

SCIENCE IMAGING SYSTEMS

# Application Note No.10

## Two-dimensional Protein Electrophoresis and SYPRO® Orange Staining

FLA-2000

### Introduction

The FLA-2000 fluorescent image analyzer can read SYPRO® Orange stained gels with high sensitivity. SYPRO® Orange, a fluorescence staining reagent, makes protein detection in gel electrophoresis easier than with Coomassie Brilliant Blue (CBB) staining and offers detection sensitivity comparable to silver staining. It is a useful reagent for either first- or second-dimension SDS-PAGE of proteins.

Although two-dimensional (2-D) electrophoresis of proteins provides tremendous amounts of information, it fails to reach its full potential with CBB staining, owing to inadequate sensitivity, and its appeal diminishes with silver staining because of the complexity of the staining procedure and the failure of some spots to stain efficiently. The combination of the FLA-2000 with SYPRO® Orange changes all of this.

In this Application Note, researchers at the Kyoto Municipal Institute of Industrial Research detail their protocol for 2-D protein electrophoresis of brewer's yeast proteins and compare three staining methods.

### Contents

1. Introduction
2. Protocols for 2-D electrophoresis of proteins
3. Comparison of protein detection methods
4. References

### Summary

- SYPRO® Orange and the FLA-2000 ; a combination with comparable or higher sensitivity than silver staining.
- With SYPRO® Orange staining, unlike with silver staining, no loss of stained spots is experienced at high concentration.
- The conventional CBB staining and silver staining methods can be applied to the same sample after SYPRO® Orange staining.

# 1 Introduction

The 2-D electrophoresis method developed by O'Farrell et al. (Reference 1) has attracted considerable attention for its ability to separate over 1,000 proteins, and even to recover small amounts of protein, in a single operation. Although advances in molecular biology and genetic methodologies now make the approach from the DNA side more effective than protein analysis in many settings, protein analysis is still a very effective method.

It is, for instance, a powerful tool for clarification of gene expression mechanism when the sample amount is large.

Although differential display is more often than not the method of choice for this purpose, chiefly because it enables direct recovery of the expressed gene from an extremely small sample, this method requires sophisticated condition settings. It is also expensive.

In the following, the protocols used in 2-D electrophoresis are explained and a staining method using SYPRO® Orange is described, taking yeast as an example.

## Differential display method

A method utilizing reverse transcription and PCR to identify differences in expressed mRNA.

# 2 Protocols for 2-D electrophoresis of proteins

## Isoelectric focusing (First dimension)

### • Preparation of reagents

#### Lysis buffer

Urea	4.8 g
Nonidet P-40	0.2 ml
Carrier ampholite (pH 3.5-10)* <sup>1</sup>	0.2 ml
2-Mercaptoethanol	0.5 ml

Make up to 10 ml with distilled water.

#### Acrylamide solution for isoelectric gel

Acrylamide (monomer)* <sup>2</sup>	28.4 g
N,N'-Methylenebisacrylamide	1.6 g

Make up to 100 ml with distilled water.

### • Preparation of isoelectric disk gel

Urea	4.8 g
Acrylamide solution (Acrylamide 28.4%, Bis 1.6%)	1.10ml
Distilled water	2.90ml
Carrier ampholite (pH 3.5-10)* <sup>1</sup>	0.25ml
Carrier ampholite (pH 5-7)* <sup>1</sup>	0.25ml
10% Nonidet P-40	2.0 ml
10% APS solution	30µl
Mix well with stirrer; when urea has dissolved add	
TEMED	30µl

Then pour gently into glass tubes sealed at the lower end with parafilm.  
Add distilled water on top of acrylamide solution.

\*<sup>1</sup> Ampholine used.

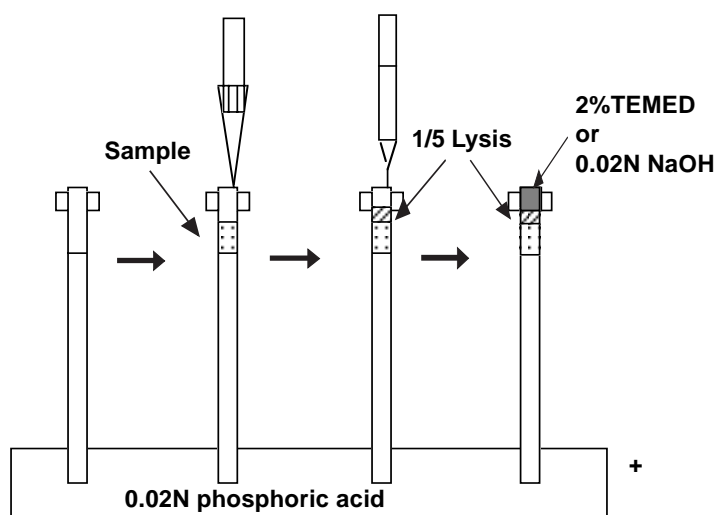
\*<sup>2</sup> Highly purified reagent required.  
(e.g., SP-grade isoelectric products from Bio Rad or Nacalai Tesque)

## APS

Ammonium persulfate

• **Isoelectric focusing**

- (1) Remove water on gel.
- (2) Fill lower electrode reservoir (positive side) with anode electrolyte (0.02N phosphoric acid).
- (3) Remove parafilm from glass tubes and place bottoms of tubes in electrode reservoir.
- (4) Attach upper electrode reservoir (negative side) and pour in sample.
- (5) Add 20 ml of Lysis Buffer diluted fivefold on top of sample.
- (6) Fill glass tubes to top with cathode electrolyte (0.02N NaOH or 2% TEMED).
- (7) Fill upper electrode reservoir (negative side) with cathode electrolyte.
- (8) For 15-cm gel, apply 200 V for 1 hr, 400 V for 16-18 hr and 600 V for 3 hr.



• **Confirming first-dimension isoelectric electrophoresis (Confirmation against isoelectric standard etc.<sup>\*3</sup>)**

- (1) Soak one removed isoelectric gel in 15% trichloroacetic acid solution for one hour. (The gel will turn cloudy white.)
- (2) Wash surface gently with distilled water, then stain with CBB.<sup>\*4</sup>

<sup>\*3</sup> Protein Test Mixture for Pi determination pH 3-10 (product of Serva Feinbiochemica GmbH & Co.) currently used.

**Trichloroacetic acid (CCl<sub>3</sub>COOH)**

Protein denaturant

<sup>\*4</sup> Gel used for confirmation cannot be used for 2-D electrophoresis.

<sup>\*5</sup> Nacalai Tesque SP used.

**SDS electrophoresis (Second dimension)**

• **Preparation of isoelectric gel treatment buffer**

0.5M Tris-HCl (pH 6.8)	25 ml
SDS <sup>*5</sup>	5 g

Make up to 200 ml with distilled water.

<sup>\*6</sup> Isoelectric gel treatment is carried out to replace the buffer in the first-dimension gels containing carrier ampholite.

• **Treatment of isoelectric gel<sup>\*6</sup>**

- (1) Take each isoelectric gel out of its glass tube and place it in a 100-ml beaker, then add 10 ml of the treatment buffer and 500 ml of 2-mercaptoethanol to the beaker and shake for 20 min.
- (2) Remove the solution from (1), add 10 ml of fresh isoelectric gel buffer and shake for 20 min. Remove the treatment buffer, add 10 ml of fresh isoelectric gel buffer and shake for 20 min.<sup>\*7</sup>

**2-mercaptoethanol (CH<sub>2</sub>(SH)CH<sub>2</sub>(OH))**

Reducing agent; used as antioxidant and to cleave S-S bonds in protein.

<sup>\*7</sup> Buffer was repeatedly changed because residual carrier ampholite increases background caused by SYPRO® Orange staining.

## • Preparation of SDS electrophoresis reagents

### Reagents for gel preparation

- 30% acrylamide, 0.135% bisacrylamide solution
- 30% acrylamide, 0.8% bisacrylamide solution
- Separating gel buffer (1M Tris, 0.27% SDS pH 8.8)
- Stacking gel buffer (0.5M Tris, 0.4% SDS pH 6.8)

### Electrode solution

- Tris-HCl 3.0 g
- Glycine 14.4 g
- SDS 1.0 g

Dissolve in distilled water to 1000 ml.

### 1% agarose for adhering isoelectric gel

- Agarose for electrophoresis\*<sup>8</sup> 1 g
- 0.5M Tris-HCl (pH 6.8) 12.25 ml
- SDS 2.5 g

Add Bromphenol Blue (BPB) dissolved in methanol.\*<sup>9</sup> Dilute to 100 ml with distilled water. Melt in microwave oven and pour into per-use lots.

## • Gel preparation

SDS separating gel (20 X 20 cm/slab)\*<sup>10</sup>

Composition	10 %	12 %	15 %	17 %	18 %
Acrylamide solution (ml)	6.6	8.0	10.0	11.3	12
1M Tris-HCl (0.27% SDS pH 8.8) (ml)	7.5	7.5	7.5	7.5	7.5
Distilled water (ml)	5.9	4.5	2.5	1.1	0.5
10% APS solution (ml)	60	60	60	60	60
TEMED (ml)	30	30	30	30	30

### SDS stacking gel

- Acrylamide solution 2 ml
- 0.5M Tris-HCl (0.4% SDS pH 6.8) 3 ml
- Distilled water 7 ml
- 10% APS solution 30 ml
- TEMED 20 ml

## • SDS electrophoresis

- (1) Assemble gel plates.
- (2) Prepare separating gel of suitable composition (see table above), then pour between gel plates.
- (3) Gently overlay with water.
- (4) When gel has set, discard water and wash upper surface of gel once with fresh, distilled water.
- (5) Prepare stacking gel of composition shown above, then pour between gel plates.
- (6) Thoroughly eliminate bubbles from gel surface using a pipette.
- (7) Apply small amount of 1% agarose (isoelectric gel adhesive) melted in microwave oven to top of gel.
- (8) Place first-dimension gel in firm contact with 1% agarose.

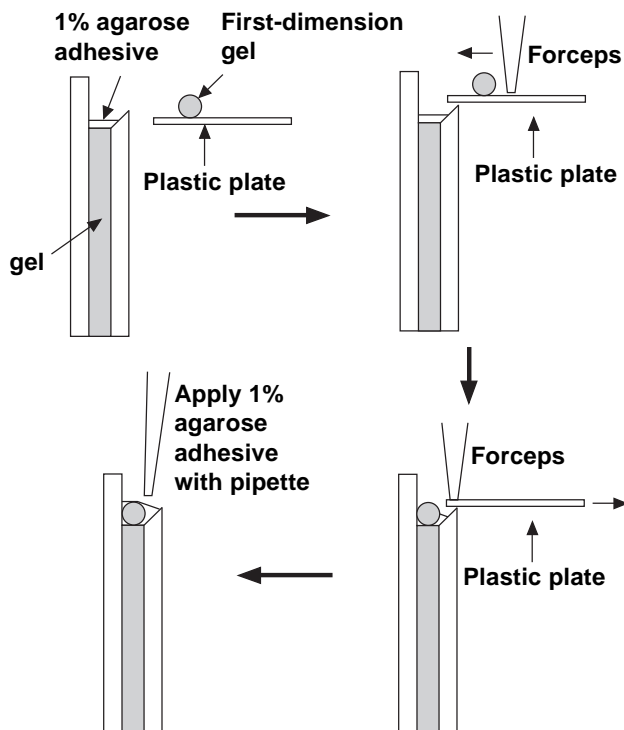
General-purpose acrylamide is sufficient. (Nacalai Tesque SP)

\*<sup>8</sup> Agarose ME (Nacalai Tesque) used.

\*<sup>9</sup> Should be kept to absolute minimum when using SYPRO® Orange. 1/8 of micro-spatula was used here. Large amount of BPB increases background. BPB remaining in gel is detected by FLA-2000.

\*<sup>10</sup> Gel concentration is selected depending on sample to be analyzed. Gel with ratio of acrylamide to bis of 30:0.135 is considered to provide optimum blotting efficiency

- (9) Attach second-dimension gel and first-dimension gel by the 1% agarose, ensuring that no bubbles get in between.



- (10) Electrophoresis.<sup>\*11</sup>

## Spot detection using SYPRO® Orange

- Transfer electrophoresed gels to vat.<sup>\*12</sup>
- Stain gels for 30 min in well-blended 5000:1 mixture of 7.5% acetic acid and SYPRO® Orange.
- Wash gels in distilled water for 15 min. Repeat. Use as much distilled water as possible each time. When staining with silver or CBB, go to step (5).
- Read image with FLA-2000.<sup>\*13</sup>
- Silver staining**<sup>\*14</sup>:

Prepare 50:20:30 mixture of ethanol:acetic acid:water as destaining solution.

Soak gels in destaining solution for 20 min with shaking.

Carry out silver staining procedure.

### CBB staining

Prepare 50:20:30 mixture of ethanol:acetic acid:water as first destaining solution.

Soak gels in first destaining solution for 20 min with shaking.

Prepare 20:5:75 mixture of ethanol:acetic acid:water as second destaining solution.

Soak gels in second destaining solution for 20 min with shaking.

Soak gels in CBB staining solution and carry out ordinary CBB staining procedure.

<sup>\*11</sup> Since SYPRO® Orange stain binds SDS, the best results are obtained if the SDS front is allowed to run off the gel prior to staining. In addition, for thick gels the manufacturer recommends a 15-minute prewash of the gel in 7.5% acetic acid, prior to staining. This prewash decreases the SDS concentration in the gel, reducing the possibility of background staining due to the presence of this detergent.

<sup>\*12</sup> Avoid gel overlapping, which causes portions of the spots with high protein concentration to be migrated from one gel to the other. Also be sure to avoid using polystyrene made vat. Instead, polypropylene is recommended. Most soft plastics tend to adsorb dyes and most glass will also.

<sup>\*13</sup> For optimum sensitivity, set the FLA-2000 excitation wavelength to 473nm and select the Y520 read-out filter. Higher sensitivity than with silver staining can sometimes be obtained by setting the reading sensitivity to F1000.

<sup>\*14</sup> Nacalai Tesque's high-sensitivity silver staining kit for protein electrophoresis used.

## 3 Comparison of protein detection methods

---

### ■ Protein detection by various staining methods

#### **CBB staining**

The widely practiced CBB staining method has a number of excellent features, including the good linearity between sample quantity and stained density value, enables N terminal sequencing after staining, can detect all types of proteins, and exhibits low variance in detection sensitivity with type of peptide. Among its drawbacks are lower sensitivity than silver staining (see in Figs. 3-1 and 3-3) and longer staining and destaining times.

#### **Silver staining**

Among various non-RI detection methods, silver staining stands out for its exceptionally high sensitivity. This method has a disadvantage, however, in that spots containing large amounts of protein tend to whiten. The spot at the upper left in Fig. 3-1, for example, is on the verge of disappearance. This is a particular problem in 2-D electrophoresis, which has a diversity of spots concentrations in the same gel. When the detection focuses on light spots, spots with very large quantities of protein disappear, or lose linearity of the results. Another drawback is that detection sensitivity is poor for some type of proteins, sometimes so poor that no spot is detected at all for that protein type. Silver staining requires stricter control than CBB staining regarding sample quantity, staining procedure and other factors.

#### **RI labeling**

In RI labeling, proteins produced by microorganisms or cultured cells are directly labeled by adding  $^{14}\text{C}$  or  $^{35}\text{S}$  labeled amino acid to the culture medium.

Although this method can detect proteins with high sensitivity irrespective of its type, use of radioisotopes is a prohibitive factor for the widespread adoption of this technique.

#### **Fluorescence staining of proteins with SYPRO® Orange**

Fluorescence staining with SYPRO® Orange is comparable to silver staining in sensitivity but does not have the problem of spot whitening (Figs. 3-1 and 3-2). Moreover, the dynamic range of the detection can be markedly expanded by using a laser scanner for read-out. This in turn significantly increases the spread between the lower and upper spot detection limits.

In silver staining, the spot detection result varies with the amount of added sample and the staining conditions. To obtain comparable results consistently every time, the condition settings have to be strictly controlled. Depending on the sample, this may be difficult.

The FLA-2000 produces 16-bit digital images with 65,536 gradations. With the contrast adjustment function, images of optimum quality can be reproducibly obtained. This is in sharp contrast to the silver staining method. SYPRO® Orange staining is less troublesome than silver staining and is as easy as the CBB staining method.

## ■ Examples of sample analysis

The 2-D electrophoresis gels in the figures below are the results obtained when identical amounts of sake brewing yeast were used in spot detection by CBB staining, silver staining and SYPRO® Orange staining.

< Sample quantity matched to CBB staining >

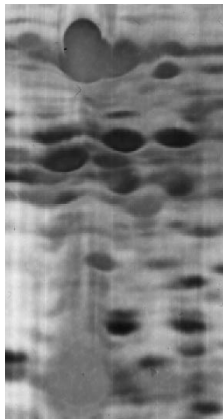


Fig. 3-1

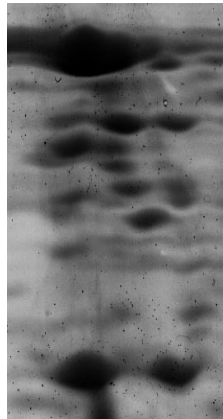


Fig. 3-2



Fig. 3-3

**Fig. 3-1 2-D electrophoresis image (silver staining)**

**Fig. 3-2 2-D electrophoresis image (SYPRO® Orange staining)**

FLA-2000 read-out conditions  
Gradation: 65536 (16 bit)  
Resolution: 100µm  
Sensitivity: F1000  
Latitude: 5  
Sample Mode: Fluor. 473nm  
Y520 Filter

**Fig. 3-3 2-D electrophoresis image (CBB staining)**

< Sample quantity matched to silver staining >

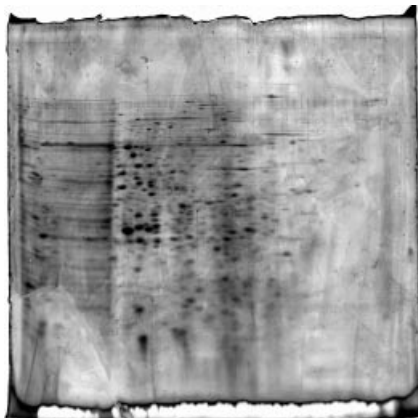


Fig. 3-4

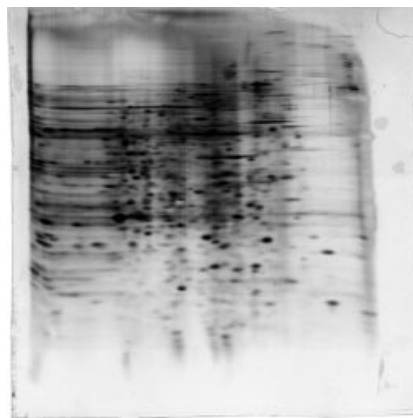


Fig. 3-5

**Fig. 3-4 2-D electrophoresis image (SYPRO® Orange staining)**

FLA-2000 read-out conditions  
Gradation: 65536 (16 bit)  
Resolution: 100µm  
Sensitivity: F1000  
Latitude: 5  
Sample Mode: Fluor. 473nm  
Y520 Filter

**Fig. 3-5 2-D electrophoresis image (silver staining)**

## ■ Conclusion

While the sensitivity of silver staining is markedly greater than that of CBB staining, large spots detectable by CBB staining whiten and disappear or fade in silver staining. Viewed against this backdrop, the SYPRO® Orange staining method is notably higher in sensitivity than the CBB staining method, is also capable detecting high-concentration spots. The combination of SYPRO® Orange staining and FLA-2000 read-out provides a broad dynamic range in excess of that obtained with the silver staining method and comparable to that obtained with the CBB staining method.

The CBB staining and silver staining methods can be easily applied to the same sample after SYPRO® Orange staining. Therefore, by combining SYPRO® Orange staining with a conventional detection method, conventional data can also be secured for the same sample.

As a consequence of these features, the SYPRO® Orange staining methodology can be effectively utilized as a detection method combining sensitivity comparable to silver staining with characteristics similar to CBB staining.

## 4 References

- 1) Patrick H. O'Farrell, High resolution two-dimensional electrophoresis of proteins, The Journal of Biological Chemistry; Vol. 250, No. 10, 4007-4021 (1975)

### About the author

Yoshihiro Yamamoto

- 1965 Born in Fukui Prefecture
- 1991 Master of Science. Faculty of Agriculture Kyoto Prefectural University
- 1994 Researcher at Applied Fermentation Laboratory, Applied Chemistry Division, Kyoto Municipal Institute of Industrial Research

Interests: Making the rounds of the junk electronic part dealers in the Nihonbashi district of Osaka. Rebuilding notebook computers.

Special skills: Linguist proficient in everything from Z80 machine language to Sun SPARC assembler (but can speak only Japanese).

### Writers

Yoshihiro Yamamoto

Kiyoo Hirooka

Nobuo Tsutsui

(Kyoto Municipal Institute of Industrial Research)

### Editor

Kenji Miura, Ph.D.

Naofumi Hora

Makiko Nagashima

(Fuji Photo Film Co., Ltd.)

This work was supported in part by a Grant-in-Aid from the Small and Medium Enterprise Agency of Japan.

SYPRO® is a registered trademark of Molecular Probes, Inc.

September 1998



**FUJI PHOTO FILM CO., LTD.**

SCIENCE SYSTEMS, EQUIPMENT PRODUCTS DIVISION

26-30, NISHIAZABU 2-CHOME, MINATO-KU, TOKYO 106-8620, JAPAN

Telephone: +81-3-3406-2201

Facsimile: +81-3-3406-2158

E-mail: sginfo@tokyo.fujifilm.co.jp

©1997-1999 Fuji Photo Film Co., Ltd.