

Complete Bacterial Proteome Extraction Kit

Cat. No. 539770

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 Complete Bacterial Proteome Extraction Kit (C-PEK) that allows sample preparation from both gram-negative and gram-positive bacterial cells in two steps in a microcentrifuge tube minimizing protein loss. 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 *E. coli* is shown in figure 1.

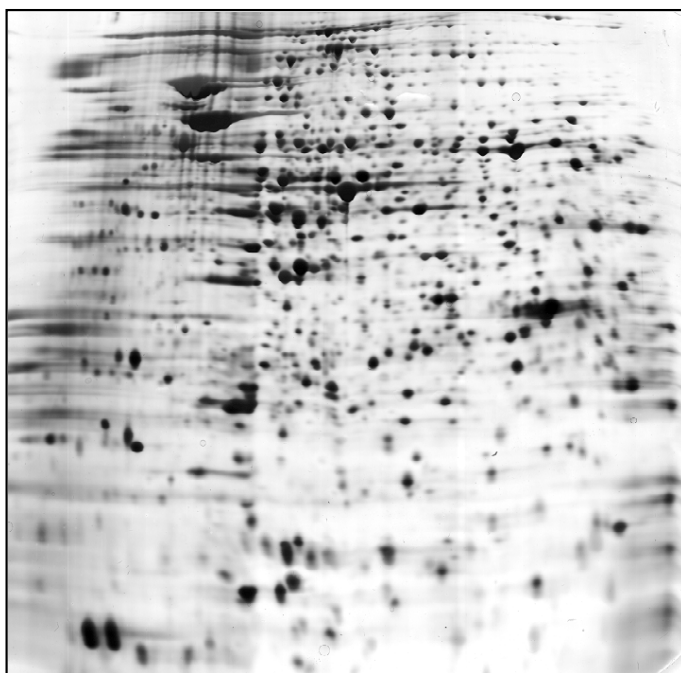


Figure 1: 2DE-Gel with 200 µg Protein from *E. coli* JM 109. Cells from *E. coli* JM 109 were collected by centrifugation and sample preparation with was performed. 200 µg proteins of the whole cell extract were separated by 2DE. First dimension was done by isoelectric focusing from pH 4 to pH 7 in an immobilized pH gradient gel by application of 50.000 Vh. The second dimension separation was by SDS-PAGE in a 12 % polyacrylamide gel. Proteins were visualized by silver staining.

C-PEK is designed to extract virtually all proteins from a given bacterial cell. 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 extensively for extraction and 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 be visualized on 2DE-gels. For degradation of nucleic acids, Benzonase®, a proprietary protease-free non-specific nuclease,

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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 are frozen and thawed by resuspension in the Resuspension Buffer. Subsequently, proteins are solubilized 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 most gram-negative bacteria are satisfactorily disrupted by the combination of freezing and resuspension in a hypotonic buffer, gram-positive cells require a more vigorous disruption using a bead mill. In order to minimize laborious optimization work, special extraction procedures for both gram-negative and gram-positive bacteria have been developed using the Bacterial Complete Proteome Extraction 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 gram-negative and gram-positive bacterial cells.

Material	Protein concentration [mg/ml] [§]
<i>E. coli</i> [#]	10
<i>S. Aureus</i> [#]	2

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

[#] Please note that different types of bacteria 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 carbamylation 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. Furthermore we found sonication of both gram-negative and gram-positive bacteria using a standard equipment to not improve the extraction result as compared with the presented protocols.

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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** (TBS): 1 vial
 - 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 12 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 and shipped at ambient temperature. Upon arrival, the buffer should be stored at +4 °C.

- **Resuspension Buffer**

The Resuspension Buffer is provided ready-to-use. Upon arrival, the buffer should be transferred to -20 °C for storage. After reconstitution, the buffers must be stored frozen at -20 °C, 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 should 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 and shipped at ambient temperature. Upon arrival, the vial should be stored at +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) should be stored at -20 °C immediately upon arrival. The enzyme must be kept on ice during the sample preparation procedure.

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 12 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.

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5. Kit Components Needed for One Extraction

- 4 ml Wash Buffer
- 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 gram-positive bacteria)

Supplied reagents are sufficient for 20 extractions (depending on the cell type).

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., LB, SOC, NB, CASO-Bouillon).
- 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.)

7. Extraction of proteins from gram-negative bacteria

Please use the following protocol for the extraction of proteins from gram-negative bacterial 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.

Abbreviations used: RT: Room temperature

1. Preparation of a gram-negative bacterial cell pellet

- 1.1 Grow a 5 ml over-night culture of e.g. *E. coli* at +37 °C and 200 rpm with rotary shaking. In most cases a liquid culture is started from a single colony on a plate preferably of the same composition as the liquid media to be used.
- 1.2 Dilute 0.5 ml over-night culture with 100 ml fresh media and continue growth at +37 °C and 200 rpm.
- 1.3 Growth is stopped in the late midlog exponential phase at an OD₆₀₀ of 0.8, more or less after 3 h, corresponding to about 2×10^{11} cells in the 100 ml culture. The culture vessel is placed on ice water and chilled immediately.
- 1.4 The *E. coli* culture is transferred into two precooled 50 ml microcentrifuge tubes and cells are collected by centrifugation at 10,000 x g for 15 min at +4 °C.
- 1.5 The supernatant is discarded and the pellets resuspended in 1 ml Wash Buffer each to remove residual media components.
- 1.6 The cell suspensions are combined completely into one 2 ml microcentrifuge tube and cells are collected by centrifugation at 10,000 x g for 15 min at +4 °C.
- 1.7 The supernatant is removed and the cell pellet is resuspended in 2 ml fresh Wash Buffer.
- 1.8 Cells are collected by centrifugation at 10,000 x g for 15 min at +4 °C. After centrifugation be careful to remove all the liquid from the washed cell pellet.
- 1.9 The washed cell pellet is frozen at -20 °C or colder for 10 min. The cell pellets may be stored under these conditions

2. Protein extraction from the gram-negative bacterial pellet

- 2.1 To the frozen cell pellet prepared in 1.9, add 150 µl ice-cold Resuspension Buffer and allow cells to thaw on ice.
- 2.2 Carefully resuspend cells by pipetting up and down with a 200 µl tip. An appropriate suspension is obtained after pipetting 30 times.
- 2.3 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.
- 2.4 Add 1.5 µl (≥ 375 U) Benzonase[®]. Proteins are solubilized by pipetting up and down 15 times with a 1 or 2 ml tip. Viscosity of the suspension will be reduced upon pipetting.

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- 2.5 Incubate sample at RT for 30 min with gentle agitation to achieve maximum protein solubilization. If available, the use of a thermomixer at RT and 500 rpm for 30 min would be advantageous.
- 2.6 Pellet insoluble components 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 30 min at +10 °C would be advantageous.
- 2.7 Transfer supernatant in a fresh microcentrifuge tube and recover as cell extract.

8. Extraction of proteins from gram-positive bacteria

Please use the following protocol for the extraction of proteins from gram-positive bacterial 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.

Abbreviations used: RT: Room temperature

1. Preparation of a gram-positive bacterial cell pellet

- 1.1 Grow a 5 ml over-night culture of e.g. *Staphylococcus Aureus* at +37 °C and 200 rpm with rotary shaking. In most cases a liquid culture is started from a single colony on a plate preferably of the same composition as the liquid media to be used.
- 1.2 Dilute 0.5 ml over-night culture with 100 ml fresh media and continue growth at +37 °C and 200 rpm.
- 1.3 Growth is stopped in the late midlog exponential phase at an OD₆₀₀ of 0.8. The culture vessel is placed on ice-water and chilled immediately.
- 1.4 The culture is transferred to precooled 50 ml microcentrifuge tubes and cells are collected by centrifugation at 10,000 x g for 15 min at +4 °C.
- 1.5 The supernatant is discarded and the pellets resuspended in 1 ml Wash Buffer each to remove remaining medium components.
- 1.6 The cell suspension is combined completely into one 2 ml microcentrifuge tube and cells are collected by centrifugation at 10,000 x g for 15 min at +4 °C.
- 1.7 The supernatant is removed and the cell pellet is resuspended in 2 ml fresh Wash Buffer.
- 1.8 Cells are collected by centrifugation at 10,000 x g for 15 min at +4 °C. After centrifugation be careful to remove all the liquid from the washed cell pellet.
- 1.9 Freeze the sample at -20°C or colder for 10 min. The cell pellets may be stored under these conditions.

2. Protein extraction from the gram-positive bacterial pellet

- 2.1 Add 150 µl ice-cold Resuspension buffer to the frozen cell pellet and allow cells to thaw on ice.
- 2.2 Carefully resuspend cells by pipetting up and down with a 200 µl tip. An appropriate suspension is obtained after pipetting 30 times.
- 2.3 Dilute the suspension with 800 µl room temperature Extraction Reagent and 50 µl Reducing Agent. Homogenize the suspension by pipetting up and down 15 times with a 1 ml tip.
- 2.4 Add 0.5 ml glass balls to the suspension. Vortex vigorously for 1 min. Incubate 1 min in ice to avoid overheating of the sample. Repeat vortexing-cooling cycle for four times to achieve optimal cell lysis.
- 2.5 Incubate on ice for 1 min to allow glass beads to settle down at the bottom of the tube. Carefully aspirate the supernatant and transfer completely into a fresh microcentrifuge tube. Be careful not to transfer glass beads.

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- 2.6 Add 1.5 μ l (\geq 375 U) Benzonase[®] and mix gently by inverting the tube 5 times.
- 2.7 Incubate sample at RT for 30 min with gentle agitation to achieve maximum protein solubilization. If available, the use of a thermomixer at RT and 500 rpm for 30 min would be advantageous.
- 2.8 Pellet insoluble components 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 30 min at +10 °C would be advantageous.
- 2.9 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.

<u>Question</u>	<u>Answer</u>
1. How do I handle reagents and samples during protein extraction with C-PEK?	<p>Do not warm reagents and samples above +30 °C to avoid protein carbamylation 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.</p> <p><i>Important:</i> 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.</p>
2. How do I prepare cell extracts generated with C-PEK for one-dimensional SDS-PAGE?	<p>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.</p> <p><i>Important:</i> 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.</p>
3. How do I load equal amounts of protein from cell extracts prepared with C-PEK on IPG-strips for comparative experiments?	<p>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.</p> <p>We recommend to determine the protein concentration in the extract solution (see below) prior to 2DE.</p>

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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 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 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. 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 formulations 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 (see box):

800 µl 1.25 x Extraction Reagent
+ 50 µl Reducing Agent
+ 150 µl <u>high quality water</u>
= 1000 µl 1 x Reagent for Sample dilution

Important: However 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.

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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 (Amersham Pharmacia, Biorad or equivalent)	Max 20 µl	A concentration of 0.2-0.5 % (w/v) carrier ampholytes is recommended
400 - 420 µl		

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)	280 µl
Carrier Ampholytes (Amersham Pharmacia, Biorad or equivalent)	5 µl stock solution (40 % w/v)
	405 µl

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10. Literature

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

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