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# In-gel total protein quantification using a ninhydrin-based method

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**Abstract** Precise in-gel quantification of total protein amount of bands or spots in gels is the basis of subsequent biochemical, molecular biological and immunological analyses. Though several methods have been designed to evaluate relative amounts of proteins, these methods are of limited reliability because (semi-) quantifications depend on the amount of protein migrating into the gel and different proteins may lead to different absorptions/intensities of stained bands or spots. In the present study, we described a method to quantify both, hydrophilic and hydrophobic proteins using in-gel digestion with proteinase K, subsequent extraction and acid hydrolysis followed by the use of the ninhydrin reaction. The protocol is accurate and compatible with mass spectrometric characterization of proteins. Reproducible in-gel protein quantification was performed from SDS-PAGE and IEF/SDS-PAGE gels using bovine serum albumin as a standard protein. Bacteriorhodopsin separated on SDS-PAGE gel was quantified in addition in order to show that the method is also suitable for quantification of hydrophobic protein. This protocol for reliable in-gel protein quantification, which not only provides "arbitrary units of optical density", can also be completed in a minimum of 4 days or maximum 1 week depending on the type of electrophoresis with the disadvantage of being time consuming.

**Keywords** Gel-based proteomics · In-gel protein quantification · In-gel proteolytic digestion · Acid hydrolysis · Ninhydrin-based quantification

S. U. Kang and S. Heo contributed equally to this work.

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## Introduction

Gel-based methods are valuable tools for biochemical work, but so far the reliability of measurements of proteins in a gel is limited (Bradford 1976; Gornall et al. 1949; Lowry et al. 1951; Smith et al. 1985). The majority of methods for in-gel determination of protein amounts of a band or spot rely on staining with a large series of different dyes with subsequent densitometric determination of absorption. Absorption of the dye–protein complex, however, may depend on the primary or secondary structure of proteins, hydrophobicity, isoelectric point, as well as on protein modifications, post-translational modifications or compounds bound to proteins, including metals or lipids (Kessler and Fanestil 1986). Different absorption at different wavelengths or intensity depending on the above-mentioned facts may be expected. It is well known and documented that one and the same staining procedure cannot be used for visualization of different proteins in an SDS gel (Schleicher and Watterson 1983).

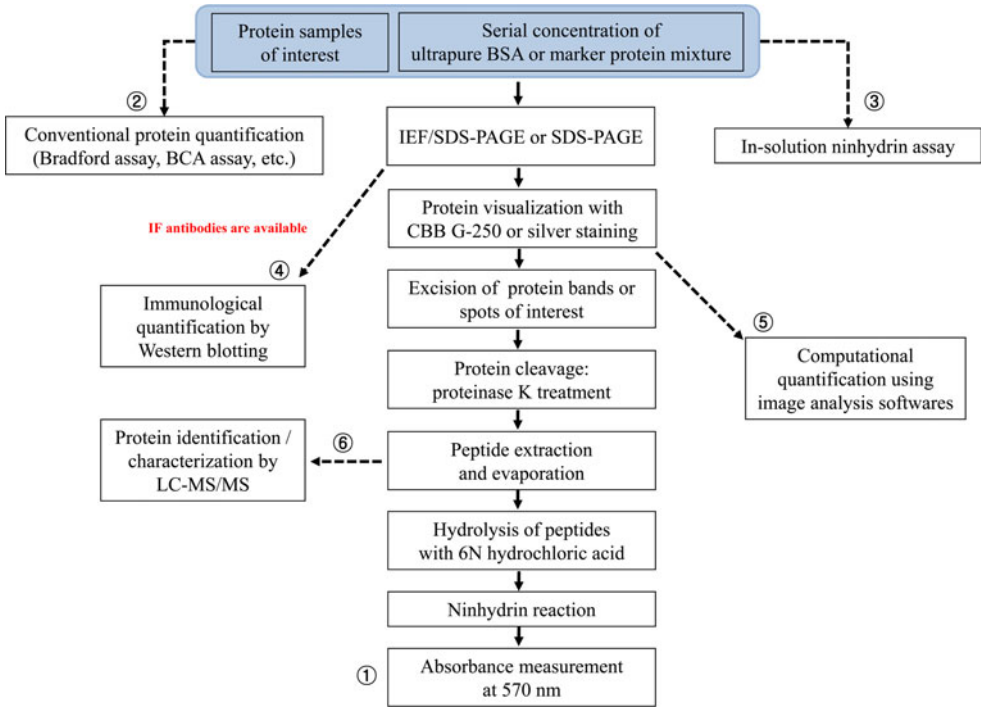


**Fig. 1** Schematic overview of the entire protocol of in-gel protein quantification. Protein samples and marker proteins are resolved on to 1D:SDS-PAGE or 2D:IEF/SDS-PAGE. Marker proteins need to be diluted sequentially to prepare standard curves. Gels are visualized with Coomassie Brilliant Blue G-250 or conventional or MS compatible silver staining method. Target protein bands or spots of interest are excised and subsequently in-gel digested with proteinase K. Digested peptides are extracted and evaporated to be hydrolyzed with 6 N hydrochloric acid. ① Hydrolyzed amino acids are reacted with ninhydrin reagents and determined at 570 nm. The amount of in-solution protein samples and marker proteins can be measured using ② conventional colorimetric assay (e.g., Bradford assay, BCA assay, etc.) or ③ in-solution ninhydrin assay. ④ Proteins of interests can be detected and quantified using Western blotting. ⑤ Stained gels can be analyzed and quantified using image analysis softwares for gel-based proteomic approaches. ⑥ Extracted peptides from in-gel digestion can be analyzed using mass spectrometry combined with HPLC for protein identification and characterization. *Arrow with solid line* indicates the procedures described in this protocol. *Arrow with dashed line* indicates other applicable experiments

It is also known that intensity of Coomassie blue staining, widely used for visualization of proteins, strongly depends and correlates with the presence of certain amino acids as lysine, arginine or histidine in a given protein (Tal et al. 1985). Differences between absorption of the protein–dye complex may even be seen by the naked eye as, e.g., the reddish color developing from the Coomassie blue–collagen complex (Duhamel et al. 1980). Moreover, buffers or solvents, etc., used may interfere with the absorption intensity or wavelength(s) of a specific protein–dye complex (Garfin et al. 1990). These inherent problems are also relevant for the use of fluorescent dyes and information on staining of gels has been reviewed by Miller et al. (2006).

It was therefore the aim of the study to develop a stain-independent in-gel protein quantification method and follow a basic chemical principle of hydrolysing proteins (Heidelbaugh et al. 1975) with subsequent total amino acid quantification using the ninhydrin reaction. The primary amino group of amino acids reacts with ninhydrin to give purple-colored products and this reaction can be used for quantitative colorimetric assay by measuring the absorbance at 570 nm using a spectrophotometer. Ninhydrin reactions were previously used to determine the amino acid composition of stained proteins separated by SDS-PAGE (Sreekrishna et al. 1980), but there were several disadvantages including: (1) loss of basic amino acid residues due to excess ammonia and (2) chemical adducts from hydrolysis of polyacrylamide gels. In a previous study (Kang and Lubec 2011), several technical problems were solved, and herein a colorimetric assay for quantification of proteins after gel-based protein separation combined with in-gel proteolytic digestion of the proteins and acid hydrolysis of resulting peptides followed by the evaporation of acidic solution and colorimetric reaction with ninhydrin is proposed.

The advantage is that protein quantification can be verified when antibodies against proteins of interest are not available. In-gel protein quantification can avoid unnecessary expense to buy commercially available antibodies or to generate own antibodies when the antibodies are not available. Another advantage is that the ninhydrin-based method after in-gel proteolytic digestion and acid

hydrolysis is compatible with both, hydrophilic and hydrophobic proteins. Usage of routine chemicals prevents the budget waste for purchasing antibodies to verify protein quantifications. Application of in-gel proteolytic digestion for maximizing the efficiency of protein/peptide extraction from gels has another advantage to use mass spectrometry-based protein identification and characterization.

The disadvantage is the time-consuming procedures for in-gel proteolytic digestion and acid hydrolysis. Additional gels with mixture of protein markers are required. Careful standardization is needed to obtain reliable standard curves. The usage of vacuum evaporator compatible with samples containing strong acidic chemicals is required. Due to high sensitivity of ninhydrin reactions, it is mandatory to be careful with contaminants, e.g., glycine, urea, ammonium bicarbonate, acrylamide, iodoacetamide, dithiothreitol, CHAPS, EGTA.

A schematic overview of work flow is shown in Fig. 1.

## Experimental design

### Protein samples

Ultrapure BSA (66.0 kDa) and eight standard proteins widely used were chosen as markers in SDS-PAGE and IEF/SDS-PAGE because of their different molecular weights: β-galactosidase (135.0 kDa), phosphorylase B (97.0 kDa), glutamic dehydrogenase (55.0 kDa), albumin (45.0 kDa), glyceraldehyde-3-phosphate dehydrogenase (36.0 kDa), carbonic anhydrase (29.0 kDa), trypsinogen (24.0 kDa) and α-lactalbumin (14.2 kDa). Each protein concentration was reconstituted to 3.0 μg/μL in 25 mM Tris–HCl (pH 7.5).

The amount of protein must not exceed 1.0 μg per protein because 1.0 mm gel thickness in SDS-PAGE and 1.5 mm gel thickness in IEF/SDS-PAGE can tolerate a maximum of 1.0 μg as a protein band and spot. Use of higher amounts than 1.0 μg per protein generates protein aggregation or streaking.

The main principle of the protocol is to quantify and thus measure the quantity of hydrophobic protein as well as hydrophilic protein from gel bands on 1D SDS-PAGE or spots on 2D IEF/SDS-PAGE by the ninhydrin reaction.

### IEF/SDS-PAGE (two-dimensional gel electrophoresis)

Two-dimensional gel electrophoresis, IEF/SDS-PAGE, has been widely used for, e.g., protein profiling, complex separation, detection of protein isoforms and survey of differences in protein expression levels. For comparison of expression levels image softwares as, e.g., Proteomeweaver™ (Bio-Rad, Vienna, Austria), ImageMaster™ (Amershampharmacia Biotech, Freiburg, Germany) and PDQuest (Bio-Rad, Vienna, Austria) after coomassie blue staining but not silver staining were used, because intensity of protein bands (spots) depends on time, temperature and concentration of chemicals during the development procedure.

### Enzymatic digestion of proteins and extraction

Following the electrophoresis steps, bands or spots of interest are excised from SDS-PAGE or IEF/SDS-PAGE. Before enzymatic digestion of the proteins, in-gel reduction and alkylation steps have to be carried out to prevent disulfide cross-linkage formation and therefore protein aggregation. Application of proteinase K displays strong proteolytic activity on denatured proteins and on native proteins as well; therefore, it was proven that proteins could be digested efficiently without reduction and alkylation steps.

### Colorimetric assay (ninhydrin assay)

Ninhydrin will react with alpha-amino group, $NH_2$–C–COOH. This group is present in all amino acids, peptides, and proteins. While the decarboxylation reaction takes place in a free amino acid, this will not occur in peptides and proteins. Thus, theoretically only amino acids will lead to the color development. However, one should always check out the possible interference from peptides and proteins by performing blank test, for example using small blank gel pieces or especially when such solutions are readily available. Recently, we reported that chemical compounds other than amino acids also yield positive results (Kang and Lubec 2011). Maximum compatible chemical concentrations are given in supplemental table 1. Therefore, successful experiment must be promised by complete hydrolysis steps of peptides and proteins with blank test.

## Materials

### Reagents

- Dithiothreitol (DTT) (Bio-Rad, http://www.bio-rad.com, cat. no. 161-0611).
- Ethylenediaminetetraacetic acid (EDTA) (Sigma, http://www.sigmaaldrich.com, cat. no. 431788-100G).
- Iodoacetamide (IAA) (Bio-Rad, http://www.bio-rad.com, cat. no. 163-2109).
- Urea (Sigma, http://www.sigmaaldrich.com, cat. no. 431788-100G).

- Glycine (Sigma, http://www.sigmaaldrich.com, cat. no. G8790-1 KG).
- Sodium dodecyl sulfate (SDS) (Sigma, http://www.sigmaaldrich.com, cat. no. L6026-250G).
- Acrylamide (Bio-Rad, http://www.bio-rad.com, cat. no. 161-0108).

! **CAUTION** Acrylamide is toxic. When handling these chemicals, wear gloves and use a pipetting aid.

- Piperazine-di-acrylamide (PDA) (Bio-Rad, http://www.bio-rad.com, 161-0202).

! **CAUTION** PDA is toxic. When handling these chemicals, wear gloves and use a pipetting aid.

- IPG buffer, pH 3–11 NL (GE Healthcare, http://www.gelifesciences.com, cat. no. 17-6004-40).
- Bromophenol Blue sodium salt—for electrophoresis (Sigma, http://www.sigmaaldrich.com, B5525-10G).
- Glycerol (Sigma, http://www.sigmaaldrich.com, cat. no. G8773-1L).
- Potassium hexacyanoferrate (III) (Sigma, http://www.sigmaaldrich.com, cat. no. P8131).
- Sodium thiosulfate (Sigma, http://www.sigmaaldrich.com, cat. no. 13481).
- 2-Propanol (Sigma, http://www.sigmaaldrich.com, cat. no. 34095).
- 2-mercaptoethanol (Bio-Rad, http://www.bio-rad.com, cat. no. 161-0710).
- Precision Plus Protein Standards (Bio-Rad, http://www.bio-rad.com, cat. no. 161-0363).
- Colloidal Coomassie blue staining kit (Invitrogen, http://www.invitrogen.com, cat. no. LC6025).
- Tributylphosphine (TBP) (Sigma, http://www.sigmaaldrich.com, cat. no. T49484-5ML).
- Ammonium bicarbonate (Sigma, http://www.sigmaaldrich.com, cat. no. 40867-50G-F).
- Proteinase K (Promega, http://www.promega.com, cat. no. V3021).
- LC–MS CHROMASOLV® methanol (Sigma, http://www.sigmaaldrich.com, cat. no. 34966-1L).
- LC–MS CHROMASOLV® acetonitrile (ACN) (Sigma, http://www.sigmaaldrich.com, cat. no. 34967-1L).

! **CAUTION** ACN is toxic. When handling, wear gloves and use a pipetting aid.

- LC–MS CHROMASOLV® water (Sigma, http://www.sigmaaldrich.com, cat. no. 39253-1L-R).
- HPLC-grade formic acid (Sigma, http://www.sigmaaldrich.com, cat. no. 27001-500ML-R).
- β-Galactosidase from *Escherichia coli* (Sigma, http://www.sigmaaldrich.com, cat. no. 48275-5MG).
- Phosphorylase B from rabbit muscle (Sigma, http://www.sigmaaldrich.com, cat. no. P4649-1VL).
- L-Glutamic dehydrogenase from bovine liver—Type II, buffered aqueous glycerol solution (Sigma, http://www.sigmaaldrich.com, cat. no. G2626-50MG).
- Albumin from chicken egg white—lyophilized powder (Sigma, http://www.sigmaaldrich.com, cat. no. A7641-50MG).
- Glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle (Sigma, http://www.sigmaaldrich.com, cat. no. G5262-5MG).
- Carbonic anhydrase from bovine erythrocytes (Sigma, http://www.sigmaaldrich.com, cat. no. C2273-1VL).
- Trypsinogen, PMSF treated from bovine pancreas (Sigma, http://www.sigmaaldrich.com, cat. no. T9011-1VL).
- α-Lactalbumin from bovine milk (Sigma, http://www.sigmaaldrich.com, cat. no. L6385-1VL).
- Bacteriorhodopsin (BR; kindly provided by Dr. Elena Kharnaukhova, FDA, Bethesda, MD, USA).
- Tin(II) chloride dehydrate (Sigma, http://www.sigmaaldrich.com, cat. no. 208035-100G).
- PlusOne™ Silver Staining Kit (GE Healthcare Life Sciences, Freiburg, Germany, 17-1150-01).
- Colloidal Blue Staining Kit (Invitrogen, Carlsbad, CA, USA, LC6025).
- Ninhydrin (Sigma, http://www.sigmaaldrich.com, cat. no. 151173-100G).
- Ready Gels 10 % Tris–HCl (Bio-Rad, http://www.bio-rad.com, cat. no. 161-1155).
- Hydrochloric acid solution—~6 M in $H_2O$ for amino acid analysis (Sigma, http://www.sigmaaldrich.com, cat. no. 84429-10X2ML).
- Sodium acetate trihydrate (Sigma, http://www.sigmaaldrich.com, cat. no. S8625-500G).
- Ethylene glycol—spectrophotometric grade, ≥99 % (Sigma, http://www.sigmaaldrich.com, cat. no. 293237-1L).
- Glacial acetic acid (Merck, http://www.merck-chemicals.com, cat. no. 1000562500).
- Ultrapure bovine serum albumin (BSA; Ambion, http://www.ambion.com, cat. no. AM2616).

Equipment

- Protein Lobind tube (Eppendorf, http://www.eppendorf.com, cat. no. 0030 108.094).
- Eppendorf microcentrifuge 5417C (Eppendorf, http://www.eppendorf.com, cat. no. 5417 000.315).
- PROTEAN II xi/XL vertical electrophoresis system (Bio-Rad, http://www.bio-rad.com).
- Mini-PROTEAN 3 electrophoresis module (Bio-Rad; http://www.bio-rad.com; cat. no. 165-3302).
- MELAG Type 221 dry-heat sterilizer (MELAG Medical technology, http://www.melag.com).
- Immobiline™ DryStrip Gels (Immobilized pH 3–10 nonlinear gradient strips) (GE Healthcare, http://www.gelifesciences.com, cat. no. 17-1235-01).

- Ettan™ IPGphor™ III IEF system (GE Healthcare Life Sciences, http://www.gelifesciences.com, cat. no. 11-0033-64).
- Eppendorf SpeedVac Concentrator 5301 (Eppendorf, http://www.eppendorf.com, cat. no. 5305 000.215).
- iMark™Microplate Absorbance Reader (Bio-Rad, http://www.bio-rad.com, cat. no. 168-1135).
- Eppendorf Thermomixer comfort (Eppendorf, http://www.eppendorf.com, cat. no. 5355 000.011).
- NUNC MaxiSorp microtiter 96-well plate (Sigma, MO, USA, M9410).

## Reagent setup

*4M sodium acetate buffer* 544 g of sodium acetate trihydrate in 100 mL of glacial acetic acid and add water up to final volume 500 ml (pH would be 5.5).

*2× concentrated Laemmli sample buffer* 126 mM Tris–HCl, 20 % glycerol, 4 % SDS, 0.02 % bromophenol blue, pH 6.8 (make aliquots and stable for 1 year if stored at −20 °C).

*30 % acrylamide/PDA* 29.25 g acrylamide, 0.75 g PDA in 100 ml. Can be kept at 4 °C for 4 weeks.

*200 mM TBP* 5 µl TBP in 95 µl isopropanol (stable for 1 year, if stored at −20 °C).

*Staining solution* 0.1 % (wt/vol) Colloidal Coomassie blue. Prepare fresh on the day of use.

*Rehydration buffer* 8 M urea, 4 % CHAPS (wt/vol), 10 mM DTT, 0.5 % IPG buffer (wt/vol).

*Equilibration buffer I* 6 M urea, 2 % SDS (wt/vol), 0.5 M Tris HCl (pH 8.8), 20 % glycerol (wt/vol), 2 % DTT (wt/vol).

*Equilibration buffer II* 6M urea, 2 % SDS (wt/vol), 0.5M Tris HCl (pH 8.8), 20 % glycerol (wt/vol), 2.5 % IAA (wt/vol), 0.5 % bromophenol blue.

*SDS-PAGE electrophoresis buffer* 25 mM Tris–HCl and 192 mM glycine and 0.1 % (wt/vol) SDS, pH 8.3. Prepare fresh on the day of use.

*Fixation solution for Colloidal blue staining* 50 % methanol (vol/vol), 10 % glacial acetic acid (vol/vol).

*Fixation solution for silver staining* 40 % ethanol (vol/vol), 10 % glacial acetic acid (vol/vol).

*Sensitizing solution for silver staining* 30 % ethanol (vol/vol), 0.2 % sodium thiosulfate (wt/vol), 17 g sodium acetate in 250 ml. Add 1.25 ml glutaraldehyde (stock solution: 25 % wt/vol).

*Silver solution for silver staining* 0.25 % silver nitrate (wt/vol) in 250 ml of water.

*Developing solution for silver staining* 6.25 g sodium sodium acetate in 250 ml of water. Stir vigorously to dissolve the sodium carbonate. Add 0.2 ml of formaldehyde (stock solution, 37 % wt/vol).

*Stop solution for silver staining* 3.65 g EDTA-$Na_2{\cdot}2H_2O$ in 250 ml of water.

*Destaining solution for silver stained gels* 50 mM potassium hexacyanoferrate and 300 mM sodium thiosulfate.

*Washing solution for silver stained gels* 50 % methanol (vol/vol) and 10 % glacial acetic acid (vol/vol).

*Stannous chloride solution* 50 mg of Tin(II) chloride dehydrate in 500 µL of ethylene glycol. A cloudy stannous chloride solution should be mixed well. Prepare fresh on the day of use.

## Equipment setup

*iMark™Microplate Absorbance Reader* Microplate Manager 6 software is designed to collect, analyze and output absorbance data from Bio-Rad's microplate readers. Once it is installed on a computer, first the type of reader should be specified, then subsequently following manufacturer instruction, type of performance and the layout of microplate are required to measure 96-well microplate at 570 nm. Read at 570 nm with 'End point Reads' mode to acquire a single absorbance reading from each well.

## Procedure

Schematic overview of this protocol is shown in Fig. 1.

### *Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of ultrapure bovine serum albumin (BSA)* ● ***TIMING*** *1 d*

1. Prepare 0.03125—1.0 µg serial concentration (0.03125, 0.0625, 0.125, 0.25, 0.5, 0.75 and 1.0 µg) of BSA.
2. Mix the protein with 2× concentrated Laemmli sample buffer (126 mM Tris–HCl, 20 % glycerol, 4 % SDS, 0.02 % bromophenol blue, pH 6.8) in a ratio of 1:1.
3. Boil the mixtures at 95 °C for 5 min with vortexing.
4. Chill the samples on ice then load on to the 10 % Ready Gels with 3 µl of a precision plus protein standards.
5. Perform electrophoresis to set the voltage to 50 V for 20 min, and then increase to 100 V (15–20 mA/gel) until the bromophenol blue dye front reaches the bottom of the gel.
6. Fix the gels for 12 h in 50 % methanol and 10 % acetic acid for Colloidal Coomassie blue staining (for silver staining, see "Silver staining of proteins").
7. Stain the gels with Colloidal Coomassie blue for at least 3 h (up to 12 h) and destain the gels with Milli-Q water until the desired bands intensities are observed.

*Two-dimensional gel electrophoresis* ● ***TIMING*** *3 d*

8. Prepare a mixture of 0.03125–1.0 μg serial concentration (0.03125, 0.0625, 0.125, 0.25, 0.5, 0.75 and 1.0 μg) samples with eight protein standards, β-galactosidase (135.0 kDa), phosphorylase B (97.0 kDa), glutamic dehydrogenase (55.0 kDa), albumin (45.0 kDa), glyceraldehyde-3-phosphate dehydrogenase (36.0 kDa), carbonic anhydrase (29.0 kDa), trypsinogen (24.0 kDa) and α-lactalbumin (14.2 kDa).

9. Add 322.5 μl rehydration buffer and 3.5 μl of 200 mM TBP to final 24 μl of different concentration of protein standard mixtures, and apply them on 18 cm immobilized pH 3–10 nonlinear gradient strips.

10. Set the IEF electrophoresis steps as following: rehydration at 30 V for 12 h, 200 V for 1 h, 500 V for 1 h, 1,000 V for 1 h, increase the voltage gradually to 8,000 V for 30 min and keep constant for a further 6 h (in total, approximately 54,560 V h).

11. Equilibrate IPG strips in equilibration buffer with 2 % DTT for 15 min and then in equilibration buffer with 2.5 % IAA instead of DTT for another 15 min.

12. Perform second-dimensional separation on a 7.5–17.5 % gradient SDS-PAGE. After running at 50 V for 30 min, keep the running voltage at 200 V for about 7 h until the dye front line reaches the edge of the gel. Load 7 μl of a precision plus protein standards ranging from 10 to 250 kDa to determine molecular masses.

▲ CRITICAL STEP Fix the parameters of gel casting system to get a reproducible gradient. No air bubble should exist between strip and gel surface to obtain high-quality gel patterns.

13. Fix the gels for 12 h in 50 % methanol and 10 % acetic acid for Colloidal Coomassie blue staining (for silver staining, see "Silver staining of proteins").

14. Stain gels with Colloidal Coomassie blue for 8 h and wash away excess of dye with distilled water.

■ **PAUSE POINT** Gels can be kept in 0.02 % sodium azide and stored for several months at 10 °C by sealing in a plastic bag.

*Silver staining of proteins* ● ***TIMING*** *2 h*

(This protocol follows a manufacturer instruction from GE Healthcare Life Sciences *PlusOne*$^{TM}$ *Silver Staining Kit*).

15. Soak the gels in fixation solution (40 % ethanol, 10 % glacial acetic acid) for 30 min.

16. Remove the fixing solution. Add sensitizing solution [250 ml of following solutions; 30 % ethanol, 0.2 % sodium thiosulfate, 17 g sodium acetate, 1.25 ml of 25 % (wt/vol) glutardialdehyde] and leave shaking for at least 30 min.

▲ CRITICAL STEP Add glutardialdehyde just before use.

17. Remove the sensitizing solution. Add distilled water and wash three times for 5 min each time.

18. Add silver nitrate solution [250 ml of following solutions; 0.25 % silver nitrate, 0.1 ml of 37 % (wt/vol) formaldehyde] and leave shaking for 20 min.

19. Remove the silver nitrate solution. Rinse twice in distilled water for 1 min each time.

20. Add developing solution [250 ml of following solutions: 6.25 g sodium carbonate, 0.05 ml of 37 % (wt/vol) formaldehyde] and leave shaking for 2–5 min.

▲ CRITICAL STEP Stir vigorously to dissolve sodium carbonate.

21. Transfer the gels to stopping solution (250 ml of following solutions; 3.65 g EDTA-$Na_2$·$H_2O$) when the protein bands/spots have reached desired intensity. Leave the gels shaking in stopping solution for 10 min.

22. Remove the stopping solution. Add distilled water and wash three times for 5 min each time.

■ **PAUSE POINT** Gels can be kept in 0.02 % sodium azide and stored for several months at 10 °C by sealing in a plastic bag.

*Spot (band) excision* ● ***TIMING*** *0.5 h*

23. Cut the gel pieces of interest into small pieces to increase surface and put into a 1.5 ml tube.

▲ CRITICAL STEP Specific bands from SDS-PAGE were cut out to result in a final gel area 8 × 2 mm which in turn was cut out into more than eight small pieces. Cutting into small pieces smaller than 1.5 $mm^3$ to increase surface is very important to maximize enzyme efficiency and peptide elution yield after in-gel digestion.

▲ CRITICAL STEP Prepare blank gel piece as a control experiment and check any contaminants against ninhydrin reaction.

**? TROUBLE SHOOTING**

*Option 1: destaining of colloidal coomassie blue stain* ● ***TIMING*** *1.5 h*

24. Wash gel pieces two times with 50 % 50 mM ammonium bicarbonate/50 % ACN for 30 min with occasional vortexing.

25. Discard the washing solution at the end of each step. Then add 100 μl of 100 % ACN to each tube to cover the gel piece completely and incubate for at least 5 min.

26. Dry the gel pieces completely in a Speedvac Concentrator 5301.

■ **PAUSE POINT** Samples can be kept at −20 °C for several weeks if wrapped tightly with parafilm.

*Option 2: Destaining of silver stained gel* ● ***TIMING*** *1 h*

27. Prepare 100 mM potassium hexacyanoferrate and 600 mM sodium thiosulfate.

28. Prepare destaining mixture by combining 100 mM potassium hexacyanoferrate: 600 mM sodium thiosulfate in a ratio of 1:1.

29. Add 100 µl of destaining mixture to the microcentrifuge tubes containing excised silver stained gel spots (200 µl for bands). Incubate gel spots with vortexing until brown color disappears.

▲ CRITICAL STEP Do not let the excised gel spots sit in the destaining mixture too long.

30. Remove the destaining mixture. Immediately wash the destained gel spots in 50 % methanol and 10 % glacial acetic acid for 5 min and repeat this step four times.

31. Additionally wash with 50 % 50 mM ammonium bicarbonate/50 % ACN for 20 min with occasional vortexing.

32. Discard the washing solution at the end of each step. Then add 100 µl of 100 % ACN to each tube to cover the gel piece completely and incubate for at least 5 min.

33. Dry the gel pieces completely in a Speedvac Concentrator 5301.

■ **PAUSE POINT** Samples can be kept at −20 °C for several weeks if wrapped tightly with parafilm.

*In-gel digestion and peptides preparation for ninhydrin reaction* ● ***TIMING*** *1 d*

34. Prepare 4 ng/µl Proteinase K solution buffered in 50 mM Tris–HCl pH 8.0.

35. Add 30 µl of Proteinase K solution to each tube.

36. Incubate the protein standards for longer than 4–24 h at 37 °C.

**? TROUBLE SHOOTING**

37. Transfer supernatants to new 1.5-ml tubes.

38. Extract peptides from gel pieces with 80 µl of 1 % formic acid for 30 min in a sonication bath and pool supernatants in previous tubes.

39. Add 80 µl of 20 % methanol and extract peptides from gel pieces for 30 min in a sonication bath. Then pool supernatants into previous tubes.

40. Add 80 µl of 40 % methanol and extract peptides from gel pieces for 30 min in a sonication bath. Then pool supernatants into previous tubes.

41. Reduce final volume to <10 µl in a Speedvac Concentrator 5301 and undergoing acid hydrolysis and ninhydrin reaction.

■ **PAUSE POINT** Samples after being completely dry can be kept at −20 °C for several weeks if wrapped tightly with parafilm. At this point, if you have mass spectrometric system, you can apply your peptide samples to mass spectrometry after adding 10 µl of HPLC-grade water.

*Ninhydrin assay* ● ***TIMING*** *1 d 2 h*

42. Hydrolyze samples with 150 µl of 6 N HCl at 99 °C for 24 h in ThermoMixer comfort with vortexing.

43. Completely evaporate hydrolyzed samples in a Savant SpeedVac concentrator at 60 °C.

44. Re-dissolve the dried samples in 50 µl of distilled water.

45. Prepare 4 M sodium acetate buffer by dissolving 544 g of sodium acetate trihydrate in 100 mL of glacial acetic acid then add distilled water up to 500 ml (pH should be 5.5).

46. Prepare stannous chloride solution by dissolving 50 mg of Tin(II) chloride dehydrate in 500 µl of ethylene glycol. A cloudy stannous chloride solution should be mixed well before using.

47. Dissolve 200 mg of ninhydrin in a 7.5 ml of mixture of ethylene glycol and 2.5 ml of 4 M sodium acetate buffer. Add 250 µl of stannous chloride solution.

48. Add 200 µl of ninhydrin reagent to 0.031251—1.0 µg of hydrolyzed protein standards in a flat-bottom NUNC MaxiSorp microtiter plate. Incubate the plate in a MELAG thermo incubator at 100 °C for 20 min.

**? TROUBLE SHOOTING**

49. The plate was removed from incubator and the absorption was measured on a iMark™ Microplate Absorbance Reader at 575 nm. The products after ninhydrin reaction have a rather broad range of absorption spectra between maximum 560 and 580 nm.

▲ CRITICAL STEP The more volume of samples you have, the longer time it takes to get maximum color changes.

▲ CRITICAL STEP An incubator was used to heat up the microplate and it was observed that heating for at least 20 min is required to provide sufficient heating for all ranges of sample volumes.

▲ CRITICAL STEP Samples in wells located at boundary of microplates develop color sooner than inner wells, but within 20 min all wells have reached maximum color.

▲ CRITICAL STEP Color development continues even after cooling to RT. However, the rate of development at RT is sufficiently low that no significant error is introduced if all absorbance measurements are made within a 10-min period.

▲ CRITICAL STEP Dimethyl sulfoxide, methyl cellosolve, and ethylene glycol were all good solvents to dissolve ninhydrin and gave satisfactory color yields. But the

stability of the reagent over time was greatest when ethylene glycol was used as a solvent (Starcher 2001).

*Data processing* ● ***TIMING*** *1 h*

50. Coefficient of variation (CV) from each sample and correlation coefficients ($R^2$) were calibrated from more than five independent experiments.

● ***TIMING***

Steps 1–7 **SDS-PAGE of ultrapure BSA** 1 d.
Steps 8–14 **Two-dimensional gel electrophoresis** 3 d.
Steps 15–22 **Silver staining of proteins** 2 h.
Step 23 **Spot (band) excision** 30 min.
Steps 24–26 **Destaining of Colloidal Coomassie blue stain** 1 h 20 min.
Steps 27–33 **Destaining of silver stained gel** 1 h.
Steps 34–41 **In-gel digestion and peptides preparation for ninhydrin reaction** 1d.
Steps 42–49 **Ninhydrin assay** 1d 2 h.
Step 50 **Data processing** 1 h.

**? TROUBLE SHOOTING**

| Step | Problem | Possible reason | Solution |
|---|---|---|---|
| Step 23 | Low elution rate<br>Low intensity of ninhydrin reaction | Size of gel pieces | Cut gel pieces into smaller than 1.5 $mm^3$ |
| Step 36 | Low elution rate<br>Low intensity of ninhydrin reaction | Short incubation time | Incubate samples at 37–56 °C overnight because enzyme activity to generate sufficient enzyme cleavage was randomly different, depending on specificity of proteins |
| | | Inappropriate choice of proteolytic enzyme | Optimize the experimental condition of proteolytic digestion of proteins using different proteases and bioinformatic tools to predict cleavage sites |
| Step 48 | Low intensity of ninhydrin reaction | Short incubation time<br>Low temperature | Incubate longer than 20 min at 100 °C |

Anticipated results

The detectable range of protein quantity with ninhydrin-based method was determined using ultrapure BSA as a standard model protein. As shown in supplemental Fig. 1, the lower detection limit was 50 ng. In-gel protein quantification of a serial dilution of ultrapure BSA and marker proteins mixture from 0.03125 to 1.0 μg was carried out by measuring arbitrary unit of optical density (OD). As shown in Fig. 2 and supplemental Fig. 2, sequentially diluted ultrapure BSA and marker proteins mixture were separated on SDS-PAGE and IEF/SDS-PAGE, respectively, and visualized using Colloidal Coomassie blue staining and silver staining. A linear function was obtained when protein amounts and arbitrary units of optical density (OD) were plotted; the correlation of coefficient ($R^2$) of ultrapure BSA separated on SDS-PAGE gel was 0.9970 from Colloidal Coomassie stained gel and 0.9950 from silver stained gel (Table 1a). The correlation of coefficient values of marker proteins mixture separated on IEF/SDS-PAGE were obtained within the range of 0.9918 and 0.9993 (Table 1b). The results represent a clear linear function and a high correlation. From our previous study, we performed optimizing experiment to find the appropriate wavelength for measurement in order to rule out impurities and to find the maximum absorption of the end product of ninhydrin reaction. Maximum intensity was observed at 570 nm and no impurities were detected (Kang and Lubec 2011).

Ninhydrin-based protein quantification was performed from in-solution as well as in-gel protein samples to determine the extraction (recovery) yield from gel matrix. As shown in Table 2, high extraction (recovery) yield from gel matrix was observed compared to total amount of protein applied on to gels which was measured from in-solution samples of marker proteins. Highest extraction yield was 94 % (phosphorylase B, rabbit muscle) and lowest extraction yield was 88.5 % (trypsinogen, bovine pancrease). Loss of total protein amount which was determined by comparing the amount between in-solution and in-gel protein samples is thought to be caused by protein loss during gel electrophoresis due to different characteristics of proteins, such as hydrophobicity, or incomplete extraction of digested peptides or insufficient digestion due to inappropriate choice of proteolytic enzymes.

Compatibility of ninhydrin-based in-gel protein quantification with hydrophobic proteins was determined using bacteriorhodopsin. Total amount of bacteriorhodopsin separated on SDS-PAGE was determined via ninhydrin-based method using BSA as standard protein (supplemental

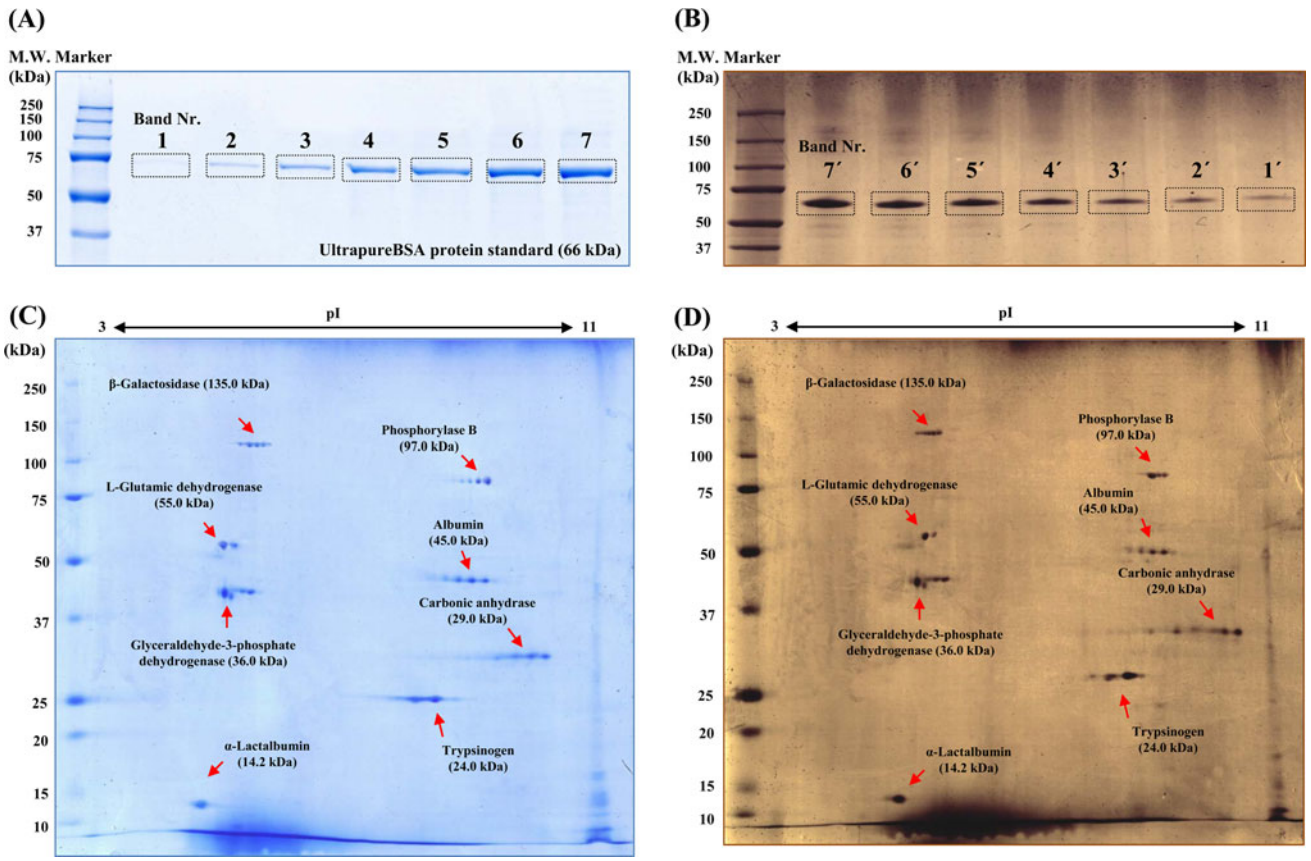


**Fig. 2** Separation of ultrapure BSA and marker protein mixture on SDS-PAGE and IEF/SDS-PAGE. 0.015625–1.0 μg series concentration of BSA was loaded onto 10 % homogenous Tris–HCl gels for SDS-PAGE and visualized with CBB G-250 (**a** band nos. 1–7) and conventional silver staining methods (**b** band nos. 1′–7′). In addition, 0.015625–1.0 μg series concentration of mixture from eight different proteins, β-galactosidase (135.0 kDa), phosphorylase B (97.0 kDa), glutamic dehydrogenase (55.0 kDa), albumin (45.0 kDa), glyceraldehyde-3-phosphate dehydrogenase (36.0 kDa), carbonic anhydrase (29.0 kDa), trypsinogen (24.0 kDa) and α-lactalbumin (14.2 kDa), were applied on IEF/SDS-PAGE and representatively shown 1.0 μg of each protein mixture. IEF/SDS-PAGE gels were stained with Coomassie blue (**c**) and silver staining (**d**)

Fig. 3). Total protein amount within a range of 0.0625 and 1.5 μg did not show significant aggregation or smear bands at the entrance of or within stacking gel for both BSA and bacteriorhodopsin, which means that this protocol is compatible with hydrophilic and hydrophobic proteins amount up to 1.5 μg without significant protein loss during electrophoresis. Higher amount of proteins needs to be optimized. Quantification of hydrophobic protein between initial amount and actual amount that migrated into gel shows discrepancy due to loss of proteins during electrophoresis. This can result in inconsistency of verification of protein quantification by Western blotting. Application of in-gel quantification of proteins by ninhydrin-based method can be an alternative to overcome problems mentioned above.

Taken together, in-gel protein quantification using ninhydrin-based method reveals a high percentage of reproducibility and this method is applicable to hydrophobic as well as hydrophilic proteins. This method is applicable for hydrophobic proteins with poor solubility and can be combined with gel-based proteomic approaches for protein identification and characterization to obtain information on sequence and modifications. A previous study has shown that different proteases can be used for in-gel digestion followed by mass spectrometrical analysis (Kang and Lubec 2011). The combination of in-gel quantification and gel-based proteomic approaches can be considered as a useful method to overcome the antibody availability problem and verification of protein quantification by Western blotting.

**Table 1** Correlation coefficients 'Linearity' of ninhydrin assay on SDS-PAGE with series concentration of BSA and on IEF/SDS-PAGE with series concentration of protein mixture. (**A**) In-gel ninhydrin assay and measurement of correlation coefficients were performed using SDS-PAGE with 0.03125–1.0 μg series concentration of BSA protein based on both two different staining methods, coomassie blue staining (band nos. 1–7) and silver staining (band nos. 1′–7′) from Fig. 2a, b. (**B**) Additionally, same protocol using different type of polyacrylamide gel, IEF/SDS-PAGE, was applied by loading 0.03125–1.0 μg series concentration of eight different proteins from Fig. 2c, d. Coefficient of variation (CV) from each sample and correlation coefficients ($R^2$) were calibrated from five independent experiments

| Staining methods[a] | Amount of protein (μg) | BSA protein standard | | | | | | | Correlation coefficients ($R^2$) |
|---|---|---|---|---|---|---|---|---|---|
| | | Blank<br>0 | 1/1′<br>0.03125 | 2/2′<br>0.0625 | 3/3′<br>0.125 | 4/4′<br>0.25 | 5/5′<br>0.5 | 6/6′<br>0.75 | 7/7′<br>1.0 | |
| (A) | | | | | | | | | | |
| C | Absorbance at 570 nm (CV[b]) | 0 | 0.051 (17.4) | 0.089 (14.6) | 0.154 (10.4) | 0.249 (9.95) | 0.557 (10.8) | 0.769 (12.6) | 1.094 (14.7) | 0.9970 |
| S | | 0 | 0.052 (13.9) | 0.081 (13.6) | 0.148 (12.1) | 0.225 (10.7) | 0.513 (9.94) | 0.701 (9.78) | 1.003 (12.3) | 0.9950 |

| Protein marker | Staining methods[a] | Protein quantity (μg) | 0 (blank) | 1.0 | 0.75 | 0.5 | 0.25 | 0.125 | 0.0625 | 0.03125 | Correlation coefficients ($R^2$) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| (B) | | | | | | | | | | | |
| β-Galactosidase | C | Absorbance | 0 | 0.816 (12.1) | 0.604 (11.6) | 0.417 (12.9) | 0.202 (13.0) | 0.129 (15.1) | 0.074 (14.8) | 0.043 (13.2) | 0.9987 |
| | S | At 570 nm | 0 | 0.811 (11.3) | 0.621 (10.3) | 0.418 (9.95) | 0.210 (10.1) | 0.126 (12.4) | 0.065 (11.9) | 0.046 (13.6) | 0.9993 |
| Phosphorylase B | C | (CV[b]) | 0 | 1.049 (9.98) | 0.778 (11.5) | 0.511 (12.7) | 0.234 (10.3) | 0.148 (10.3) | 0.074 (10.9) | 0.051 (11.4) | 0.9985 |
| | S | | 0 | 1.028 (10.8) | 0.765 (11.1) | 0.523 (10.0) | 0.228 (12.4) | 0.139 (11.7) | 0.069 (11.6) | 0.048 (10.5) | 0.9987 |
| L-Glutamic dehydrogenase | C | | 0 | 0.884 (12.4) | 0.626 (12.4) | 0.480 (11.2) | 0.228 (10.9) | 0.120 (11.4) | 0.069 (10.7) | 0.045 (9.97) | 0.9963 |
| | S | | 0 | 0.883 (9.53) | 0.617 (13.2) | 0.459 (15.0) | 0.221 (10.6) | 0.124 (12.2) | 0.063 (12.3) | 0.044 (11.9) | 0.9970 |
| Albumin | C | | 0 | 1.092 (13.7) | 0.790 (13.2) | 0.533 (11.8) | 0.277 (11.0) | 0.162 (10.2) | 0.087 (14.1) | 0.059 (14.5) | 0.9987 |
| | S | | 0 | 1.034 (8.98) | 0.757 (9.72) | 0.571 (10.9) | 0.261 (10.4) | 0.164 (11.6) | 0.080 (11.0) | 0.053 (12.1) | 0.9967 |
| Glyceraldehyde-3-phosphate dehydrogenase | C | | 0 | 1.104 (10.0) | 0.808 (12.7) | 0.538 (12.0) | 0.269 (14.6) | 0.160 (16.9) | 0.079 (15.7) | 0.063 (18.2) | 0.9989 |
| | S | | 0 | 1.101 (11.8) | 0.799 (15.2) | 0.524 (10.2) | 0.371 (12.2) | 0.149 (11.8) | 0.077 (13.2) | 0.056 (13.3) | 0.9918 |
| Carbonic anhydrase | C | | 0 | 1.151 (8.84) | 0.834 (8.67) | 0.589 (11.0) | 0.288 (10.4) | 0.158 (12.3) | 0.085 (12.7) | 0.068 (13.9) | 0.9988 |
| | S | | 0 | 1.122 (7.96) | 0.841 (10.1) | 0.583 (9.93) | 0.279 (9.91) | 0.155 (12.4) | 0.082 (12.7) | 0.064 (12.2) | 0.9993 |
| Trypsinogen | C | | 0 | 1.152 (11.2) | 0.811 (13.0) | 0.571 (12.5) | 0.294 (12.8) | 0.168 (10.7) | 0.096 (10.6) | 0.069 (11.8) | 0.9976 |
| | S | | 0 | 1.117 (10.4) | 0.794 (10.1) | 0.546 (9.94) | 0.280 (9.98) | 0.161 (11.4) | 0.098 (10.7) | 0.062 (10.7) | 0.9980 |
| α-Lactalbumin | C | | 0 | 1.128 (13.6) | 0.797 (10.9) | 0.549 (11.7) | 0.237 (11.3) | 0.156 (11.4) | 0.077 (14.6) | 0.061 (12.9) | 0.9965 |
| | S | | 0 | 1.124 (10.6) | 0.801 (11.1) | 0.552 (9.95) | 0.240 (12.0) | 0.139 (10.7) | 0.081 (13.2) | 0.062 (11.6) | 0.9972 |

[a] Staining methods: *C* coomassie staining, and *S* silver staining

[b] Coefficient of variation (CV) is a normalized measure of dispersion of a probability distribution. It is defined as the ratio of the standard deviation to the mean value (CV = standard deviation × 100 ÷ the arithmetic mean (%))

**Table 2** Observation of molecular weight and optical density of 1.0 μg of standard marker proteins on IEF/SDS-PAGE at 570 nm from in-gel and in-solution protein quantification using ninhydrin reaction with extraction (recovery) yield

| Protein name | Observed molecular weight (kDa)/PI value | OD at 570 nm (CV[a]) | | Extraction (recovery) yield[b] % |
|---|---|---|---|---|
| | | In-solution (%) | In-gel (%) | |
| β-Galactosidase, *E. coli* | 135.0/5.3 | 0.902 (6.32) | 0.816 (12.1) | 90.5 |
| Phosphorylase B, rabbit muscle | 95.0/8.7 | 1.116 (5.72) | 1.049 (9.98) | 94.0 |
| L-Glutamic dehydrogenase, bovine liver | 55.0/5.0 | 0.969 (5.50) | 0.884 (12.4) | 91.2 |
| Albumin, chicken egg | 45.0/8.5 | 1.198 (7.01) | 1.092 (13.7) | 91.2 |
| Glyceraldehyde-3-phosphate dehydrogenase, rabbit muscle | 39.0/5.2 | 1.227 (4.99) | 1.104 (10.0) | 90.0 |
| Carbonic anhydrase, bovine erythrocytes | 28.5/9.0 | 1.285 (6.68) | 1.151 (8.84) | 89.6 |
| Trypsinogen, bovine pancreas | 25.5/8.0 | 1.301 (7.00) | 1.152 (11.2) | 88.5 |
| α-Lactalbumin, bovine milk | 14.2/4.4 | 1.223 (5.47) | 1.128 (13.6) | 92.2 |

Extraction (recovery) yield of marker proteins separated on IEF/SDS-PAGE gel was determined by comparing total protein amount between in-solution and in-gel protein determination using ninhydrin reaction

[a] Coefficient of variation (CV) is a normalized measure of dispersion of a probability distribution. It is defined as the ratio of the standard deviation to the mean value [CV = standard deviation × 100 ÷ the arithmetic mean (%)]

[b] Extraction (recovery) yield (%) = mean value of in-gel (OD./in-solution (OD) × 100

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## References

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Duhamel RC, Meezan E, Brendel K (1980) Metachromatic staining with Coomassie Brilliant Blue R-250 of the proline-rich calf thymus histone, H1. Biochim Biophys Acta 626(2):432–442

Garfin DE, Dunbar DS, Kimura H, Timmons TM, Merril CR, Harrington MG (1990) Guide to protein purification. In: Deutscher MP (ed) Methods in enzymology, vol 182, 1st ednAcademic Press, Inc., San Diego, pp 425–498

Gornall AG, Bardawill CJ, David MM (1949) Determination of serum proteins by means of the biuret reaction. J Biol Chem 177(2): 751–766

Heidelbaugh ND, Huber CS, Bednarczyk JF, Smith MC, Rambaut PC, Wheeler HO (1975) Comparison of three methods for calculating protein content of foods. J Agric Food Chem 23(4):611–613

Kang SU, Lubec G (2011) Determination of in-gel protein concentration by a ninhydrin-based method. Proteomics 11(3):481–484

Kessler RJ, Fanestil DD (1986) Interference by lipids in the determination of protein using bicinchoninic acid. Anal Biochem 159(1):138–142

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193(1):265–275

Miller I, Crawford J, Gianazza E (2006) Protein stains for proteomic applications: which, when, why? Proteomics 6(20):5385–5408

Schleicher M, Watterson DM (1983) Analysis of differences between coomassie blue stain and silver stain procedures in polyacrylamide gels: conditions for the detection of calmodulin and troponin C. Anal Biochem 131(2):312–317

Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC (1985) Measurement of protein using bicinchoninic acid. Anal Biochem 150(1):76–85

Sreekrishna K, Jones CE, Guetzow KA, Prasad MR, Joshi VC (1980) A modified method for amino acid analysis with ninhydrin of Coomassie-stained proteins from polyacrylamide gels. Anal Biochem 103(1):55–57

Starcher B (2001) A ninhydrin-based assay to quantitate the total protein content of tissue samples. Anal Biochem 292(1):125–129

Tal M, Silberstein A, Nusser E (1985) Why does Coomassie Brilliant Blue R interact differently with different proteins? A partial answer. J Biol Chem 260(18):9976–9980