polyacrylamide gel electrophoresis (SDS-PAGE) involves solubilization, denaturation and reduction to completely break any interactions between the proteins and to remove non-protein sample components. Ideally, to avoid protein losses, one would achieve complete sample solubilization in a single step and thus eliminate unnecessary handling. The prerequisite of a successful proteome analysis is a standardized and reproducible sample preparation procedure for the biological sample of choice.
Having this in mind, we have developed the Mammalian Complete ProteoExtract Kit (C-PEK) that allows sample preparation from both tissue culture cells and tissues in two steps in a microcentrifuge tube minimizing protein loss. Extraction from tissue culture cells and tissues with C-PEK results in a total protein sample solubilized in the presence of ultrapure chemicals that is ready for two-dimensional separation with high reproducibility. The complete cell extract is ideally suited for overview gels and for fast screening of the influence of experimental parameters on protein expression or modification by means of e.g. 2DE-Western-Blotting or autoradiography of cell extracts. A representative 2D gel of proteins extracted from a tissue culture cell line is shown in figure 1.

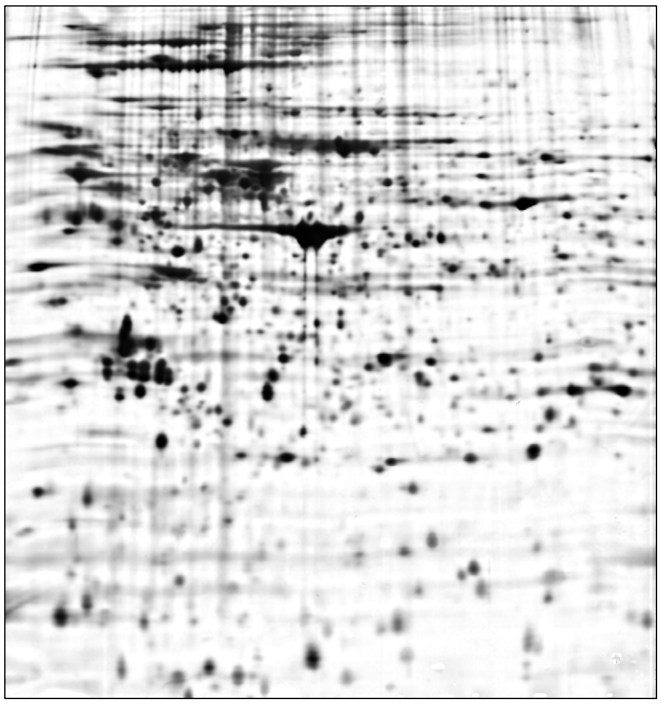

Figure 1: 2DE-Gel with 250 µg Protein from *HepG2* liver carcinoma cells. Cells were collected by centrifugation and sample preparation was performed. 200 µg proteins of the whole cell extract were separated by 2DE. First dimension was done by isoelectric focusing from pH 4 to pH 7 in an immobilized pH gradient gel by application of 50.000 Vh. The second dimension separation was by SDS-PAGE in a 12 % polyacrylamid gel. Proteins were visualized by silver staining.

C-PEK is designed to extract virtually all proteins from a given tissue culture cell or mammalian tissue. It includes a hypotonic buffer for cell resuspension and lysis and a denaturing extraction reagent for the solubilization of proteins. The Extraction Reagent contains compounds that have previously been used for extraction as also new reagents with increasing solubilization power that improve

solubilization of proteins prior to 2DE resulting in an increased total number of spots that can to be visualized on 2DE-gels. For degradation of nucleic acids, Benzonase®, a proprietary protease-free non-specific nuclease, is included, which leads to a clarified, non-viscous protein solution showing higher resolution in 2DE-gels. 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 complete proteome extraction procedure. Furthermore, besides being 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]. The addition of a protease inhibitor cocktail during cell extraction was found to be unnecessary as the overall protease activity was sufficiently inhibited by the Extraction Reagent in tested cases.

For cell lysis and protein extraction, cells or tissues are frozen and thawed by resuspension in the Resuspension Buffer. Subsequently, proteins are extracted with the Extraction Reagent. The lysis conditions are very important for the success of the extraction procedure and strongly depend on the cell type. While tissue culture cells are satisfactorily disrupted by the combination of freezing and thawing in a hypotonic buffer, tissues require a more vigorous disruption using a bead mill for efficient disruption and homogenization. In order to minimize laborious optimization work, special extraction procedures for tissue culture cells and for tissues have been developed using the Mammalian Complete ProteoExtract Kit and are described below. 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 C-PEK procedure for selected tissue culture cells and tissues.

Material	Protein concentration [mg/ml][$]
***Baby Hamster Kidney* Cells**[#]	10
***Hep G2* Liver Carcinoma Cells**[#]	8
Rat Liver[*]	20
Rat Brain[*]	10

[$] Protein concentrations are rounded mean values from independent experiments.

[#] Results were obtained using 1.5 x 10^8 cells. Please note that different types of cells and different cell numbers may yield considerably different amounts of protein per unit.

[*] Please note that different tissues or tissues from different organisms may yield considerably different amounts of protein per unit.

During protein extraction and sample preparation, it is very 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. The use of a hypotonic buffer for cell resuspension renders cell disruption by sonication unnecessary thus avoiding any impairment of the poor temperature control during this procedure with the result.

2. Kit Components (for 20 reactions)*

* Please note that the Extraction Reagent 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.

- **Wash Buffer:** 2 vials
  - 100 ml / vial
  - Supplied ready-to-use

- **Resuspension Buffer** (Imidazole/Sucrose): 1 vial
  - 3 ml / vial
  - Supplied ready-to-use

- **Extraction Reagent** (Urea/Thiourea/Detergent): 1 vial
  - 25 ml / vial
  - Supplied as dry powder**, to be reconstituted by the addition of 11 ml high quality water**

- **Reducing Agent:** (DTT): 1 vial
  - 2.27 ml / vial
  - Supplied as dry powder, **to be reconstituted by the addition of 2 ml high quality water**

- **Benzonase®**: 1 vial

- **Glass Beads (0.4 - 0.6 mm ϕ)**: 1 vial

6 items

3. Storage Conditions for Reagents

- **Wash Buffer**
  The Wash Buffer is provided ready-to-use. Upon arrival, the buffer should be transferred to +4 °C for storage.

- **Resuspension Buffer:**
  The Resuspension Buffer is provided ready-to-use. Upon arrival, the buffer should be stored at -20 °C. After the first use store preferably in aliquots of convenient volume (see Chapter 5) to avoid repeated freezing and thawing.

- **Extraction Reagent:**
  The Extraction Reagent is supplied as dry powder and can be stored unopened at +4 °C. The Extraction Reagent should be reconstituted just prior to use as described below. After reconstitution, the Extraction Reagent must be stored frozen at –20 °C, preferably in aliquots of convenient volume (see Chapter 5) to avoid repeated freezing and thawing.

- **Reducing Agent:**
  The reducing agent DTT is supplied as dry powder. Upon arrival, the vial should be transferred to +4 °C for storage. The reducing agent should be reconstituted just prior to use as described below. After reconstitution, aliquot the unused material in 60 µl volumes and freeze at –20 °C for further use. Avoid repeated freezing and thawing.

- **Benzonase®:**
  Benzonase® (≥ 250 U/µl) is delivered at ambient temperature, but should be transferred to –20 °C upon arrival.

4. Preparation Instructions for Reagents

Reducing Agent (One Vial)

Add 2 ml of high quality water to the contents of the vial and mix by vortexing. Swirl the vial gently until the content is completely dissolved. The reconstituted reagent consists of 2.27 ml 1 M DTT.

Extraction Reagent (One Vial)

Add 11 ml of high quality water to the content of the vial and mix by vortexing. The solution will become cold to the touch and needs to be warmed to 25°C for complete solubilization. During sample preparation the Extraction Reagent must be kept at RT to prevent precipitation of chaotropes and detergents. **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. Store at –20 °C for further use. It is recommended to aliquot the Extraction Reagent in desired volumes to avoid repeated freezing and thawing.

5. Kit Components Needed for One Extraction

- 4 - 8 ml Wash Buffer (depending on the sample)
- 50 µl Reducing Agent
- 150 µl Resuspension Buffer
- 800 µl Extraction Reagent
- 1.5 µl (≥ 375 U) Benzonase®
- 0.5 ml Glass Beads (in case of tissue extraction)

Supplied reagents are sufficient for 20 extractions.

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.)
- *For tissue extraction*: Mortar and pestle made from porslin, small spatula. These instruments must be cooled to –70 °C or colder prior to be used in the C-PEK procedure!

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 1 for adherent cells
Follow protocol 2 for suspension grown cells.

Abbreviations used: RT: Room temperature

1. Preparation of cell pellet from adherent tissue culture cells

1.1 Grow cells in appropriate media until 1 - 1.5 x 10^8 of sub-confluent cells is reached.
1.2 Aspirate the growth medium without disturbing the cell monolayer. Be careful to remove the entire medium from the cell monolayer.
1.3 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.
1.4 Carefully suspend the attached cells in fresh Wash Buffer (1 ml/75 cm^2 flask) using a rubber policeman.
1.5 Transfer the cell suspension into a 2 ml-microcentrifuge tube and pellet cells at 150 x g for 10 min at +4 °C.
1.6 Discard the supernatant. Be careful to remove all liquid from the cell pellet after centrifugation.
1.7 Freeze the washed cell pellet at –20 °C or colder for 10 min. The cell pellet may be stored under these conditions.
1.8 Continue with step 3.1 of the protocol

2. Preparation of cell pellet from suspension-grown tissue culture cells

2.1 Grow cells in appropriate media until 1 - 1.5 x 10^8 cells in logarithmic growth phase (viability by e.g. trypan blue exclusion should not be below 90%) are reached.
2.2 Isolate the cells by centrifugation at 150 x g for 10 min at +4°C.
2.3 Carefully aspirate the medium and resuspend cells in Wash Buffer (2 ml/10^8 cells).
2.4 Isolate the cells by centrifugation at 150 x g for 10 min at +4°C
2.5 Aspirate the supernatant without disturbing the cell pellet. Discard the supernatant.
2.6 Resuspend the cell pellet in Wash Buffer (2 ml/10^8 cells) by pipetting up and down with a 1 ml tip.
2.7 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.

2.8 Discard the supernatant. Be careful to remove all liquid from the cell pellet after centrifugation.
2.9 Freeze the washed cell pellet at –20 °C or colder for 10 min. The cell pellet may be stored under these conditions.
2.10 Continue with step 3.1 of the protocol

3. Protein extraction from the tissue culture cell pellet

3.1 Add 150 µl ice-cold Resuspension Buffer to the frozen cell pellet and allow cells to thaw on ice.
3.2 Resuspend cells by pipetting up and down with a 200 µl tip. An appropriate solution is obtained after pipetting 15 times.
3.3 Add 800 µl of room temperature Extraction Reagent and 50 µl Reducing Agent. Mix gently by inverting the tube 5 times. The suspension will become very viscous.
3.4 Add 1.5 µl (≥ 375 U) Benzonase®. Pipetting up and down 20 times with a 1 ml tip solubilizes the proteins. Upon pipetting, viscosity of the suspension will be reduced.
3.5 Incubate suspension at RT for 30 min with gentle agitation for maximum solubilization of proteins. If available, the use of a thermomixer would be advantageous at +25 °C and 1000 rpm for 30 min.
3.6 Pellet remaining insoluble components for 30 min at 25,000 x g and +10 °C. If available, the use of an ultracentrifuge for 30 min at 75,000 x g and +10 °C would be advantageous.
3.7 Transfer supernatant in a fresh microcentrifuge tube and recover as cell extract.

8. Extraction from tissues

Please use the following protocol for the extraction of proteins from tissue. 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

1. Preparation of tissue pellet

1.1 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 achat or from glass. Glass was found to be more stable in liquid nitrogen.
1.2 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.
1.3 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.
1.4 Transfer 50-100 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 may be stored at -20°C or colder.

2. Protein extraction from the tissue

2.1 Add 150 µl ice-cold Resuspension Buffer to the frozen tissue powder and allow to thaw on ice. Vortex thoroughly for 1 min to suspend the tissue pieces completely.
2.2 Add 800 µl room temperature Extraction Reagent and 50 µl Reducing Agent in that order.
2.3 Add 0.5 ml glass balls to the suspension.
2.4 Vortex vigorously for 1 min. Incubate 1 min on ice to avoid overheating of the sample.
2.5 Repeat step 2.4 for four times to achieve optimal tissue disruption and cell lysis.
2.6 Incubate for 1 min on ice to allow glass beads to settle at the bottom of the tube. Carefully aspirate the supernatant from the glass beads and transfer into a fresh microcentrifuge tube. Do not transfer glass beads.
2.7 Add 1.5 µl (375 U) Benzonase® and mix gently by inverting the tube 5 times.
2.8 For maximum solubilization of proteins, incubate at RT for 1 h under gentle agitation. If available, the use of a thermomixer may be advantageous at +25 °C and 1,000 rpm for 1 h.
2.9 Insoluble components are sedimented by centrifugation for 30 min at 25,000 x g and +10 °C. Ultracentrifugation for 30 min at 75,000 x g and +10 °C would be advantageous.
2.10 Transfer supernatant in a fresh microcentrifuge tube and recover as cell extract.

9. Technical Appendix

Important: Please understand the following information as guideline for specific technical questions encountered when preparing cell extracts generated with the C-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 samples during protein extraction with C-PEK?	**Do not warm reagents and samples above +30 °C to avoid protein carbamoylation by urea.** Do not cool extraction reagents and samples 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 components may sometimes 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 and sample components occurs, please warm the sample immediately to +25 °C under gentle agitation until content is completely dissolved.
2. How do I prepare cell extracts generated with C-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 the boiling step must be omitted in case 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 cell extracts prepared with C-PEK on IPG-strips for comparative experiments?	You may generate extracts from equal starting numbers of cells and load equal volumes of extract solutions on IPG-strips. Please note that 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 to determine the protein content in the extract solution (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 an analytical gel (17-18-18 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 (17-18-18 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 cell extracts?

Due to the detergents, reducing agents and chaotropes in the samples 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 of extract solutions. Please use the information given by the manufacturers for further guidelines.
Alternatively you may try to 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*:** Dilution of extracts 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 do I prepare cell extracts obtained by C-PEK for IPG-2DE

The extracts are 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.
The cell extracts may need to be diluted with the Extraction Reagent used for the sample preparation. We recommend the addition of low concentrations of carrier ampholytes by the user in order to help prevent the precipitation of the protein of interest during the isoelectric focusing step.
We recommend to add carrier ampholytes in a concentration between 0.2 and 0.5 % (w/v). Table 2 will give a guideline for preparation of cell extracts for IPG using reswelling of IPG strips with the protein solution.
***Important*:** Please note that the Extraction Reagent provided is a 1.25 x formulation and needs to be both supplemented with Reducing Agent from the provided stock solution and diluted to 1 x with water before being used for sample dilution:

800 µl 1.25 x Extraction Reagent
\+ 50 µl Reducing Agent
\+ 150 µl high quality water
= 1000 µl 1 x reagent for sample dilution

	Important: Despite the fact that extract solutions are of low conductivity, it cannot completely be ruled out that in some cases certain samples introduce higher levels of conducting substances or other contaminants that interfere with the application of high voltages during IEF when preparative sample loads are being applied. If this is experienced repeatedly with your sample of choice, we recommend to precipitate the sample using TCA/Acetone (Yuan et al., 2002) and to resolubilize the proteins in the appropriate volume of 1 x Extraction Reagent for sample dilution (see above box).
7. What conditions must I apply during first dimension isoelectric focusing	For information on focusing conditions please refer to the instruction manual of your instrument provider. Focusing conditions are dependent on the equipment used and of both the presence/absence of ampholytes in the sample and the pI range of the used IEF gel. The instrument suppliers provide helpful guidelines for choosing focusing conditions that can be applied to most samples without modifications.

Table 2:Scheme for preparation of cell extracts obtained by C-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	-
1 x Extraction Reagent for sample dilution (as prepared according to point 6 in **Technical Appendix** above)	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		

***** We recommed to use Serva ampholytes (Servalyt®)

***Example*:** If your protein concentration is 10 mg/ml in the cell extract obtained with C-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 (10 mg/ml)	20 µl
1 x Extraction Reagent for sample dilution (as prepared according to point 6 in **Technical Appendix** above)	380 µl
Carrier Ampholytes (Servalyt®)	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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