# Sensitivity and Protein-to-Protein Consistency of Flamingo™ Fluorescent Gel Stain Compared to Other Fluorescent Stains

Tom Berkelman, Life Science Group, Bio-Rad Laboratories, Inc., Hercules, CA 94547

## Introduction

Proteomics research often involves 1-D or 2-D electrophoretic separation of protein mixtures in polyacrylamide gels. Following electrophoresis, the gels are stained for detection and quantitation of the separated proteins. Fluorescent stains are commonly used for this purpose due to the high sensitivity and wide dynamic range possible using fluorescent reagents. Quantitation is most reliable when the fluorescence response is linear with respect to the amount of protein over the widest possible range. Ideally, a fluorescent stain should also respond similarly to diverse proteins. There should be minimal specificity with respect to the physical or chemical characteristics of an individual protein.

A number of fluorescent protein stains are now available. The current study was undertaken to compare the performance of four commercial staining products: Flamingo fluorescent gel stain (Bio-Rad Laboratories), SYPRO Ruby protein gel stain (Invitrogen Corp., also distributed by Bio-Rad Laboratories), Deep Purple total protein stain (GE Healthcare), and Krypton protein stain (Pierce Biotechnology).

Detection sensitivity is related to the fluorescence intensity of a given quantity of stained protein. It also depends on the magnitude of background fluorescence. These values were determined for the different stains under identical imaging conditions. The limit of sensitivity and the linearity of response for each stain were determined using visible-light laser imaging under conditions optimal for each stain. The limits of sensitivity were also determined for an imaging system using UV transillumination. Differential protein-to-protein staining was examined using a collection of proteins selected to represent diversity in charge, size, and posttranslational modification.

## Methods

Criterion™ Tris-HCl 4–20% gels with eighteen 5 mm wide wells were used for SDS-PAGE. Gel images generated by laser scanning were produced using the Molecular Imager® PharosFX™ system. Gels were scanned using either 532 nm or 488 nm excitation as indicated. Fluorescence emission on all scans was filtered through a 605 nm bandpass filter. Except when indicated, the photomultiplier tube (PMT) setting for low-intensity samples was used. Gel images generated by UV transillumination were produced using the VersaDoc™ 3000 imaging system with 300 nm bulbs, 520 nm longpass emission, and the camera aperture fully open. All images were analyzed using Quantity One® software.

Experiments to determine relative signal and background levels, visual limits of sensitivity, and linearity used gels loaded with a dilution series of broad range SDS-PAGE standards (unstained). Dilutions were prepared to give the following sample load (for each protein) in a loading volume of 5 µl: 960 ng, 480 ng, 240 ng, 120 ng, 60 ng, 30 ng, 15 ng, 8 ng, 4 ng, 2 ng, 1 ng, 0.5 ng, 0.25 ng, 125 pg, 60 pg, 30 pg.

The proteins selected to evaluate protein-to-protein variability in staining intensity were resolved as single bands by SDS-PAGE. Constant 240 ng loads based on $A_{280}$ in a guanidine solution (Gill and von Hippel 1989) were applied to SDS-PAGE and stained as described. All of the proteins were purchased from Sigma.

Gels were stained according to protocols accompanying each product as summarized in Table 1.

**Table 1. Summary of gel staining protocols.**

| Flamingo Fluorescent Gel Stain | SYPRO Ruby | Deep Purple | Krypton Protein Stain |
|---|---|---|---|
| 1. Fix 2 hr in 40% ethanol, 10% acetic acid | 1. Fix 2x 30 min in 50% methanol, 7% acetic acid | 1. Fix 1 hr in 10% methanol, 7% acetic acid | 1. Fix 2x 30 min in 40% ethanol, 10% acetic acid |
| 2. Stain overnight (15–18 hr) in stain solution | 2. Stain overnight (15–18 hr) in stain solution | 2. Wash 30 min in 200 mM sodium carbonate | 2. Wash 5 min in water |
| 3. Wash 30 min in 0.1% (v/v) Tween 20 | 3. Destain 30 min in 10% methanol, 7% acetic acid | 3. Stain 1 hr in stain solution | 3. Stain 2 hr in stain solution |
| | 4. Wash 2x 5 min in water | 4. Destain 2x 15 min in 7.5% acetic acid | 4. Destain 5 min in 5% acetic acid |
| | | | 5. Wash 2x 15 min in water |

## Results

### Fluorescence Intensity and Background

Flamingo fluorescent gel stain is excited in the visible range with an excitation peak intermediate between the 532 nm and 488 nm lasers used by the Pharos FX imager. Therefore, both excitation wavelengths were tested on gels stained with Flamingo stain as well as on gels stained with SYPRO Ruby stain. Gels stained with Flamingo fluorescent gel stain had the highest protein-associated fluorescence intensity. Gels stained with Krypton and Deep Purple stains had considerably lower background than those stained with either Flamingo or SYPRO Ruby stains but had correspondingly lower protein-associated fluorescence. Flamingo stain gave the highest difference between signal and background.

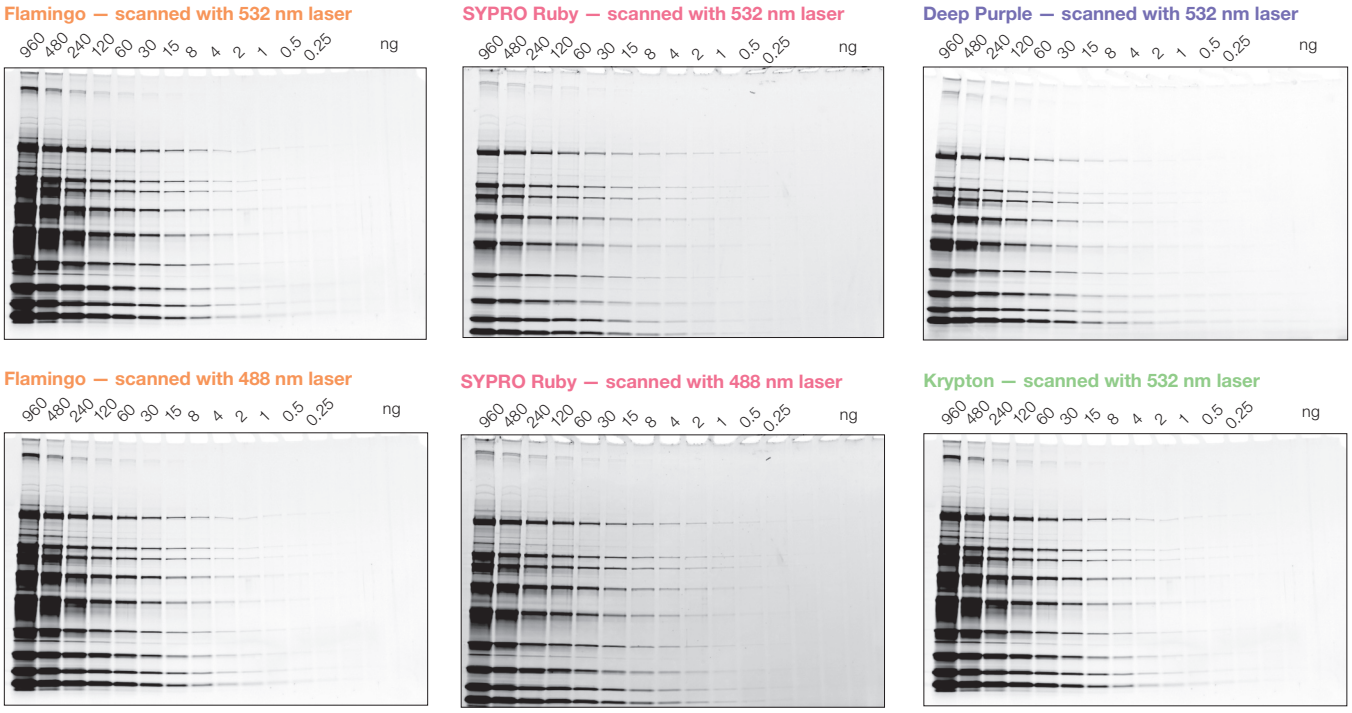



**Fig. 1. Images of gels stained with different fluorescent stains generated with the PharosFX system.** Gels stained with Flamingo and SYPRO Ruby stains were imaged under both 488 nm (blue) and 532 nm (green) laser light. Gels stained with Deep Purple and Krypton stains were imaged only under 532 nm (green) laser light. All images were subjected to the same image transformation.

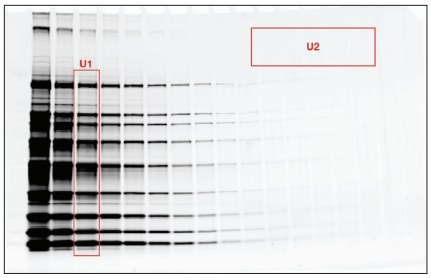



**Fig. 2. Determination of fluorescent signal associated with protein and background fluorescence (example).** A box encompassing all of the standards loaded at 240 ng (U1), and a box of equal area around an empty region (U2) were drawn on each gel. The total pixel intensity in each region was determined. Results are shown in Table 2. The example gel shown was stained with Flamingo fluorescent gel stain.

**Table 2. Protein-associated and background fluorescence.** Fluorescence intensity values are given in arbitrary relative units. Protein-associated fluorescence equals pixel intensity of protein lane minus background pixel intensity.

| Gel Stain | Pixel Intensity | | | Protein–Associated Fluorescence |
|---|---|---|---|---|
| | Excitation Wavelength | Protein Lane | Background | |
| Flamingo | (532 nm) | 49.74 | 4.36 | 45.92 |
| Flamingo | (488 nm) | 43.63 | 3.76 | 39.87 |
| SYPRO Ruby | (532 nm) | 22.56 | 4.44 | 18.12 |
| SYPRO Ruby | (488 nm) | 43.03 | 7.67 | 35.36 |
| Deep Purple | (532 nm) | 24.91 | 1.01 | 23.89 |
| Krypton | (532 nm) | 26.26 | 0.72 | 25.54 |

### Sensitivity With Visible Light Laser Scanning

Flamingo fluorescent gel stain gave the lowest limit of detection with laser scanning.

**Table 3. Limit of detection with visible laser light excitation for each staining method.** The value given is the lowest amount of protein for which all nine of the protein standards are visible.

| | Gel Stain | | | |
|---|---|---|---|---|
| | Flamingo | SYPRO Ruby | Deep Purple | Krypton |
| Wavelength | 532 nm | 488 nm | 532 nm | 532 nm |
| Protein amount | 30 pg | 500 pg | 125 pg | 125 pg |

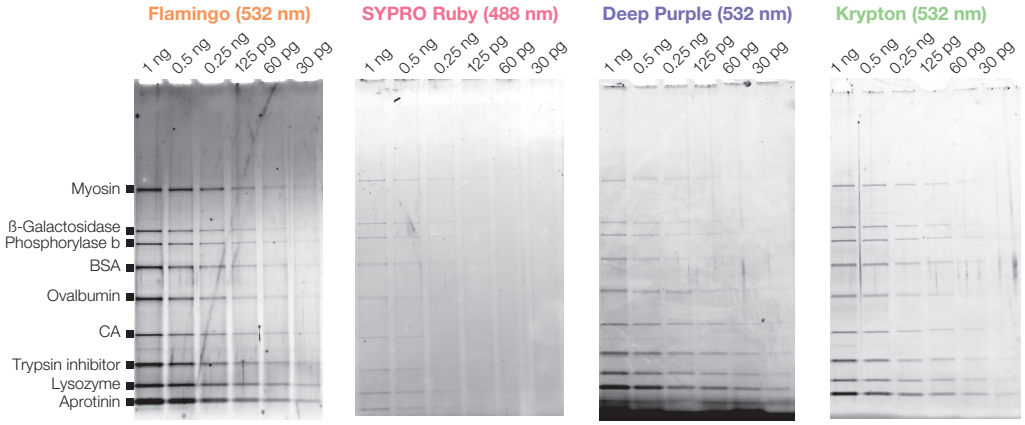
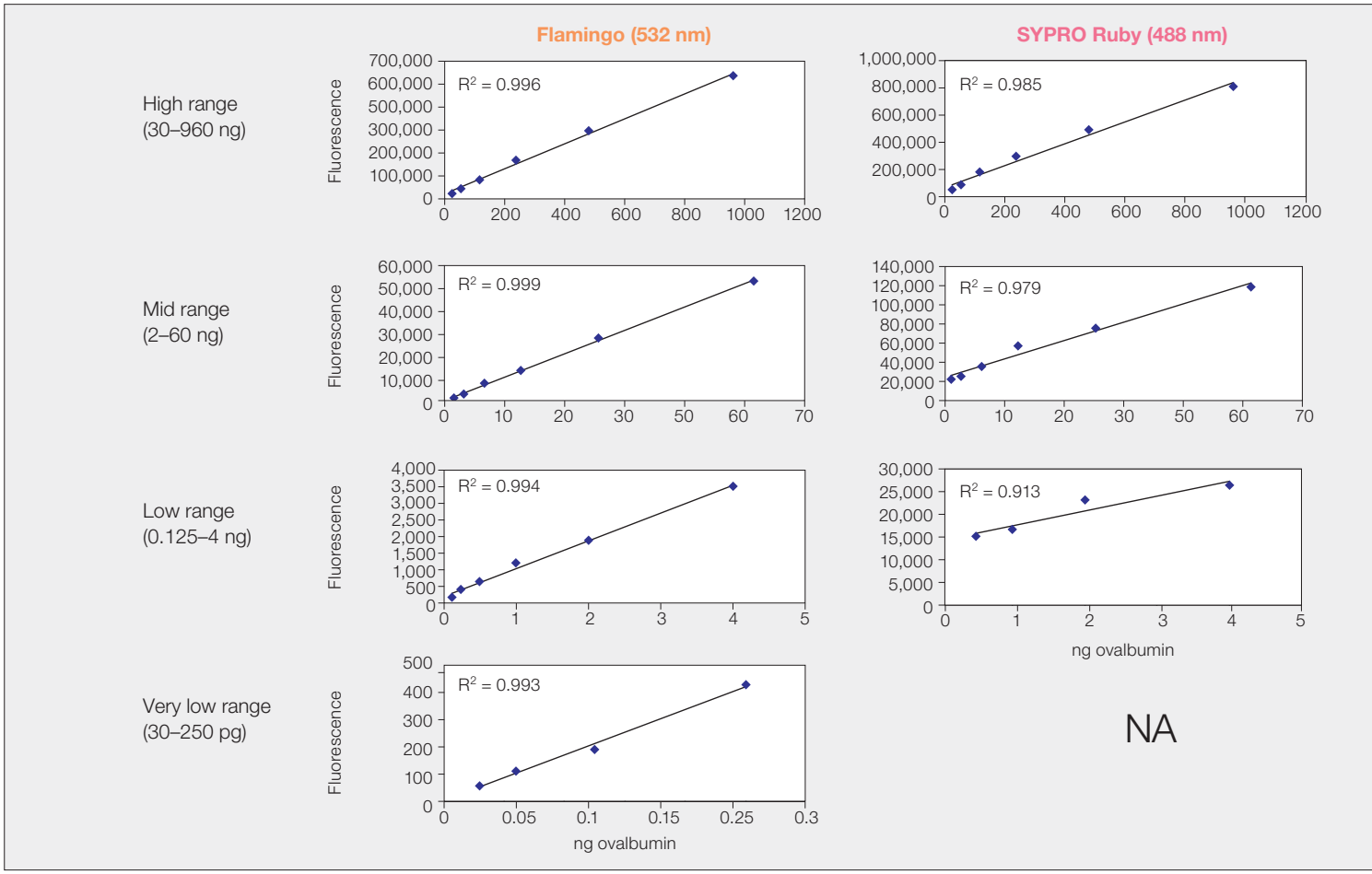



**Fig. 3. Gel images generated by laser scanning.** Gels containing a dilution series of protein standards were stained either with Flamingo, SYPRO Ruby, Deep Purple, or Krypton stains as described and scanned using the PharosFX system. The images were transformed to show the lowest possible visual limit of sensitivity. Only the portion of each gel showing protein loads of 1 ng or lower is shown. Table 3 summarizes the results.

### Linearity of Staining

Flamingo fluorescent gel stain exhibited the best linearity over the widest range of quantities, with $R^2$ >0.99 over the entire range tested (Figure 5).



**Limit of Sensitivity With UV Transillumination**

SYPRO Ruby fluorescence was considerably brighter than the other stains under UV transillumination and needed a shorter exposure to prevent image saturation. Flamingo stain gave the lowest limit of sensitivity with UV transillumination.

**Table 4. Limit of sensitivity with uv transillumination for each staining method.** The value given is the lowest amount of protein at which all nine of the protein standards are visible.

| | Gel Stain | | | |
|---|---|---|---|---|
| | Flamingo | SYPRO Ruby | Deep Purple | Krypton |
| Protein amount | 125 pg | 500 pg | 500 pg | 250 pg |

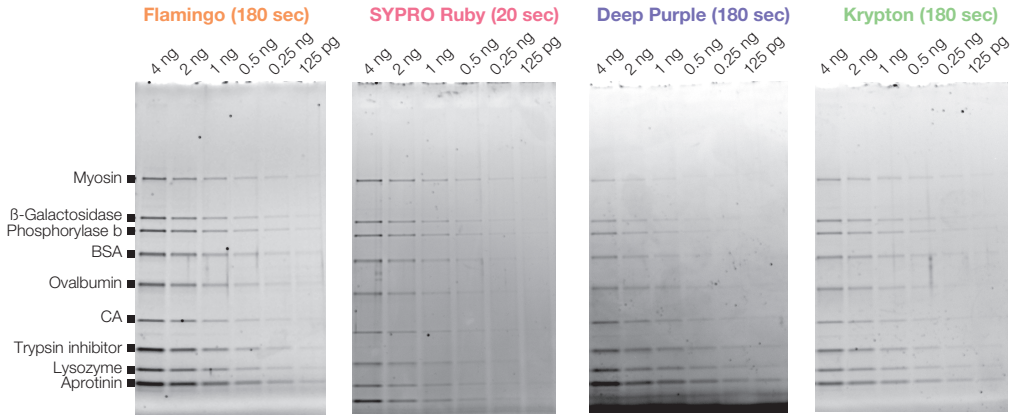



**Fig. 4. Gel images generated by UV transillumination.** Gels containing dilution series of protein standards were stained either with Flamingo, SYPRO Ruby, Deep Purple, or Krypton stains as described and imaged using the VersaDoc 3000 system with exposure times as indicated. The images were transformed to show the lowest possible visual limit of sensitivity. Only the portion of each gel showing protein loads of 4 ng or lower is shown. Table 4 summarizes the results.

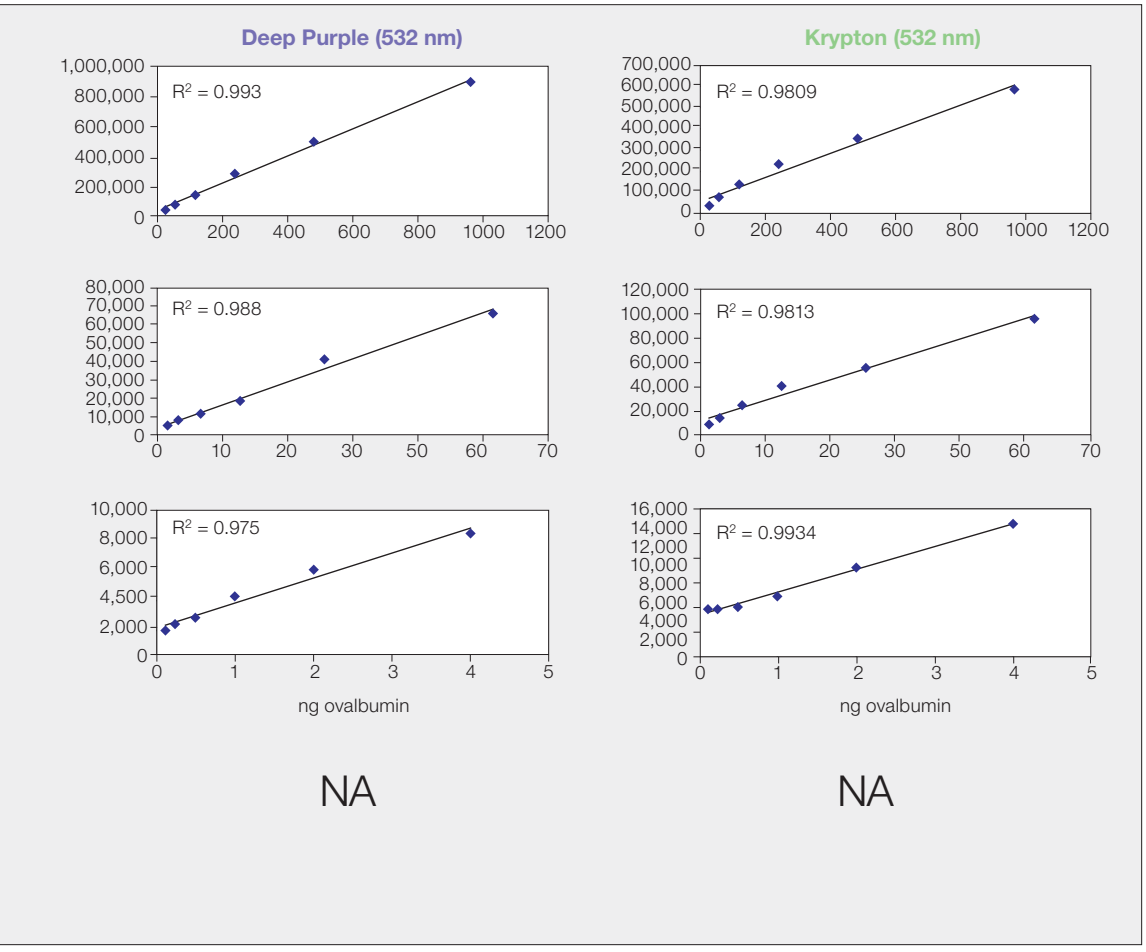



**Fig. 5. Linear dynamic range of staining intensity.** Gels containing dilution series of protein standards were stained either with Flamingo, SYPRO Ruby, Deep Purple, or Krypton stains as described and scanned at 532 nm or 488 nm using the PharosFX system. The Flamingo-stained gel was scanned using the instrument setting for medium-intensity samples (45% maximum PMT voltage). All other gels were scanned using the instrument setting for low-intensity samples (55% maximum PMT voltage). The total pixel intensity following background subtraction associated with the ovalbumin band was determined across the entire visible range of the dilution series. Fluorescence is given in arbitrary relative units. Results are graphed over four concentration ranges.

### Protein-to-Protein Differential Staining

All of the stains tested exhibited significantly reduced staining intensity with the very acidic protein pepsin (Figure 6). Otherwise, Flamingo, SYPRO Ruby, and Krypton stains did not exhibit any readily discernible staining bias based on size or isoelectric point (pI). None of the stains tested appeared to exhibit any bias related to posttranslational modification. All reacted similarly with RNase A and RNase B, which only differ by the presence of glycosylation on RNase B. Deep Purple stain exhibited reduced staining intensity with the acidic protein calmodulin and almost no staining with the very acidic protein pepsin. Flamingo, SYPRO Ruby, and Krypton stains exhibited similar average deviations from mean staining intensity, with Flamingo stain showing the lowest average deviation. Deep Purple exhibited a significantly greater average deviation from mean staining intensity than did the others.

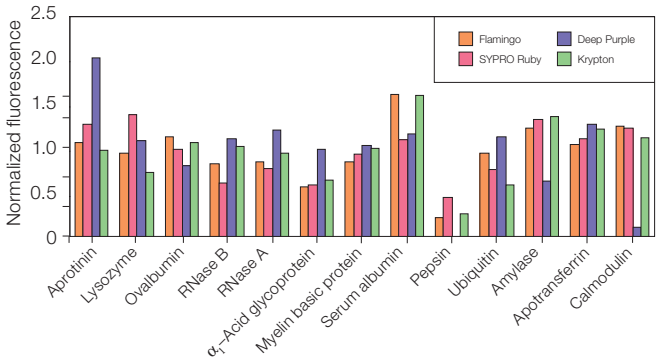



**Fig. 6. Consistency of staining response among diverse proteins.** A group of proteins was selected to represent diverse properties (described in Table 5). A constant amount (240 ng) of each protein was resolved by SDS-PAGE. Gels were stained either with Flamingo, SYPRO Ruby, Deep Purple, or Krypton stains and scanned with the PharosFX system using the 488 nm laser for SYPRO Ruby and the 532 nm laser for the other stains. Total fluorescence associated with each protein band was determined, and the value was normalized by dividing by the average total fluorescence associated with each protein band for each gel.

**Table 5. Proteins selected for differential staining experiment.**

| Protein | Special Properties |
|---|---|
| Aprotinin (bovine) | Small (MW = 6,500)<br>Basic (pI = 9.24) |
| Lysozyme (chicken) | Basic (pI = 9.32) |
| Ovalbumin (chicken) | Phosphoprotein<br>Glycoprotein |
| RNase B (bovine) | Basic (pI = 8.64)<br>Glycoprotein |
| RNase A (bovine) | Basic (pI = 8.64) |
| $\alpha_1$-Acid glycoprotein (ovine) | Acidic (pI ~ 4)<br>Glycoprotein |
| Myelin basic protein (bovine) | Very basic (pI = 11.28)<br>Phosphoprotein |
| Serum albumin (bovine) | |
| Pepsin (porcine) | Very acidic (pI = 3.24)<br>Phosphoprotein |
| Ubiquitin (bovine) | Small (MW = 8,600) |
| Amylase (*Bacillus amyloliquifaciens*) | |
| Apotransferrin (human) | Glycoprotein |
| Calmodulin (bovine) | Acidic (pI = 4.09) |

**Table 6. Average deviation from mean staining intensity within the group of proteins tested.**

| | Gel Stain | | | |
|---|---|---|---|---|
| | Flamingo | SYPRO Ruby | Deep Purple | Krypton |
| Protein amount | 23.5% | 25.2% | 35.5% | 25.0% |

## Discussion

Of the stains tested, Flamingo fluorescent gel stain had the brightest intrinsic fluorescence when scanned with the PharosFX laser-based imager regardless of whether 488 nm or 532 nm excitation was used. A lower scanner PMT setting was therefore necessary in order to prevent image saturation when evaluating the linear range of Flamingo fluorescent gel stain. When this measure was taken, Flamingo fluorescent gel stain exhibited a significantly wider linear range for quantitation, and better linearity across the entire range than did the other stains tested.

SYPRO Ruby protein gel stain was notable in that its optimal exposure time for imaging by UV transillumination was considerably shorter than that required for imaging the other stains (20 sec vs. 180 sec). This is consistent with the reported high relative UV absorbance of SYPRO Ruby (Berggren et al. 2000). Nonetheless, SYPRO Ruby was no more sensitive with UV transillumination than were the other tested stains when their relative lack of brightness was compensated for with a longer exposure. Both Flamingo fluorescent gel stain and Krypton protein stain proved more sensitive with UV transillumination, with Flamingo stain showing the highest sensitivity. This may be due to the relatively high background observed with SYPRO Ruby. However, SYPRO Ruby may be preferable in instances where equipment limitations prevent the relatively long exposure times required by the other stains.

Factors considered as possibly influencing staining behavior were charge (pI) and the presence of posttranslational modifications (glycosylation and phosphorylation). Two small proteins (aprotinin and ubiquitin) were also included in the study to test for any possible bias towards or against small proteins. The only bias observed for all stains was poor staining of pepsin. Pepsin is exceptional among proteins for its very low pI and its low content of basic amino acids. It therefore displays little positive charge, suggesting that electrostatic interaction between negatively charged dye and positive charges on proteins may be a staining mechanism shared among the stains tested. Deep Purple total protein stain was notable in its almost complete inability to stain pepsin and its relative lack of response to calmodulin, another very acidic protein. This may be explained by the reported requirement for interaction between the dye in Deep Purple and the primary amine of lysine residues (Coghlan et al. 2005). The other acidic protein tested, $\alpha_1$-acid glycoprotein, stained quite strongly with Deep Purple. This acidic glycoprotein owes its low pI to the presence of sialic acid rather than a lack of basic amino acids, suggesting that the mechanistic basis of Deep Purple staining is more related to amino acid composition than overall charge.

## Conclusions

Of the stains tested:

- Flamingo fluorescent gel stain has the highest fluorescence with visible light laser scanning and gives the highest signal to background ratio
- Flamingo stain is the most sensitive for use with visible light laser scanning
- Flamingo stain is the most sensitive for use with UV transillumination, provided that a relatively long exposure time is used
- Flamingo stain exhibits the best signal vs quantity linearity
- Flamingo, SYPRO Ruby, and Krypton stains all exhibit relatively low protein-to-protein staining variability, with Flamingo stain showing the least. Deep Purple total protein stain exhibits more variability

If the primary concerns in choosing a stain are sensitivity, linearity, and protein-to-protein consistency, Flamingo stain is the preferred one.

## References


Berggren K et al., Background-free, high sensitivity staining of proteins in one-and two-dimensional sodium dodecyl sulfate-polyacrylamide gels using a luminescent ruthenium complex, Electrophoresis 21, 2509–2521 (2000)

Coghlan DR et al., Mechanism of reversible fluorescent staining of protein with epicocconone, Org Lett 7, 2401–2404 (2005)

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