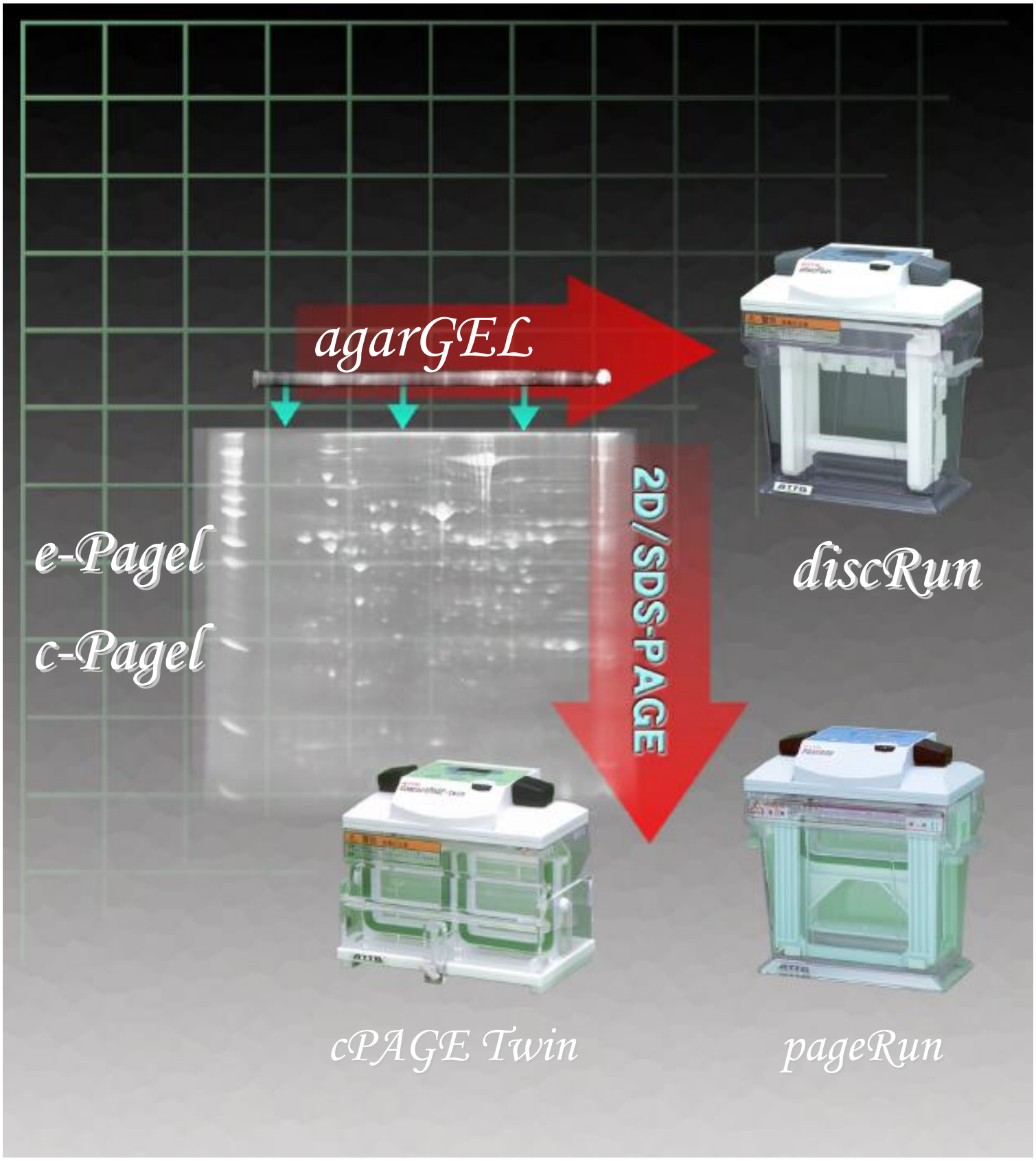
MEMO

Tips for 2-D Electrophoresis



Introduction

Electrophoresis is an essential technique for protein separation and analysis. There are various types and methods of electrophoresis, and here, 2-dimensional electrophoresis, which has been closely reexamined in recent years due to the proteome boom, is discussed. The advantage of 2-dimensional electrophoresis is **high resolution**, and because of this, 2-dimensional electrophoresis has been used for comprehensive analysis of proteins. However, because electrophoresis has to be performed twice, 2-dimensional electrophoresis is slightly complicated and time-consuming, and is occasionally considered to be cumbersome. This technical manual introduces **fast**, **small**, **inexpensive** and **simple** compact/mini-agarose gel 2-dimensional electrophoresis systems and provides tips for performing electrophoresis. The present 2-dimensional electrophoresis systems consist of first-dimension agarose isoelectric focusing (IEF) electrophoresis and second-dimension SDS-PAGE.

2-D Compact System

2-D Mini-slab System

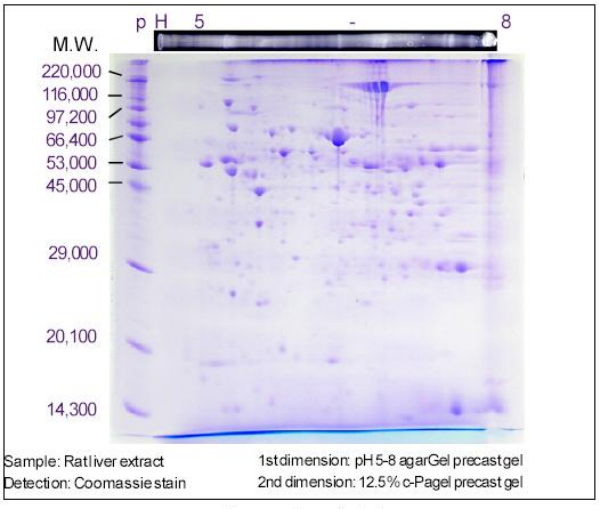
Goals

Ideal 2-dimensional electrophoresis patterns are:

- Cleanly separated spots
- Reproducible results
- Low (high S/N) and even background noise
- Accurate results

In order to satisfy these conditions, machine functions and procedural tips are important. This technical manual summarizes the tips for obtaining clean patterns and accurate results.

With ATTO 2-D Compact/Mini Systems (Agarose Gel 2-dimensional electrophoresis), because agarose gel is used in first-dimension electrophoresis, it is possible to apply a large amount of a sample and separate large molecular weight samples (i.e., transcription factors and membrane proteins). In addition, because of the superior operability of the electrophoresis system and peripheral devices, results can be obtained in as little as 1 day. ATTO 2-D Compact/Mini Systems are ideal for 2-dimensional electrophoresis.



Example of data

Experiment Flow

With ATTO 2-D Compact/Mini-slab Type Systems (agarose gel 2-dimensional electrophoresis), **2-dimensional electrophoresis can be completed in a day**, and the present technical manual explains the experimental procedures (for product information, please refer to the catalogue).

The following "Experiment Flow and Points of Caution" figure lists the main points in each step of 2-dimensional electrophoresis (vital points, points of caution and tips).

In the following pages, the procedures and points of caution in each step are explained in greater detail.

For the handling and composition of electrophoresis solutions, please refer to the instruction manual supplied with each product.

Experiment Flow and Points of Caution

Sample preparation

- As surface active agents, amphoteric CHAPS and nonionic Triton X-100 are used.
- As a reducing agent, DTT is used (the reducing power of mercaptoethanol is weak)
- Prevent protein degradation with a protease inhibitor
- Modify thiol groups (to prevent the reassembly of disulfide bonds)
- Eliminate precipitates and suspended matter

1st-dimensional electrophoresis (Agarose IEF)

- Use of a precast gel is recommended
- When preparing a hand-cast gel, do so very carefully
- Minimize the volume of sample solutions
- Use proper electrode solutions and properly set "+" and "-" terminals
- Apply 300 V of constant voltage for 150 to 210 minutes

Fixation, washing, and pretreatment

- Remove the gel from the column (Easy with agarose gel)
- Prevent protein diffusion by soaking the gel in a fixing solution (white bands are seen)
- Sufficiently wash the gel using pure water (remove ampholites)
- Soak the gel in an SDS equilibration buffer (bind SDS)

2nd-dimensional electrophoresis (SDS-PAGE)

- Use of a precast gel is recommended
- Securely adhere the first-dimension gel to the second-dimension gel
- Add a molecular weight marker (solution) by soaking it in filter paper
- Fix to the second-dimension gel using agarose
- Apply 20 - 40 mA of constant current for approximately 90 minutes

Staining and detection

- When fixed with fixing solution and stained using either the Coomassie or silver staining solution, background noise is low, and detection within a short period of time is possible.
- Stain and decolorize while stirring
- Insufficient ampholite removal leads to high background noise

Imaging and Analysis

- Use a gel documentation system
- Compare patterns
- Save analysis data

Troubleshooting

Data

Sample preparation

- Preparation of sample buffer

The optimal composition of sample buffer **depends on the type of sample analyzed**, and many studies have been conducted on this issue. The following shows the composition of a sample buffer to achieve complete dissolution and denaturation (please refer to the instruction manual for details). While referring to the composition below, adjust the composition of each sample buffer depending on sample type. Please select high-grade reagents.

- Preparation of Sample buffer

Dissolve Tris, urea *1, thiourea *1, Complete Mini EDTA-free *2, CHAPS *3 and Triton X-100 *3 in distilled water, and adjust the **pH** of the resulting solution at **8.8 - 9.0** *4. Dissolve DTT *5 immediately prior to sample preparation.

- Sample preparation

(1) Add 5 - 20 volumes of sample buffer to a sample (tissue or cells), and sufficiently dissolve the sample using devices such as a homogenizer at 4 - 10°C.
If the sample is a solution, the sample volume should not exceed 1/10 the volume of the sample buffer.
If the salt concentration is high (approximately ≥50 mM), carry out desalination first. A high salt concentration can hinder electrophoresis.
(2) Centrifuge the sample buffer at 50,000 rpm for 20 minutes at 4°C *6.
(3) Recover the supernatant. If there is a lipid layer on the liquid surface, recover the supernatant without aspirating the lipid *7.
(4) Measure the concentration of proteins as necessary *8.
(5) Add 1 M acrylamide solution or 1 M iodoacetamide (1/10 the volume of the sample buffer) and mix the resulting solution. This modifies thiol groups (-SH) to prevent the reassembly of disulfide bonds *9.

***6** With a general centrifuge, centrifugation is carried out at 15,000 rpm for 30 - 60 minutes at 4°C. For better results, ultracentrifugation is recommended. Insufficient centrifugation can lead to clogging of insoluble components in gel, thus disrupting electrophoresis patterns.

***7** Lipid contamination can lead to clogging of insoluble components, thus markedly disrupting electrophoresis patterns.

***8** With regard to the amount of sample added to gel, please refer to the section on "Electrophoresis".
While various micro-protein quantification methods and kits are available, some cannot be used due to the composition and concentration of sample buffer.
Example of use: The Amersham Biosciences "2-D Quant Kit"

***9** In order to prevent the reassembly of disulfide bonds (-S-S-) due to the reoxidation of reduced thiol groups (-SH) during electrophoresis, thiol groups are modified for better stabilization. This modifying reaction of thiol groups by acrylamide is referred to as the *Michael* reaction or *Michael* addition. Reassembly of disulfide bonds within the same protein molecules or among different protein molecules during electrophoresis can lead to the formation of homooligomer and heterooligomer spots, or can occasionally cause marked vertical and horizontal streaking on 2-D patterns (**please refer to the next page**).

ATTOPREP

Sample preparation is fundamental for favorable results.

* For desalination, concentration and separation.

***1** Urea and thiourea are mild denaturing agents, and they can improve solubility (particularly for membrane proteins).

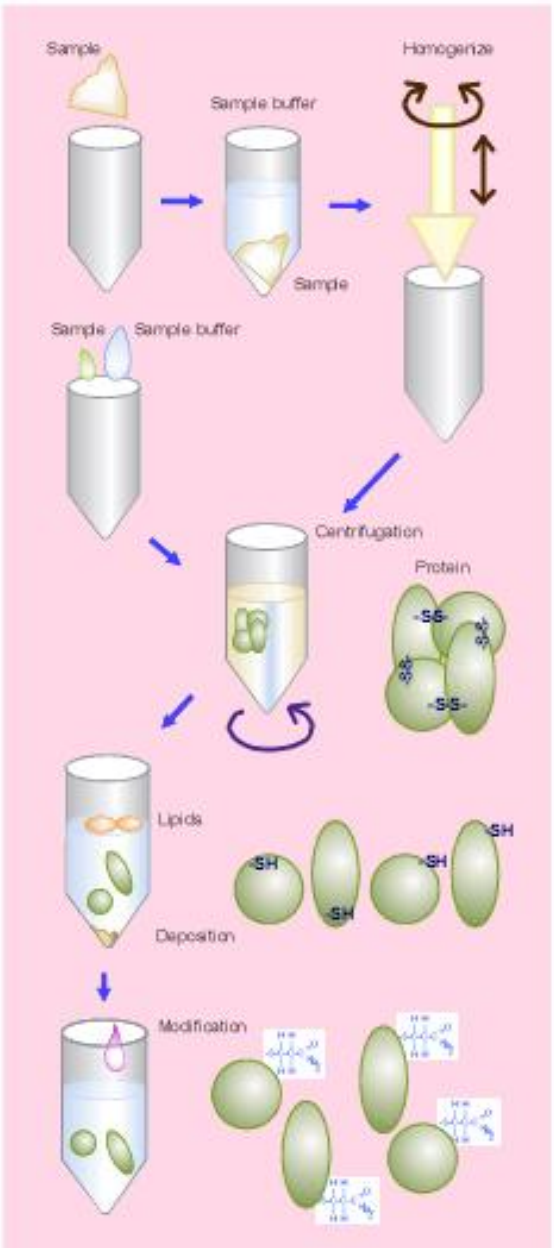
***2** Complete Mini EDTA-free is a protease inhibitor (anti-protein decomposing agent) produced by Rosh Diagnostics Inc. A reagent without EDTA is used because EDTA itself has a charge.

***3** CHAPS is an zwitterionic surface-active agent and Triton X-100 is a nonionic surface-active agent, and these compounds improve the solubility of hydrophobic proteins.

***4** The pH of 8.8 - 9.0 is important for the reduction and modification of thiol groups (see *9).

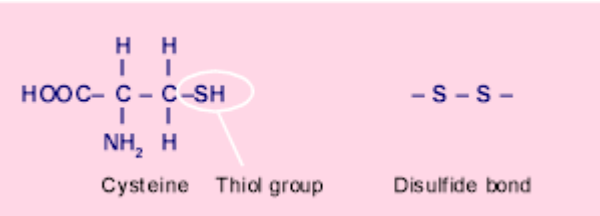
***5** DDT is a reducing agent and severs disulfide bonds (-S-S-). Mercaptoethanol is not used due to its low reducing power. In addition, sample buffers containing DTT must be stored at -20°C and used within one week.

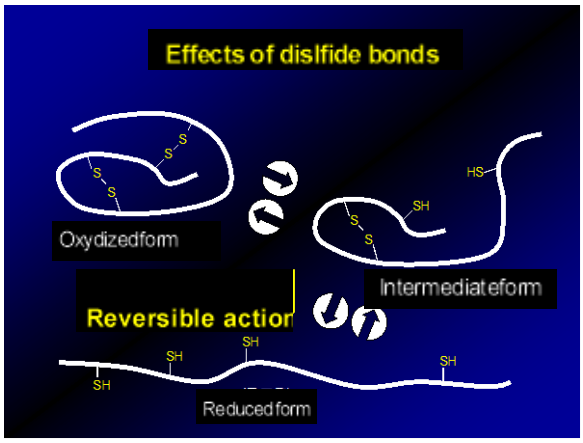
***** Sample buffer, which is prepared by dissolving and mixing Tris, urea, thiourea, Complete mini-EDTA-free, CHAPS and Triton X-100 and adjusting pH, can be stored for longer periods of time by dividing into smaller portions, storing at -20°C, and adding DTT when used.



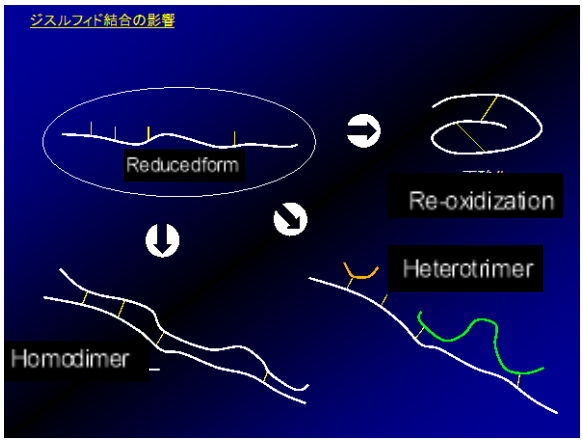
- Effects of disulfide bonds

Amino acids make up proteins, and thiol groups (-SH) found in amino acids such as cysteine are generally stable due to disulfide bonds (-S-S-).



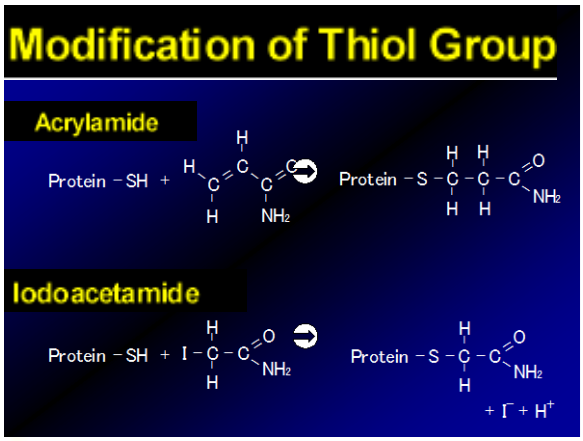


It has been clarified that even when disulfide bonds (-S-S-) are severed using a reducing agent, they may be reassembled during electrophoresis. This reassembly causes erroneous electrophoresis results. For example, when bound with proteins and peptides having other thiol groups, homooligomers and heterooligomers can form, and marked vertical and horizontal streaking can appear on 2-D patterns. Large complexes can cause gel clogging during electrophoresis.

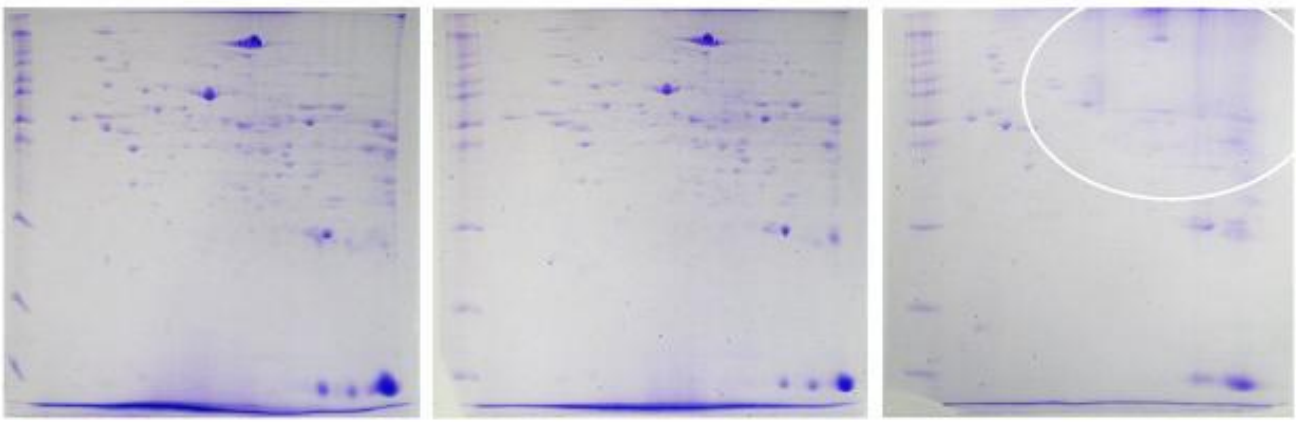


In order to prevent reassembly, a method to modify thiol groups (-SH) using either acrylamide or iodoacetamide is employed in the ATTO discRun and agarGEL protocols. The modification of thiol groups by acrylamide is referred to as the ***Michael* reaction** or ***Michael* addition**.

Naturally, the sample mass increases, but when carrying out mass spectrometry (MS), the equipment can automatically deduct the appropriate amount by entering the type of modification into the software.



Example of Data



Obtained using ATTO 2-D Compact System

Gel preparation : 1st dimentsion IEF

- Preparation of agarose gel solution

(1) Sufficiently wash and dry a gel column. *10

(2) Weigh the reagents

- Solution A: Agarose IEF, sorbitol and distilled water
- Reagent B: Urea and thiourea
- Pharmalite™ H (with an appropriate pH range)

(3) In a clear container with a tight lid (e.g., 25/50 mL conical tube), weigh and add each reagent and distilled water for Solution A, and **gently mix the resulting solution** (Solution A).

(4) Heat Solution A in a 100°C water bath *11 for approximately 20 minutes to **completely dissolve the components**. Dissolve until fine granules and crystals disappear completely.

(5) Once Solution A is dissolved, **quickly** add Reagent B. Mix the resulting solution, and **completely dissolve** Reagent B.

(6) After complete dissolution, add Pharmalite™ while gently **stirring**, and mix the resulting solution evenly. If bubbles are seen, leave the resulting solution to stand until all bubbles disappear (The resulting solution does not solidify at room temperature due to thiourea).

The first-dimension agarose gel solution is prepared in this manner.

- Preparation of gel columns

(7) Wear gloves *12. Mark thoroughly washed and dried columns using an oil-based marker 50 mm from one end for the compact-size system and 75 mm from one end for the mini-size system (gel preparation marker).

(8) Place a dialysis membrane on the end of the columns further from the gel preparation marker, and set the columns on a gel preparation rack (upper chamber).

- Injection - solidifying of the agarose gel solution

(9) Place the first-dimension agarose gel solution into a gel preparation syringe, and inject the agarose gel solution into the columns. Insert the **tip of the tube connected to the syringe to the bottom end of each** column *13, and as the liquid surface elevates, bring the tube upward.

Fill each column up to the gel preparation mark (if air bubbles are seen in the column, gently tap the column to bring the air bubbles upward). **Eliminate all air bubbles**.

(10) After injection, place 50 µL of the **overlay solution** on top of the first-dimension agarose gel solution. Using a narrow tip, **gradually** place the overlay solution along the inner wall of each column *13.

(11) Place plastic wrap over the first-dimension electrophoresis chamber, and leave the columns standing horizontally at 5 - 10°C for six hours or overnight (the agarose gel does not solidify at room temperature due to thiourea).

If the rod gels are not to be used soon after solidification, remove the columns from the gel preparation rack and seal them to **avoid drying**. Store the columns at 5 - 10°C. Rod gels in this state can be stored for 3 - 4 months.

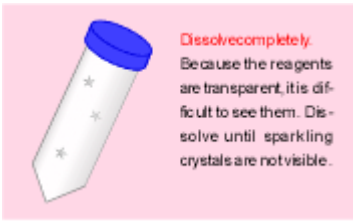
ATTO agarGEL

* Reagent unevenness, irregularities due to decreased temperature, and air bubbles lead to poor gel preparation (poor electrophoresis patterns). Gel quality markedly affects the outcome of electrophoresis.

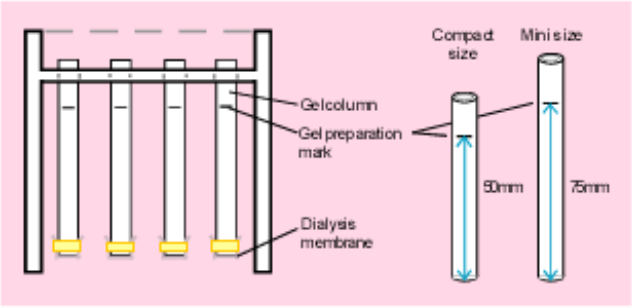
** With Pharmalite (ampholite), a pH gradient is created by electrification.

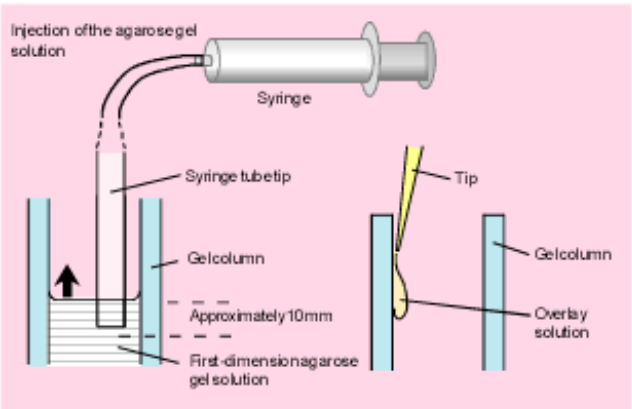
***10** Thoroughly wash and dry columns. Dirt and water droplets lead to poor electrophoresis patterns.

***11** Do not use a microwave as this can disrupt electrophoresis patterns.



***12** Do not touch the gel column, dialysis membrane, syringe, tube and tip with bare hands. A small amount of protein (e.g., keratin, etc.) can lead to the formation of false spots.





***13** If the agarose solution tube tip is not placed at the bottom of columns or the overlay solution is injected too quickly, air can clog the solution.

Electrophoresis : 1st dimentsion IEF

- Preparation for electrophoresis and sample addition

(1) To the lower electrophoresis chamber, add the lower electrode solution *14.

(2) Gently shake the gel columns to eliminate the overlay solution on top of the gel.

(3) Place the columns in the upper chamber. Plug the unused holes of the upper chamber with silicone caps.

(4) Set the upper chamber into the lower chamber, and apply an appropriate amount of sample solution to the upper end of the first-dimension agarose gel *15. Use a narrow tip, and gradually pour along the inner wall of the columns.

If CBB staining is used following second-dimension electrophoresis:

100 µg for the compact-size system (gel length: 50 mm)
200 µg for the mini-size system (gel length: 75 mm)

If silver staining is used following second-dimension electrophoresis:

1 - 2 µg for the compact-size system (gel length: 50 mm)
2 - 4 µg for the mini-size system (gel length: 75 mm)

is a rough standard for the total protein mass.

(5) Gradually place 10 µL of overlay solution *16, and then add upper electrode solution up to the upper end of the columns.

(6) Gently pour the upper electrode solution *14 into the upper chamber. Slowly add from the end without touching the column.

(7) Connect the electrode terminals and the electric power source so that the **acidic electric solution is positive (+)** *14, while the **basic electrode solution is negative (-)** *14.

- Electrification (electrophoresis)

(8) Apply **300 V of constant voltage** *17 for **150 minutes using the compact-size system (gel length: 50 mm)** and for **210 minutes using the mini-size system (gel length: 75 mm)** *17. In addition, when connecting an external power supply unit, choose a **unit that can generate a low current**. Only currents of **about 1 mA** flow.

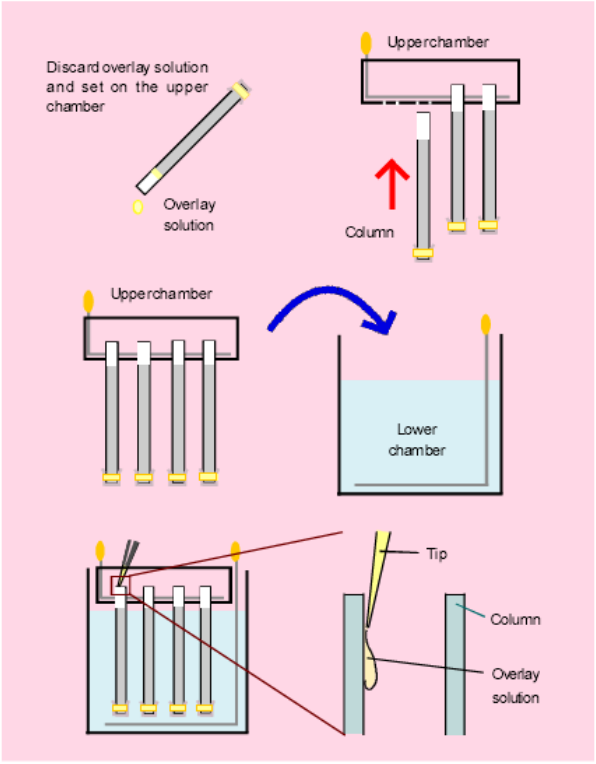
*AE-6541 discRun, an electrophoresis system equipped with an integrated power supply module, is convenient.

AE-6541 discRun
(for hand-cast gel/for precast gel)

- Marker

When using the second-dimension marker, IEF electrophoresis marker and molecular weight marker from the first-dimension electrophoresis, purchase those that **can also be used with a denaturing system (includes urea)**. Because the present specification includes urea in the gel, when using markers for an undenatured (native) system, accurate results may not be obtained. While colored markers are convenient for confirming electrophoresis, exercise caution regarding the above-mentioned issues.

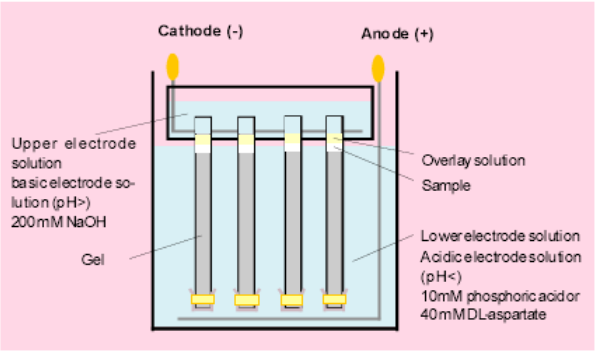
Example: BioRad 2-D Standards (second-dimension marker)



Guidelines for sample volume

Detection	Compact-size system (gel length: 50 mm)	Mini-size system (gel length: 75 mm)
CBB staining	100 µg	200 µg
Silver staining	1 - 2 µg	2 - 4 µg

*15 While the maximum sample volume is 50 µL, smaller volumes are desirable.



*14 Upper electrode solution = basic electrode solution (pH>)
= 200 mM NaOH ← cathode "-"
Lower electrode solution = acidic electrode solution (pH<)
= 10 mM phosphoric acid or 40 mM DL-aspartate ← anode "+"
Do not use L-aspartate due to its low solubility.
With some samples, "+" and "-" are reversed.
Use the above-mentioned electrode solutions regardless of pH range.

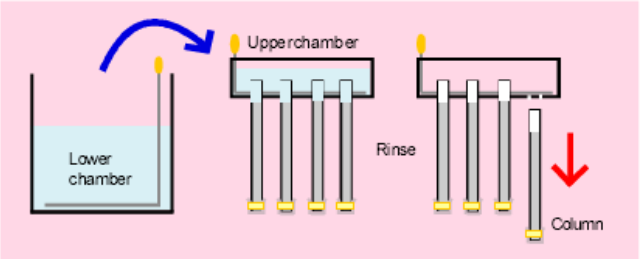
*16 This prevents sample precipitation caused by direct contact of the sample with the electrode solution. By adding BPB to a final concentration of approximately 0.01%, electrophoresis (movement of BPB inside gel towards the anode) can be confirmed.

*17 With agarose gel, there is no need to increase voltage in steps like the O'Farrell technique. Even with longer electrification, the sharpness of bands (band convergence) hardly changes. Observe the recommended time to avoid drifting of the pH gradient.
Reduce the voltage when carrying out electrophoresis for longer periods of time (overnight). Maintain the Vhr value (voltage × time) at a constant value (e.g., 300 V × 210 min = 70 V × 900 min [15 h]).

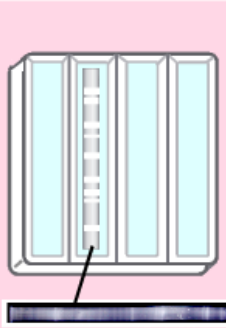
Fixation, washing and pretreatment

(1) After the end of electrophoresis, remove the upper chamber from the lower chamber, and discard the upper electrode solution. Use distilled water to wash out the upper and lower electrode solutions.
(2) Remove the columns from the rack, and without tipping out the gel, turn the column **horizontally** and remove the dialysis membrane. Tilting the columns may cause the gel to fall out.
(3) On top of a tray including the fixing solution *18, tilt each column to slide out the rod gel. Gradually immerse the gel into the fixing solution, and gently stir the gel for three minutes.
(4) After discarding the fixing solution, use distilled water to wash the gel. Discard the distilled water, and add fresh distilled water to wash the gel for one minute. Repeat this washing procedure for a total of three times.
(5) Again, discard the distilled water, and add fresh distilled water to gently stir for **2 hours** *19.
(5)' (Storage) If the second-dimension electrophoresis is not carried out immediately, gels can be stored at this stage *20.
(6) Once the preparation for second-dimension electrophoresis is completed, discard the distilled water, add 100 mL of SDS equilibration buffer *21, and gently stir for 10 minutes.

***18** Trichloroacetic acid (TCA)
***19** Washing with distilled water is performed to remove ampholines. Insufficient washing at this stage leads to higher background noise (refer to data on page 9).
***20** Gels can be stored in distilled water for a couple of days (refrigerator or room temperature). When storing gels for more than a couple of days (up to 3 months), place gels in plastic wrap and store them frozen. Be certain to thaw gels at room temperature before use.
***21** 50 mM Trs-HCl pH 6.8, 2% SDS and 0.01% BPB



Gel removal
The gel naturally slides out by tilting the gel column, but if the gel does not slide easily, use a rubber cap or micropipette to force out the gel using air pressure.



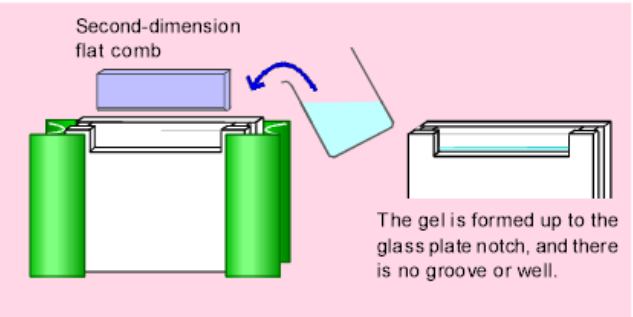
Gel image after equilibration

Gel preparation:2nd dimension (SDS-PAGE)

Because second-dimension electrophoresis is standard SDS-PAGE (Laemmli system), details are omitted.
(1) Prepare the following solutions:
- 30% acrylamide solution (acrylamide + bisacrylamide)
- Tris-hydrochloric acid (pH: 8.8) for separating gel
- Tris-hydrochloric acid (pH: 6.8) for concentrating gel
- 10% ammonium persulfate (APS), prepare when needed
- TEMED

(2) Assemble the electrophoresis plate for gel preparation.
(3) Select a gel concentration *22 matching the target molecular weight range.
(4) Prepare a separating gel solution. Place the solution in a graduated beaker, and lastly, add APS and TEMED. Pour the resulting solution into the glass plate.
(5) Place distilled water on top *23, and leave the resulting solution to stand. The solution gels *24 in approximately 40 minutes.
(6) Prepare a concentrating gel solution.
(7) After confirming the polymerization of the separating gel, discard the distilled water, and after washing the upper end of the gel using the concentrating gel solution, pour the solution. Position the second-dimension comb*, and leave the resulting solution to stand. Gelation takes approximately 40 minutes.

***22** In general, set the concentration at 5 - 20% (a molecular-weight range of about 10,000 - 400,000 Da).
***23** Because oxygen (air) hinders the gelation (polymerization) of acrylamide, distilled water is placed on top to block out air and flatten the upper end of the gel.
***24** The gel is solidified when the borderline between the gel solution and distilled water disappears and then reappears. The time for gelation differs depending on gel concentration and temperature.



* The second-dimension comb is flat (no groove or well).

* Precast gels
The "e-Pagel" and "c-Pagel" series are available.
These gels can be used straight out of the bag.

Electrophoresis: 2nd dimension (SDS-PAGE)

- Application of first-dimension gel

(1) Place the notch of the electrophoresis plate (second-dimension gel) upward and forward.

(2) When applying a molecular weight marker (solution), soak the molecular weight marker using a square piece of clean **filter paper** (3×3 mm) and place the filter paper on the top end of the gel using tweezers. Use two pieces of filter paper if the sample volume is high.

(3) Place the SDS-equilibrated first-dimension gel in front of the notch of the electrophoresis plate without damaging the gel.

(4) **Adhere** the first-dimension gel to the top of the second-dimension gel **very carefully from one end in small increments without creating air bubbles**.

(5) Heat and dissolve the agarose solution to adhere the first-dimension gel *25, and place a total of 100 µL at the contact region between the first-dimension gel and the upper end of the second-dimension gel and the contact region between the filter and the second-dimension gel.

* Insufficient adhesion between the first- and second-dimension gels causes spots to spread sideways. Air bubbles can also disturb electrophoresis patterns. This procedure must be carried out carefully and attentively.

***25** Heat and dissolve the 1% agarose (general agarose for electrophoresis)/SDS-PAGE electrophoresis buffer, and divide the resulting solution in 1 mL portions.

- Second-dimension electrophoresis

(6) Beforehand, place electrophoresis buffer *26 into the lower electrophoresis chamber of the second-dimension (slab-type) electrophoresis system.

(7) Set the electrophoresis plate (gel) with the first-dimension gel in the upper electrophoresis chamber.

(8) Place the upper electrophoresis chamber with the electrophoresis plate in the lower electrophoresis chamber.

(9) Place an appropriate amount of the electrophoresis buffer in the upper chamber. Place a safety cover over the power supply module, and connect the lead wire and the power supply module.

(10) Set the electrification conditions according to the specifications of the electrophoresis system, and begin electrophoresis.

(11) When BPB moves to within 3 - 5 mm of the gel tip, stop electrification. Electrophoresis is completed in about 35 minutes with ATTO AE-7350 cPAGE or AE-7341 cPAGE-twin and 80 - 90 minutes with ATTO AE-6531 mPAGE (pageRun) and mini-slab type systems.

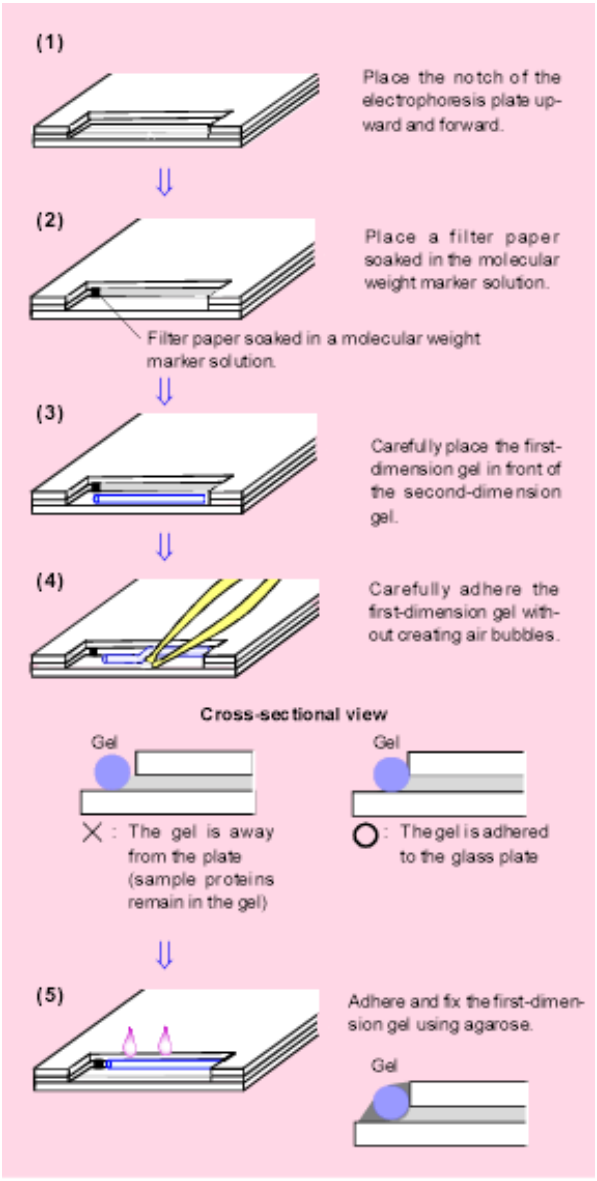
* The setting and electrification conditions vary among the different systems. Follow the instruction manual enclosed with each system.

***26** 25 mM Tris, 192 mM glycine, and 0.1% SDS

* Electrophoresis systems equipped with a power supply module, such as the "ATTO AE-7341 cPAGE Twin" and "ATTO AE-6531 mPAGE (pageRun)" are recommended.

AE-7341 cPAGE Twin

AE-6531 mPAGE (pageRun)



Electrification conditions

Electrophoresis system	Setting (mode)	Electrification time
ATTO AE-7350 CompactPAGE	Tris-Gly/PAGEL High	35 min.
ATTO AE-7341 CompactPAGE Twin	Tris-Gly/PAGEL	
ATTO AE-6531 mPAGE (pageRun)	Constant current 20 mA (1 gel sheet)	85 min.
ATTO AE-6450 Dual Mini Slab Kit	Constant current 40 mA (2 gel sheets)	

* Electrification times are guidelines and should be adjusted according to your sample, protocol, or ambient temperature.

Gel staining

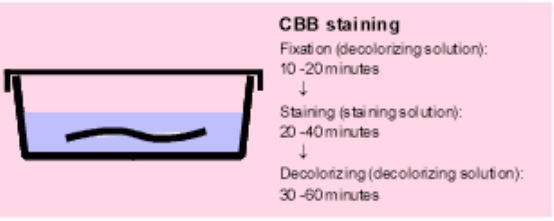
- CBB stain (Coomassie brilliant blue stain)

(1) Place decolorizing solution in a tray [*27] that is slightly larger than the gel.

(2) Remove the gel from the glass plate. Soak the gel in fresh decolorizing solution, and stir the gel to fix the proteins [*28].

(3) Discard the decolorizing solution, and stir the gel in CBB staining solution.

(4) Discard the staining solution, and stir the gel in fresh decolorizing solution. The staining procedure is completed while a slight amount of background noise remains.



*27 Select a tray that is one to several centimeters larger than the gel in order to allow the gel to be sufficiently soaked in the solutions. A tray with a lid is convenient. A commercially available plastic container is sufficient.

*28 Fixation reduces the time for staining and decolorizing. Stirring ensures that the entire gel is stained and decolorized evenly. Stir so that the gel does not remain in one place.

- Silver staining, fluorescent staining and negative staining

Follow the protocol for each stain (references and instruction manuals).

* Silver staining kit
"*EzStain Silver*"
Does not contain glutaraldehyde, which is unsuitable for mass spectrometry.


"*EzStain Silver*"
(Currently sold only in Japan)

Imaging and Analysis

2-dimensional electrophoresis patterns are recorded using a gel documentation system ("ATTO Printgraph" series, etc.), a flatbed scanner with a transmitted light source, or a digital camera. Select an appropriate device by studying your purpose to use the stored images.

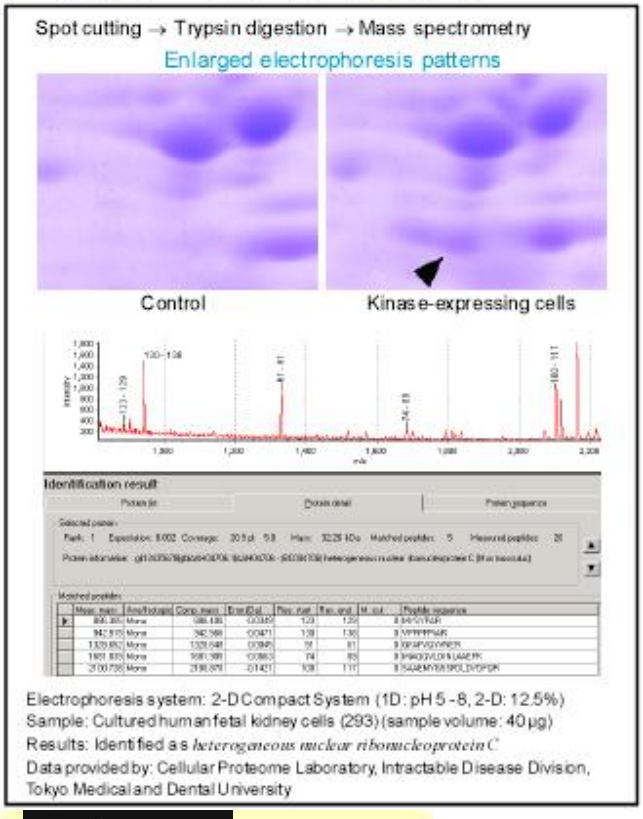
Intended use (1): Quantify the concentration of spots
Recommended device: **ATTO Printgraph Series**
Advantage: With the Printgraph, a transmitted light source is used to obtain highly quantifiable image data that are suitable for determination.
Disadvantage: Due to problems with pixel count, small spots may not be detected.

Intended use (2): Obtain high-quality images for manuscript publication
Recommended device: Flatbed scanner
Advantage: A scanner is suitable for obtaining high-quality images due to its high resolution and small image aberration.
Disadvantage: Because image data are subjected to automatic correction during scanning, quantification of low-concentration bands may be poor.

Intended use (3): Record and store electrophoresis results
Recommended device: Digital camera
Advantage: High-pixel digital cameras have a high degree of resolution, and are widely available.
Disadvantage: Because quantifiability is low, a digital camera is not suitable for determining the concentration of spots. In addition, digital cameras are not suitable for capturing patterns with dark or pale spots.

The following methods can be used to analyze the components of target spots: (1) concentration determination, (2) isoelectric and molecular weight measurement, and (3) mass spectrometry (MS).
(1) Concentration determination: The degree of staining is quantified based on the absorption rate (degree) of a light source. Image analysis software is used. The expression of a target protein is quantified, and results are compared among samples under different conditions.
(2) Isoelectric and molecular weight measurement: Based on positional data in the gel for isoelectric and molecular weight markers, pH is plotted along the X axis, while molecular weight is plotted along the Y axis for calibration. Based on the coordinates (X, Y) of target spots, isoelectric point and molecular weight are estimated using each calibration curve. Image analysis software is generally used. The physical properties of proteins, i.e., isoelectric point and molecular weight, can be quantified.
(3) Mass spectrometry (MS): Stained spots are excised and subjected to mass spectrometry. Please refer to the mass spectrometer protocols for details. The physical properties of proteins can be quantified.

An example of mass spectrometry (MS) data



Electrophoresis system: 2-D Compact System (1D: pH 5 - 8, 2-D: 12.5%)
Sample: Cultured human fetal kidney cells (293) (sample volume: 40 µg)
Results: Identified as *heterogeneous nuclear ribonucleoprotein C*
Data provided by: Cellular Proteome Laboratory, Intractable Disease Division, Tokyo Medical and Dental University


ATTO Printgraph

Troubleshooting

Refer to the following for common troubles with electrophoresis patterns and countermeasures:

- First-dimension bands are not sharp
Second-dimension spots are spread sideways

(1) Insufficient protein reduction

Use DTT, not mercaptoethanol, as a reducing agent. Even when stored at -20°C, the reducing power of dissolved DTT begins to decrease after one week.

(2) Insufficient modification of protein thiol groups

Prevent reassembly of disulfide bonds by modifying thiol groups by adding either acrylamide or iodoacetamide to the sample buffer. If pH at this stage is not within 8.8 - 9.0, the efficiency of modification is compromised.

(3) Protein degradation or deterioration

Prevent degradation by preparing samples on ice or adding a protease inhibitor to the sample buffer. Store sample buffers containing dissolved proteins at -80°C, avoid repeated freezing and thawing, and use within 2 weeks.

(4) Lipid and insoluble matter contamination

Sufficiently centrifuge sample buffers containing dissolved proteins to separate and eliminate lipids and insoluble matter.

(5) Sample overload

Set the sample volume at ≤100 µg for the compact-size system (gel length: 50 mm) and ≤200 µg for the mini-size system (gel length: 75 mm).

(6) Poorly prepared first-dimension agarose gel

If agarose IEF powder is not completely dissolved during the agarose gel preparation, sharp bands cannot be obtained. Remake agarose gel if the gel contains air bubbles or is contracted due to drying.

(7) Insufficient electrophoresis (electrification)

Apply 300 V of constant voltage for 150 minutes for the compact-size system (gel length: 50 mm) and 210 minutes for the mini-size system (gel length: 75 mm).

(8) Poor adhesion between the first and second-dimension gels

When placing the first-dimension gel on top of the second-dimension gel, a gap between the two gels causes proteins to diffuse or spots to spread sideways. Evenly place the agarose solution for adhesion on the top end of the second-dimension gel, and carefully adhere the first-dimension gel from one end without creating air bubbles.

(9) Reassembly of disulfide bonds

Commercially available anti-streaking agents appear to be effective.

- Spots are undetectable

(1) Insufficient sample volume (especially with CBB staining)

The sample volume may be insufficient if molecular weight marker bands are seen, but not sample spots. If staining is performed using CBB, restain using a silver stain. If the amount of proteins cannot be quantified, as a preliminary study, prepare a dilution series of the sample, and perform (SDS-)PAGE.

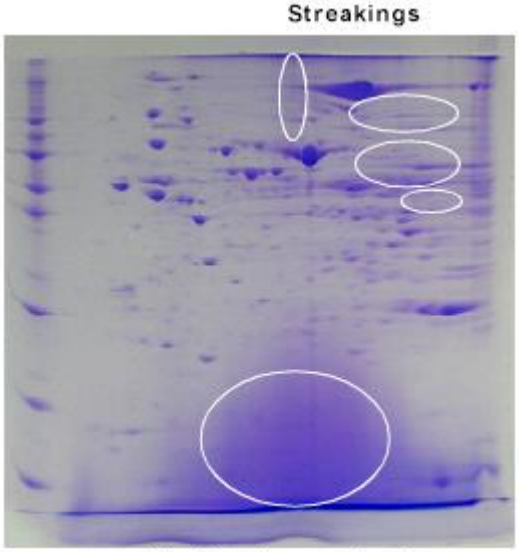
(2) Improper first-dimension electrophoresis

If stripes are not seen after fixation of the first-dimension gel or spots are not spread throughout the gel, the first-dimension electrophoresis might not have been performed properly.

(3) Poor detection (even the markers are not seen)

Following silver staining or blotting, poor detection may cause spots to be undetected. Always apply internal standards (molecular weight markers) with the sample.

- Common problems (actual data)



Streaking

The causes of streaking include poor sample preparation (dissolution, pH, degradation, -S-S- reassembly, etc.), improper first-dimension electrophoresis and poor adhesion of the rod gel to the second-dimension gel.

Horizontal streaking

The causes of horizontal streaking include, poor first-dimension electrophoresis (including sample preparation) and poor adhesion of the second-dimension gel. Confirm whether first-dimension electrophoresis was conducted properly by checking for opaque patterns following first-dimension fixation (refer to page 6). If stripes are not seen (except for when the sample volume is low), refer to the sections on sample preparation and first-dimension electrophoresis. If streaking on the basic side is notable or reproducible, refer to the section on sample preparation.

High background noise

When washing following first-dimension electrophoresis and gel fixation is insufficient, ampholites remain in the rod gel, and this increases background noise in the low molecular weight region.

- Poor second-dimension spots
Vertically streaking spots

(1) Insufficient protein reduction

Use DTT, not mercaptoethanol, as a reducing agent. In addition, even when stored at -20°C, the reducing power of dissolved DTT decreases after one week.

(2) Insufficient modification of protein thiol groups

Prevent reassembly of disulfide bonds by modifying thiol groups by adding either acrylamide or iodoacetamide to the sample buffer. If pH at this stage is not within 8.8 - 9.0, the efficiency of modification is compromised.

(3) Protein degradation or deterioration

Prevent degradation by preparing samples on ice or adding a protease inhibitor to the sample buffer. Store sample buffers containing dissolved proteins at -80°C, avoid repeated freezing and thawing, and use within 2 weeks.

- High background noise (particularly in the lower section of gel)

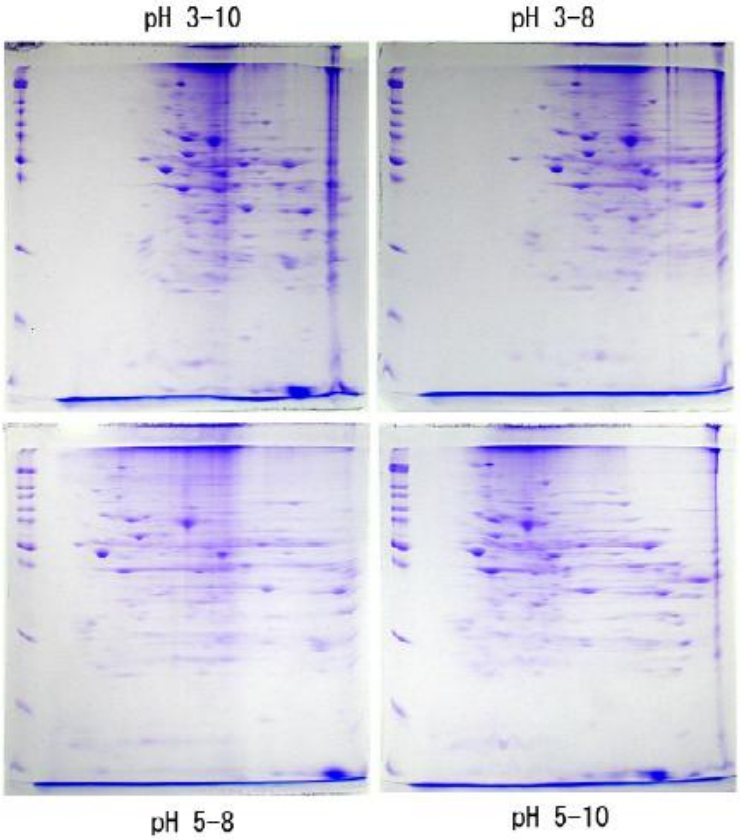
Insufficient washing of first-dimension gel

Thoroughly wash the fixed first-dimension gel following electrophoresis in distilled water. Insufficient washing causes ampholites to remain in the gel and increases background noise after second-dimension electrophoresis.

Sample Data

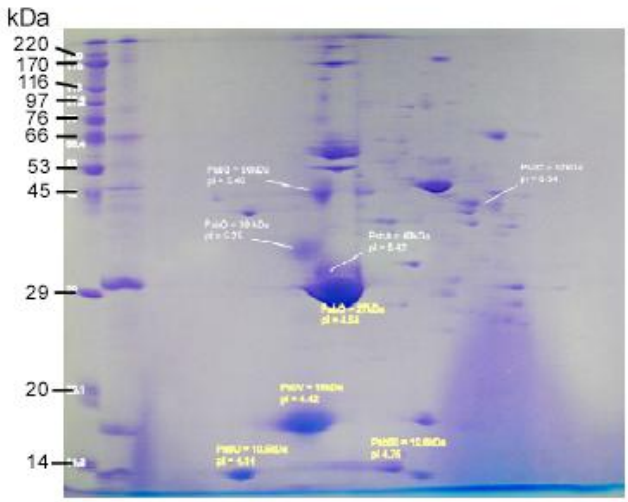
pH range variation

2D Compact System
Sample: Rat kidney
1st dimension:
Hand-cast agarose gel
2nd dimension:
12.5% hand-cast polyacrylamide gel,
60 x 60 x 0.75 mm
Detection: CBB stain

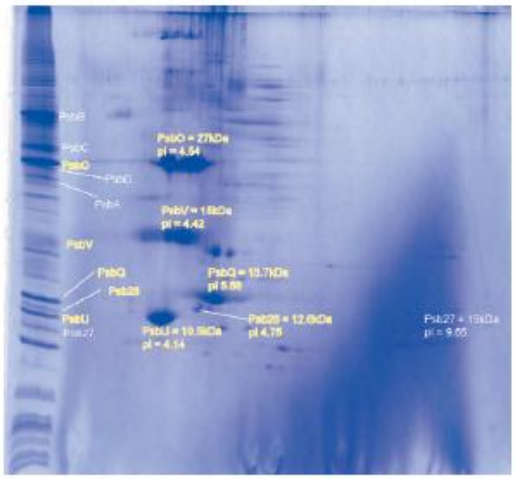


Comparison to IPG dry-strip gel

2D Mini-slab System
Sample: thylakoid membrane protein, 70 microg (In the gel data shown below, hydrophobic proteins are discribed in yellow and hydrophilic proteins are discribed in white.)
1st dimension: pH 3-10 agarGELI (2.5 mm I.D. x 75 mm)
2nd dimension: 12.5% polyacrylamide hand-cast gel, 90 (W) x 80 (H) x 1 (T) mm
Detection: CBB stain



ATTO Mini-slab system (IEF for 1st dimension)



IPG system from another manufacturer
(PAGE for 1st dimension)

* Good detection result, particulary for hydrophilic proteins (described in white)