CALBIOCHEM®

# Partial Bacterial Proteome Extraction Kit

**Cat. No. 539780**

# Product Information

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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 as also extraction. For this reason, we have developed the Partial Bacterial Proteome Extraction Kit (P-PEK) as a tool to extract proteins of variable solubility from both gram-negative and gram-positive bacteria in the presence of different detergents and chaotropes.

P-PEK is designed for a 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 bacterial 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 to be analyzed by one-dimensional SDS-PAGE (Fraction 4). A schematic representation of P-PEK as well as corresponding 2D-gels using *E. coli* 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].

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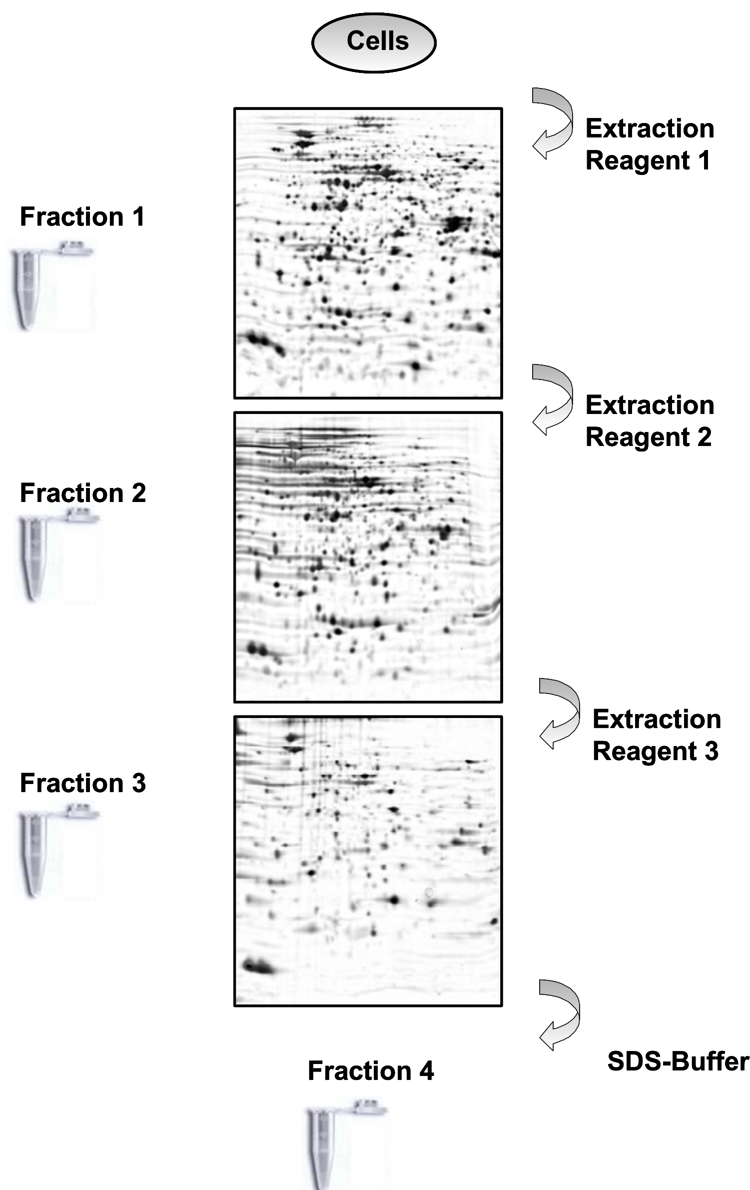


**Figure 1: Schematic view of the Partial Bacterial Proteome Extraction Procedure (P-PEK).** *E. coli* cells were collected by centrifugation and sample preparation was performed. 200 µg protein extract of fractions 1 to 3 were separated by 2DE. First dimension was done by isoelectric focusing in immobilized pH gradient gels (pI 4-7). The second dimension was carried out by SDS-PAGE in 12% polyacrylamide gels. 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, gram-negative bacteria require a vigorous disruption using a bead mill. In case of gram-positive bacteria, sonication is required to disrupt the cells. 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-protocol for gram-negative bacteria works without any sonication step to avoid impairment of the poor temperature control when sonicating small volumes. In case of extraction from gram-positive bacteria, sonication is required for efficient cell disruption in hypotonic Extraction Reagent 1. However Extraction Reagent 1 is devoid of urea, special care has to be taken be the user to follow the protocol and to avoid overheating of the sample. 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 gram-negative and gram-positive bacteria.**

| Material | Fraction | Protein concentration [mg/ml][$] |
|---|---|---|
| ***E. coli*** **Cells**[#] **(Gram-negative)** | Extraction Reagent 1<br>Extraction Reagent 2<br>Extraction Reagent 3<br>SDS-Buffer | 3.7<br>3.7<br>1<br>1.8 |
| ***S. Aureus*** **Cells**[#] **(Gram-positive)** | Extraction Reagent 1<br>Extraction Reagent 2<br>Extraction Reagent 3<br>SDS-Buffer | 4.7<br>2<br>1.7<br>2.1 |

[$] 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** (TBS): 1 vial
  - 100 ml / vial
  - Supplied ready-to-use
- **Extraction Reagent 1** (Tris): 1 vial
  - 60 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 Vial B**
- **Extraction Reagent 3 B**: 1 vial
  - 10 ml / vial
  - Supplied ready to reconstitute Extraction Reagent 3, Vial 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 Vial B**
- **SDS-Buffer B**: 1 vial
  - 6 ml / vial
  - Supplied ready to reconstitute SDS-Buffer, Vial 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 g / vial

**10 items**

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## 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 for storage.

- **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 A and 3 A**
  Extraction Reagent 2 A and 3 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 can be stored unopened at +4 °C. The 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).

- **Extraction Reagent 3 B and SDS-Buffer B**
  Extraction reagent 3 B and SDS-Buffer 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. 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 to store the Protease Inhibitor Cocktail in aliquots.

- **Benzonase®**
  Benzonase® (≥ 250 U/µl) should be transferred to –20 °C immediately upon arrival.

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## 4. Preparation Instructions for Reagents

**Extraction Reagent 1** (One Vial)

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. and 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** (Extraction Reagent 3 must be reconstituted from two Vials, 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** (SDS-Buffer must be reconstituted from two Vials, 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 the unused SDS-Buffer B after reconstitution of SDS-Buffer 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 ml Wash Buffer
- 3000 µl Extraction Reagent 1
- 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 (in case of gram-negative cells)

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., LB, SOC, NB, CASO).
- 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)
- Sonicator with Microtip in case of extraction from gram-positive bacteria (e.g. Branson Sonifier)
- *Optional:* Ultracentrifuge and rotor up to 75,000 x g for 2 ml tube size (e.g. Beckman, Sorvall etc.)

## 7. Extraction from gram-negative bacteria (exemplary *E. coli*)

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

Abbreviations used: RT: Room temperature

**Part A:** ***E. coli* cell pellet preparation (gram-negative bacteria)**

- Grow up a 5 ml overnight culture of *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.
- Dilute 0.5 ml overnight culture with 100 ml fresh media and incubate at 37 °C and 200 rpm.
- Stop culture growth in the late midlog exponential phase when $OD_{600}$ = 0.8, more or less after 3h.
- Place the culture vessel on ice water and chill immediately.
- Label two precooled 50 ml centrifuge tubes with the numbers 1 and 2. Transfer the *E. coli* culture into the centrifuge tubes (50 ml each) and pellet cells by 15 min centrifugation at 10,000 x g and +4 °C.
- Discard the supernatants without disturbing the pellet. Resuspended the cells from tube 1 in 2 ml ice-cold Wash Buffer to remove residual media components. Use the obtained suspension to resuspend the pellet from tube 2.
- Transfer the cell suspension into a 2 ml microcentrifuge tube and centrifuge at 10,000 x g for 15 min at +4 °C.
- Carefully remove the supernatant without disturbing the pellet. Discard the supernatant.
- Repeat the washing procedure once more by resuspending the cells in 2 ml fresh and ice-cold Wash Buffer. Centrifuge at 10,000 x g for 15 min at +4 °C to pellet the washed cells. Be careful to remove any wash buffer from the washed cell pellet before proceeding.
- Freeze the washed cell pellet at -20 °C or colder for 10 min. Cell pellets may be stored under these conditions.

**Part B:** **Protein extraction from gram-negative bacteria**

1 **Cell disruption and extraction of most soluble proteins with Extraction Reagent 1**

1.1. To the frozen cell pellet add first 10 µl Protease Inhibitor Cocktail and then 1000 µl of ice-cold **Extraction Reagent 1** and allow the cells to thaw on ice.
1.2. Resuspend the cells by pipetting up and down with a 1 ml pipette tip for a minimum of 30 times until the pellet is completely resuspended.

1.3. Add 0.5 ml glass beads (estimate amount by displacement of the cell suspension). Vortex thoroughly for 1 min.
1.4. Allow cooling on ice for 1 min to avoid overheating of the sample. Repeat vortexing-cooling cycle for a total of five times.
1.5. Incubate sample for 1 min on ice without shaking to allow glass beads to settle at the bottom of the tube. Carefully aspirate the cell extract from the glass beads by transfer of the suspension into a fresh, precooled microcentrifuge tube. Do not transfer glass beads.
1.6. Add 0.8 µl (≥ 200 Units) Benzonase® and mix by gently inverting the tube 5 times.
1.7. Incubate sample at +4 °C for 15 min with gentle agitation to achieve maximum protein solubilization. If available, the use of a thermomixer at +4 °C and 1000 rpm for 15 min would be advantageous.
1.8. Clarify the cell extract by centrifugation at 20,000 x g for 10 min at +4 °C. If available, the use of an ultracentrifuge at 75,000 x g for 30 min at +4 °C would be advantageous. 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 1000 µl of fresh ice-cold Extraction Reagent 1 and resuspend pellet by pipetting up and down with a 1 ml pipette tip for a minimum of 30 times until the pellet is completely resuspended.
1.10. Centrifuge at 20,000 x g for 10 min at +4 °C. If available, the use of an ultracentrifuge at 75,000 x g for 30 min at +4 °C would be advantageous.
1.11. Save the supernatant as wash fraction without disturbing the pellet.
1.12. Repeat steps 1.9 – 1.11 once. Be careful to remove all the liquid from the disrupted cell pellet before proceeding. Combine supernatant with the previously saved wash fraction. The analysis of the wash fraction is optional.

**2 Extraction of proteins of intermediate solubility with Extraction Reagent 2**

2.1. To the pellet prepared according to step 1.12 above add in the order indicated: 1 ml 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 30 times until the pellet is completely resuspended.
2.3. For maximum solubilization of proteins incubate at RT under gentle agitation for 15 min. 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 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.
2.5. Transfer the supernatant completely to a fresh microcentrifuge tube and recover as **fraction 2**. Be careful to remove all liquid from the pellet before proceeding.

**3 Extraction of 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 the pellet by pipetting up and down with a 1 ml pipette tip for a minimum of 30 times until the pellet is completely resuspended.
3.3. For maximum solubilization of proteins incubate at RT under gentle agitation for 15 min. 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 cell pellet before proceeding.

**4 Extraction of otherwise insoluble 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.
4.4 The sample is clarified by centrifugation at 20,000 x g for 30 min at +10 °C.
4.5 The supernatant is transferred completely to a fresh microcentrifuge tube and recovered as **fraction 4**.

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## 8. Extraction from gram-positive bacteria (exemplary *Staphylococcus aureus*)

Please use the following protocol for the extraction of proteins from gram-positive bacteria. 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: *S. Aureus* cell pellet preparation

- Grow up a 5 ml overnight culture of *S. 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.
- Dilute 0.5 ml overnight culture with 200 ml fresh media and incubate at 37 °C and 200 rpm again.
- Stop culture growth in the late midlog exponential phase when $OD_{600}$ = 0.8 - 1. Place the culture vessel on ice water and chill immediately.
- Label four precooled 50 ml centrifuge tubes with the numbers 1 - 4. Transfer the *S. Aureus* culture into the centrifuge tubes (50 ml each) and pellet cells by centrifugation at 10,000 x g for 15 min at +4 °C.
- Discard the supernatants without disturbing the pellet. Resuspended the cells from tube 1 in 2 ml ice-cold Wash Buffer to remove residual media components. Use the obtained suspension to resuspend the pellet from tube 2 and so on until all four cell pellets are resuspended in the 2 ml wash buffer.
- Transfer the cell suspension into a 2 ml microcentrifuge tube (Falcon, Greiner, Nalgen, etc.) and centrifuge at 10,000 x g for 15 min at +4 °C.
- Carefully remove the supernatant without disturbing the pellet. Discard the supernatant.
- Repeat the washing procedure once more by resuspending the cells in 2 ml fresh and ice-cold Wash Buffer. Centrifuge at 10,000 x g for 15 min at +4 °C to pellet the washed cells. Be careful to remove any wash buffer from the washed cell pellet before proceeding.
- Freeze the washed cell pellet at -20 °C or colder or colder for 10 min. Cell pellets may be stored under these conditions.

### Part B: Extraction of proteins from *S. Aureus*

1 **Extraction of the most soluble proteins with Extraction Reagent 1**

1.1 To one cell pellet prepared according to part A of the protocol, add first 10 µl Protease Inhibitor Cocktail and then 1000 µl of ice-cold Extraction Reagent 1 and allow the cells to thaw on ice.

1.2 Resuspend the pellet by pipetting up and down with a 1 ml pipette tip for a minimum of 30 times until the pellet is completely resuspended.

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1.3 Place container with the prepared cell suspension in an ice-water bath and allow cooling down for 5 min.
1.4 Sonicate on ice for 10 sec with a sonicator equipped with a microtip at maximum setting.
1.5 Allow cooling on ice-water for 1 min to avoid overheating the sample. Repeat sonication-cooling cycle four times.
1.6 Add 0.8 µl (≥ 200 Units) Benzonase® and mix by gently inverting the tube 5 times.
1.7 Incubate sample at +4 °C for 15 min with gentle agitation to achieve maximum protein solubilization. If available, the use of a thermomixer at +4 °C and 1000 rpm for 15 min would be advantageous.
1.8 Clarify the cell extract by centrifugation at 20,000 x g for 10 min at +4 °C. If available, the use of an ultracentrifuge at 75,000 x g for 30 min at +4 °C would be advantageous.
1.9 Transfer the supernatant completely to a fresh microcentrifuge tube and recover as **fraction 1**.
1.10 To wash away remaining proteins soluble in Extraction Reagent 1, add to the pellet 1000 µl of fresh ice-cold Extraction Reagent 1 and resuspend pellet by pipetting up and down with a 1 ml pipette tip for a minimum of 30 times until the pellet is completely resuspended.
1.11 Centrifuge at 20,000 x g for 10 min at +4 °C. If available, the use of an ultracentrifuge at 75,000 x g for 30 min at +4 °C would be advantageous.
1.12 Save the supernatant as wash fraction without disturbing the pellet.
1.13 Repeat steps 1.10 – 1.12 once. Be careful to remove all the liquid from the disrupted cell pellet before proceeding. Combine supernatant with the previously saved wash fraction. The analysis of the wash fraction is optional.

**2 Extraction of proteins of intermediate solubility with Extraction Reagent 2**

2.1 Add to the washed pellet prepared according to 1.13 above in the order indicated: 1000 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 30 times until the pellet is completely resuspended.
2.3 For maximum solubilization of proteins incubate at RT under gentle agitation for 15 min. If available the use of a thermomixer at 1000 rpm and RT for 15 min would be advantageous.
2.4 The sample is clarified by centrifugation at 20,000 x g and +10 °C for 10 min. If available, the use of an ultracentrifuge for 30 min at 75,000 x g and +10 °C would be advantageous.
2.5 The supernatant is transferred completely to a fresh microcentrifuge tube and recovered as **fraction 2**. Be careful to remove all liquid from the pellet before proceeding.

**3 Extraction of proteins of low solubility with Extraction Reagent 3**

3.1. Add to the pellet 500 µl of room temperature Extraction Reagent 3.
3.2. Resuspend the pellet by pipetting up and down with a 1 ml pipette tip for a minimum of 30 times until the pellet is completely resuspended.
3.3. For maximum solubilization of proteins incubate at RT under gentle agitation for 15 min. If available the use of a thermomixer at 1000 rpm and RT for 15 min would be advantageous.
3.4. The sample is clarified by centrifugation at 20,000 x g and +10 °C for 10 min. If available, the use of an ultracentrifuge for 30 min at 75,000 x g and +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 cell pellet before proceeding.

4 **Extraction of otherwise insoluble 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.
4.4 The sample is clarified by centrifugation at 20,000 x g for 30 min at +10 °C.
4.5 The supernatant is transferred completely to a fresh microcentrifuge tube and recovered as **fraction 4**.

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## 9. Technical Appendix

*Important:* Please understand the following information as guideline 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 **fraction 2** and **3** 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 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 the 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 since this may lead to precipitation of proteins that need high chaotrope and detergent concentrations for solubility and thus to misleading results.

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 to low for e.g. preparative loads on gels we recommend to precipitate the desired amount of protein using TCA/Acetone (Yuan *et al.*, 2002) and 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.

7. Storage and preparation of **fraction 1** and Wash fraction 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

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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.

However, **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.

However, **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.

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**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 | |

* We recommend to use Serva ampholytes (Servalyt®).

*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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