

Imaging two-dimensional protein gels stained with SYPRO Ruby

Typhoon Variable Mode Imager

Key words: proteomics, imaging, fluorescence, 2-D electrophoresis, gel stains, SYPRO Ruby, Typhoon

Two-dimensional gel electrophoresis (2-D electrophoresis) is a powerful and crucial technique widely used in proteomics studies to separate complex protein mixtures. The first-dimension step of 2-D electrophoresis is isoelectric focusing (IEF) and separates proteins based on their isoelectric points. The second-dimension step is SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and this separates proteins based on their molecular weights (1, 2).

Fluorescence imaging technology is widely used for proteome analysis due to its advantages of high sensitivity combined with wide linear dynamic range. Transition metal chelate dyes, such as SYPRO™ Ruby protein gel stain, are commonly used for staining protein gels (3, 4, 5). SYPRO Ruby has gained much popularity as a sensitive, reproducible stain that can be used for both one-dimensional (1-D) and 2-D gels. When scanned with a powerful laser-based fluorescence imager, SYPRO Ruby offers low nanogram sensitivity plus a much wider linear dynamic range than the traditional silver staining method (6, 7). SYPRO Ruby is also compatible with mass spectrometry and Edman sequencing.

Typhoon™ Variable Mode Imagers are excellent choices for imaging protein gels stained with SYPRO Ruby (8). Typhoon 8600 and 9200 series have two excitation sources for fluorescence imaging: a green (532 nm) and a red (633 nm) laser. Typhoon 9400 series has an additional blue laser with two excitation lines (457 nm and 488 nm). The green laser and the two laser lines from the blue laser are compatible with SYPRO Ruby excitation. Typhoon offers extremely high sensitivity and a wide linear range for SYPRO Ruby quantitation.

Products used

| | |
|--|------------|
| Typhoon 8600 | 63-0027-96 |
| Typhoon 9200 | 63-0038-49 |
| Typhoon 9210 | 63-0038-51 |
| Typhoon 9400 | 63-0038-53 |
| Typhoon 9410 | 63-0038-55 |
| Low Fluorescence Glass Plate, 3-mm-thick | 63-0028-92 |
| 2-D Electrophoresis using immobilized pH gradients | 80-6429-60 |
| Ettan™ DALT <i>twelve</i> Separation Unit and Power Supply/Control Unit, 115 V | 80-6466-46 |
| IPGphor™ Isoelectric Focusing Unit | 80-6414-02 |
| 18 cm DryStrip Holders | 80-6417-44 |
| 24 cm DryStrip Holders | 80-6469-88 |
| Ettan DALT Gel Caster | 80-6467-22 |
| Ettan DALT Gel Casting Cassette, 1.0 mm | 80-6466-84 |
| Ettan DALT Blank Cassette Insert | 80-6467-03 |
| Immobiline™ DryStrips, pH 4–7, 18 cm | 17-1233-01 |
| Immobiline DryStrips, pH 3–10 NL, 24 cm | 17-6002-45 |
| IPG Buffer, pH 3–10 NL | 17-6000-88 |
| IPG Buffer, pH 4–7 | 17-6000-86 |
| Equilibration Tube Set | 80-6467-79 |
| ImageMaster™ 2D Elite Software | 80-6350-56 |
| EPS 301 Power Supply | 18-1130-01 |
| Amersham Biosciences™ miniVE Vertical Electrophoresis System | 80-6418-77 |
| Phenylmethyl Sulfonyl Fluoride (PMSF) | US20203 |

PlusOne™ reagents

| | |
|---------------------------------|------------|
| Acrylamide IEF | 17-1300-02 |
| Agarose M | 17-0422-01 |
| Agarose NA | 17-0554-01 |
| Ammonium persulfate | 17-1311-01 |
| Bromophenol Blue (BPB) | 17-1329-01 |
| CHAPS | 17-1314-01 |
| Dithiothreitol (DTT) | 17-1318-01 |
| Glycerol (87% w/w) | 17-1325-01 |
| Glycine | 17-1323-01 |
| Immobiline DryStrip Cover Fluid | 17-1335-01 |
| 2-Mercaptoethanol | 17-1317-01 |
| N,N'-Methylene-bisacrylamide | 17-1304-01 |
| Sodium Dodecylsulphate (SDS) | 17-1313-01 |
| TEMED | 17-1312-01 |
| Tris | 17-1321-01 |
| Urea | 17-1319-01 |

Other materials required

- SYPRO Ruby protein gel stain (Molecular Probes)
- BSA protein standard (Molecular Probes)
- Large polypropylene trays
- 1-butanol
- Triethanolamine hydrochloride
- Trichloroacetic acid (TCA)
- Acetone
- Ethanol
- Glacial acetic acid
- Methanol
- Sample suspension buffer
 - 8 M Urea
 - 4% (w/v) CHAPS
 - 20 mM Triethanolamine hydrochloride (pH 8.0)
 - 20 mM DTT
 - 1 mM PMSF
- Rehydration solution with IPG buffer
 - 8 M Urea
 - 2% (w/v) Chaps
 - 0.5% (v/v) IPG buffer
 - trace of BPB
 - 20 mM DTT (added just prior to use)
- Gel storage solution
 - 0.375 M Tris-Cl, pH 8.8
 - 0.1% (w/v) SDS
- Electrophoresis buffer
 - 25 mM Tris-Cl, pH 8.8
 - 192 mM Glycine
 - 0.1% (w/v) SDS
- SDS equilibration buffer
 - 50 mM TrisCl, pH 8.8
 - 6 M Urea
 - 30% (v/v) Glycerol
 - 2% (w/v) SDS
 - trace of BPB
- Agarose sealing solution
 - Electrophoresis buffer
 - trace of BPB
 - 0.5% (w/v) Agarose

Protocol

1

Preparing the sample: *E. coli* extract

- 1.1. Suspend 400 mg of lyophilized *E. coli* in 10 ml of sample suspension buffer.
- 1.2. Sonicate the suspension, a few seconds per burst, and repeat until maximum clarification is observed. Chill on ice between bursts.
- 1.3. Precipitate the sonicate overnight at -40 °C with 80 ml acetone, 10 ml 100% (w/v) TCA, and 1 ml 2-mercaptoethanol. Collect the precipitate by centrifugation at 32 000 × g for 20 min.
- 1.4. Wash the pellet with the same volume of 80% acetone and 1% 2-mercaptoethanol and leave in the -40 °C freezer for a few hours.

- 1.5. Collect the precipitate by centrifugation as before. Air-dry and resuspend the pellet in 10 ml 8 M urea and 2% (w/v) CHAPS, with sonication to aid solubilization.
- 1.6. Clarify the extract by centrifugation at 32 000 × g for 30 min and collect the supernatant.

2

Performing first-dimension electrophoresis using IPGphor Isoelectric Focusing Unit

- 2.1. Dilute the *E. coli* extract (100 µg) with rehydration solution containing IPG buffer.
- 2.2. Load the IPG strips (18 cm or 24 cm) by in-gel rehydration.
- 2.3. Use the following focusing protocol:
 - Rehydration for 12 h, focusing at 500 V for 500 Vh,
 - 1 000 V for 1 000 Vh, and 8 000 V for 64 000 Vh.
- 2.4. Store the focused IPG strips at -70 °C to -80 °C.

For more experimental details, refer to the IPG strip product instruction, IPGphor user manual, and 2-D electrophoresis technical manual (1).

3

Performing second-dimension electrophoresis using the Ettan DALT*twelve* system

- 3.1. Equilibrate the focused IPG strips in SDS equilibration buffer plus 1.0% (w/v) DTT for 15 min.
- 3.2. Equilibrate the focused IPG strips in SDS equilibration buffer plus 2.5% (w/v) iodoacetamide for 15 min.
- 3.3. Load the equilibrated strips onto self-cast 12% homogenous, 1-mm Laemmli gels.
- 3.4. Seal the IPG strip in place with agarose sealing solution.
- 3.5. Perform the electrophoresis using the Ettan DALT*twelve* system at 5 W/gel for 30 min and then at 28 W/gel until the BPB dye front just leaves the gel.

For more details, refer to Ettan DALT*twelve* system user manual and the 2-D electrophoresis technical manual (1).

4

Staining

- 4.1. Fix the gel for 30 min in a mixture of 40% (v/v) ethanol and 10% (v/v) acetic acid (the fixation improves protein retention in the gel).
- 4.2. Stain the gel directly with the working stain solution (between 5- and 10-fold greater than the gel volume) for at least 12 h.
- 4.3. Rinse the gels with deionized water.
- 4.4. Destain for 30 min in 10% (v/v) methanol and 7% (v/v) glacial acetic acid.

5

Imaging

- 5.1. The gel can be imaged on a 3-mm-thick low-fluorescence glass plate using the +3 mm focal plane setting. The glass plate helps to keep the gel intact permitting future analysis

steps. The plate also helps to protect the Typhoon glass platen from temporary contamination by SYPRO Ruby, which is a problem common with this stain on all imagers. A thorough cleaning of the glass plate is recommended immediately after scanning (see 5.3).

A thin layer of deionized water between the glass plate and the platen will minimize the appearance of interference patterns. Pour sufficient deionized water onto the platen to form a pool and then gently place the low-fluorescence glass plate on top of the water. Avoid trapping any bubbles between the glass plate and the platen. Pour a small amount of deionized water onto the glass plate. Place the gel on top of the water. Avoid bubble formation between the gel and the glass plate.

Alternatively, the gel can be imaged by using “platen” for the focal plane setting and directly placing it on the platen on a small amount of deionized water. Avoid bubble formation between the gel and the platen. A thorough cleaning of the platen is recommended immediately after scanning (see 5.3).

- 5.2. Set up the Typhoon scanner control software for SYPRO Ruby detection (excitation maximum: 450 nm, emission maximum: 610 nm). Choose an appropriate laser line (457 nm, 488 nm, or 532 nm) for excitation and 610BP30 for the emission filter.

In addition, choose appropriate PMT voltage (450–800 V) and focal plane setting (see 5.1) as well as following parameters:

Pixel size – 100 μm
Sensitivity – Normal

For more details about setting up the scanner control and other practical aspects of using Typhoon imagers, please refer to *Typhoon Instrument Guide*.

- 5.3. After scanning, use a soft, lint-free cloth dampened with deionized water to rinse the glass plate, platen, and if necessary, the sample lid. Repeat the procedure with 75% ethanol. Rinse again with deionized water to remove any possible fluorescent residue from the ethanol. If the glass plate or the platen is still dirty, clean with a 5–10% hydrogen peroxide solution followed by extensive rinsing with deionized water to remove residual hydrogen peroxide.

Results

Figure 1 shows a 2-D protein gel stained with SYPRO Ruby and imaged by the Typhoon 532 nm laser line. The number of spots detected by ImageMaster 2D Elite Software was 379. The 457 nm and 488 nm laser lines were also tested for the same gel. No significant difference in the number of spots or detection sensitivity was observed for any of the three laser lines that excite SYPRO Ruby.

The 2-D gel image was analyzed using ImageMaster 2D Elite Software. ImageMaster 2D Elite software is a powerful tool for 2-D protein gel analysis and features special algorithms for spot finding and analysis routines for gel-to-gel comparisons. Other important features include data normalization, background correction, gel matching and grouping, and database input of analysis results. Figure 2 shows a snapshot of the spot detection process using this software.

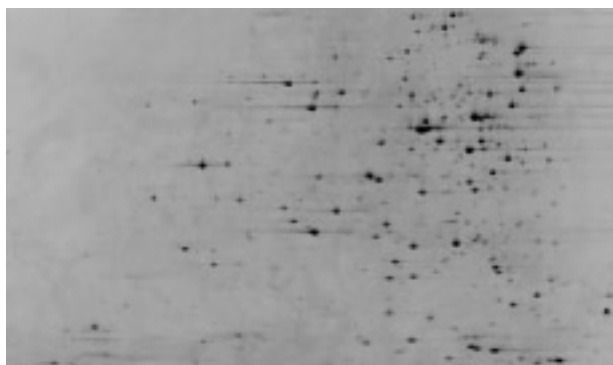


Fig 1. SYPRO Ruby staining of a 2-D gel. A total protein extract of *E. coli* (100 μg) focused on an 18 cm Immobiline DryStrip (pH 4–7) was run out in the second dimension using a 12%, 1-mm Laemmli gel using the Ettan DALT*twelve* system. The gel was stained overnight with ~ 5 volumes of SYPRO Ruby and imaged using the Typhoon 532 nm laser line.

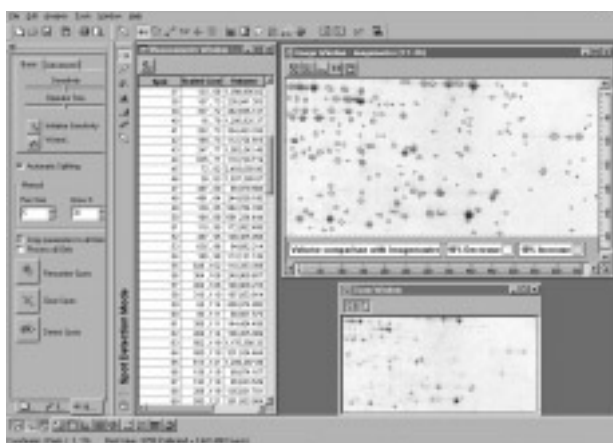


Fig 2. Spot detection of a SYPRO Ruby stained 2-D gel in progress using ImageMaster 2D Elite Software.

The absolute number of spots from a 2-D protein gel heavily depends on the sample loading amount, sample treatment conditions, both first-dimension and second-dimension electrophoresis conditions, and other experimental factors. Therefore, the number of spots detected on different gels should not be used to compare the gel staining efficiency or the imager performance. To study the quantitative aspect of SYPRO Ruby stained protein gels imaged by Typhoon; a dilution series of BSA protein standard was loaded on a 12% 1-mm Laemmli gel. This approach is widely used to evaluate the detection limit and linear range for SYPRO Ruby stained protein gels (9). It eliminates the errors from possible protein loss during the first-dimension and second-dimension electrophoresis procedures, as well as gel-to-gel variations. Using this approach, the limit of detection (LOD) was determined to be 1 ng/band for BSA protein (The LOD is the threshold at which the background-corrected signal-to-noise is at least three). The linear range was found to be 10^3 fold (see Fig 3). Similar sensitivity and linear range were found for all three laser lines that excite SYPRO Ruby — 457 nm, 488 nm, and 532 nm. It is important to note that Typhoon offers a wide linear

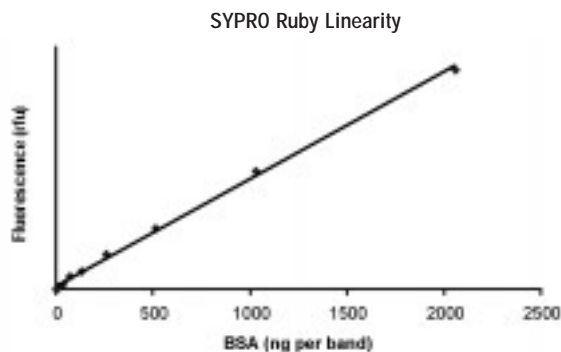


Fig 3. Fluorescence intensity of SYPRO Ruby stained BSA bands versus BSA amount (1–2060 ng per band). Fluorescence refers to integrated relative fluorescence units (rfu). The linear fit to the data has an R^2 value of at least 0.99. The linear range was determined to be 10^3 fold.

dynamic range of five orders of magnitude (from count 1 to 100 000). The major factors limiting detection limits and linear range are the background staining of the gel, the imperfect stain/protein binding stoichiometry, and other experimental factors, not the excitation wavelength (6). This also suggests that the Typhoon has the potential to offer even better results when improved fluorescent staining methods become available. Although the detection limit and linear range of the SYPRO Ruby stained 1-D gels may not represent the absolute detection limit and linear range of the 2-D gels, they can be used to achieve relative comparison among different SYPRO Ruby detection methods.

The large scan area of Typhoon (35 × 43 cm) conveniently allows imaging of large size, or multiple 2-D gels at the same time. The laser-based scanner system of Typhoon does not sacrifice resolution when imaging large gels. This is because spatial resolution does not depend on the gel size. Furthermore, the confocal optics used in Typhoon

instruments, coupled with the moving-head mechanism, offer superior image uniformity and eliminate the artifacts commonly seen with galvanometer systems (8). Typhoon also provides sensitive detection for a wide range of fluorochromes, such as Cy[™]3 and Cy5, which are suitable for 2-D protein gel detection using direct dye labelling methods (10).

Conclusion

Typhoon imagers are excellent choices for imaging SYPRO Ruby stained 2-D protein gels. When imaged with Typhoon (457 nm, 488 nm, or 532 nm laser lines), SYPRO Ruby offers high sensitivity (LOD of 1 ng/band) and a wide linear range (10^3 fold) for protein gels. With high sensitivity and uniformity, wide linear range, and large scan area, Typhoon imagers provide high quality images that are free of scanning distortion and artifacts for large 2-D gels.

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