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

Tom Berkelman

Life Science Group
6000 James Watson Drive
Hercules, CA 94547 USA

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 protein. 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 605 nm bandpass. Except when indicated, the PMT setting for low-intensity samples was used. Gel images generated by UV transillumination were produced using the VersaDoc™ 4000 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 limit 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 above. All of these proteins were purchased from Sigma.

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

Table 1. Summary of staining protocols

Flamingo	SYPRO Ruby	Deep Purple	Krypton
1. Fix 2 hr in 40% ethanol, 10% acetic acid	1. Fix 30 min in 50% methanol, 7% acetic acid	1. Fix 1 hr in 10% methanol, 7% acetic acid	1. Fix 30 min in 40% ethanol, 10% acetic acid
2. Stain overnight (15–18 hr) in stain solution	2. Fix 30 min in 50% methanol, 7% acetic acid	2. Wash 30 min in 200 mM sodium carbonate	2. Fix 30 min in 40% ethanol, 10% acetic acid
3. Wash 30 min in 0.1% (v/v) Tween 20	3. Stain overnight (15–18 hr) in stain solution	3. Stain 1 hr in stain solution	3. Wash 5 min in water
	4. Destain 30 min in 10% methanol, 7% acetic acid	4. Destain 15 min in 7.5% acetic acid	4. Stain 2 hr in stain solution
	5. Wash 5 min in water	5. Destain 15 min in 7.5% acetic acid	5. Destain 5 min in 5% acetic acid
	6. Wash 5 min in water		6. Wash 15 min in water
			7. Wash 15 min in water

Results

Fluorescence Intensity and Background

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 gave the highest difference between signal and background.

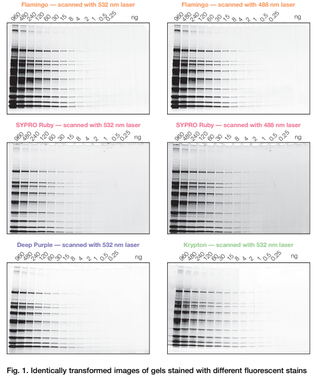

Fig. 1. Identically transformed images of gels stained with different fluorescent stains and imaged with the PharosFX system. Gels stained with Flamingo and SYPRO Ruby stains were imaged both with 488 nm (blue) and 532 nm (green) laser light. Gels stained with Deep Purple and Krypton stains were imaged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

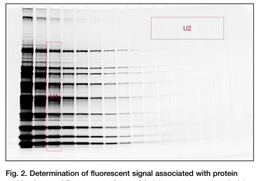

Fig. 2. Determination of fluorescent signal associated with protein and background fluorescence (example). A box encompassing all of the standards loaded at 240 ng (U1), and a box of equal area around an empty region (U2) were drawn on each gel. The total pixel intensity in each region was determined. 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 = Pixel intensity of protein lane – Background pixel intensity.

	Pixel Intensity			
Gel Stain	Excitation Wavelength	Protein Lane	Background	Protein-Associated Fluorescence
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

Linearity of Staining

Flamingo fluorescent gel stain exhibited the best linearity over the widest range, with R^2 >0.99 over the entire range tested.

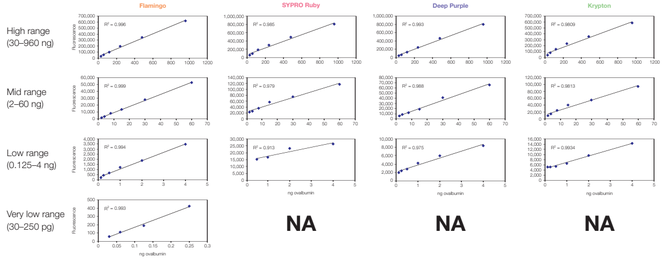

Fig. 5. Linear dynamic range of staining intensity. Gels containing dilution series of protein standards were stained either with Flamingo, SYPRO Ruby, Deep Purple, or Krypton stains as described and scanned using the PharosFX system. The Flamingo-stained gel was scanned using the instrument setting 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: high range, 30–960 ng; mid range, 2–60 ng; low range, 0.125–4 ng; very low range, 30–250 pg (Flamingo only).

Limit of Sensitivity With Laser Scanning

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

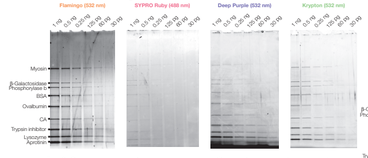

Fig. 3. Gel images generated by laser scanning. Gels containing a dilution series of protein standards were stained either with Flamingo, SYPRO Ruby, Deep Purple, or Krypton stains as described and scanned using the PharosFX system. The images were transformed to show the lowest possible visual limit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

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

	Gel Stain			
	Flamingo	SYPRO Ruby	Deep Purple	Krypton
Protein amount	30 pg	500 pg	125 pg	125 pg

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 gave the lowest limit of sensitivity with UV transillumination.

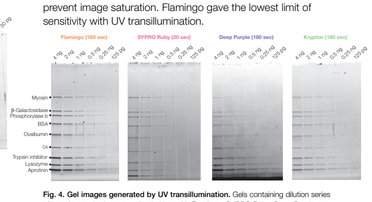

Fig. 4. Gel images generated by UV transillumination. Gels containing dilution series of protein standards were stained either with Flamingo, SYPRO Ruby, Deep Purple, or Krypton stains as described and imaged using the VersaDoc 4000 system with exposure times as indicated. The images were transformed 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.

Table 4. Limit of sensitivity with uv transillumination for each staining method. The value given is the 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

Protein-to-Protein Differential Staining

All of the stains tested exhibited significantly reduced staining intensity with the very acidic protein, pepsin. Otherwise, Flamingo, SYPRO Ruby, and Krypton stains did not exhibit any readily discernible staining bias based on size or isoelectric point. 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 exhibited similar average deviations from mean staining intensity, with Flamingo showing the lowest average deviation. Deep Purple exhibited a significantly greater average deviation from mean staining intensity than the others.

Table 5. Proteins selected for differential staining experiment.

Protein	Special Properties
Aprotinin (bovine)	Small (MW = 6,500) Basic (pI = 9.24)
Lysozyme (chicken)	Basic (pI = 9.32)
Ovalbumin (chicken)	Phosphoprotein Glycoprotein
RNase B (bovine)	Basic (pI = 8.64) Glycoprotein
RNase A (bovine)	Basic (pI = 8.64)
α_1-Acid glycoprotein (ovine)	Acidic (pI ~ 4) Glycoprotein
Myelin basic protein (bovine)	Very basic (pI = 11.28) Phosphoprotein
Serum albumin (bovine)	
Pepsin (porcine)	Very acidic (pI = 3.24) Phosphoprotein
Ubiquitin (bovine)	Small (MW = 8,600)
Amylase (*Bacillus amyloliquifaciens*)	
Apotransferrin (human)	Glycoprotein
Calmodulin (bovine)	Acidic (pI = 4.09)

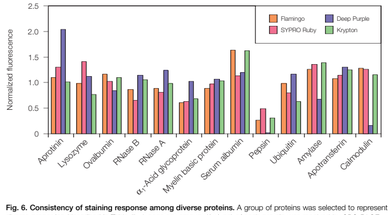

Fig. 6. Consistency of staining response among diverse proteins. A group of proteins was selected to represent diverse properties (described in Table 5). A constant amount (240 ng) of each protein was resolved by SDS-PAGE. Gels were stained either with Flamingo, SYPRO Ruby, Deep Purple, or Krypton stains 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 value for each gel.

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

	Gel Stain			
	Flamingo	SYPRO Ruby	Deep Purple	Krypton
Deviation	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. When this measure was taken, Flamingo exhibited a significantly wider linear range for quantitation, and better linearity across the entire range than the other stains tested.

SYPRO Ruby protein gel stain was notable in that it 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 the other tested stains when their relative lack of brightness was compensated for with a longer exposure. Both Flamingo and Krypton protein stain proved more sensitive with UV transillumination, with Flamingo showing the highest sensitivity. This may be due to the relatively high background observed with SYPRO Ruby. SYPRO Ruby may, however, 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 relatively 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, α_1-acid glycoprotein, stained relatively 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.

Conclusion

Of the stains tested:

- Flamingo fluorescent gel stain has the highest fluorescence with laser scanning and gives the greatest difference between signal and background
- Flamingo is the most sensitive for use with visible light laser scanning
- Flamingo is the most sensitive for use with UV transillumination, provided that a relatively long exposure time is used
- Flamingo exhibits the best linearity
- Flamingo, SYPRO Ruby, and Krypton stains all exhibit relatively low protein-to-protein staining variability, with Flamingo 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 is the preferred stain.

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)

Gill SC and von Hippel PH, Calculation of protein extinction coefficients from amino acid sequence data, Anal Biochem 182, 319–326 (1989)

SYPRO is a trademark of Molecular Probes, Inc. Deep Purple is a trademark of GE Healthcare. Krypton is a trademark of Pierce Biotechnology, Inc. Tween is a trademark of ICI Americas Inc.

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