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Visualization of proteins electro-blotted on Hybond ECL and Hybond-P using Deep Purple Total Protein Stain

key words:
Hybond ECL • nitrocellulose • Hybond-P • polyvinylidene fluoride • Deep Purple Total Protein Stain • electro-blotting

Introduction

Deep Purple™ Total Protein Stain is one of the most sensitive stains currently used for detecting and quantifying protein in 1-D and 2-D electrophoresis studies. As a gel stain, Deep Purple Total Protein Stain is characterized by its brightness, low background and artifact levels, and quantitative linearity over more than four orders of magnitude (1, 2). This application note demonstrates the use of Deep Purple Total Protein Stain as a simple, quick and highly sensitive stain for detecting and quantifying protein electro-blotted to Hybond™ ECL™ nitrocellulose and Hybond-P polyvinylidene fluoride (PVDF) membranes.

Proteins electro-blotted onto membranes can be used in a range of applications. Blotting to Hybond-P (PVDF) essentially archives protein in a dry stable state. Due to its strength and tolerance of organic solvents, Hybond-P is suitable for further analysis with antibodies, activity assays, stains, mass spectrometry and automated *N*-terminal sequencing at a time convenient to the researcher. Proteins are typically blotted to Hybond ECL (nitrocellulose) for further immunological analysis, especially Western blotting. Total protein detection on electro-blots is often desired for locating and/or confirming protein position and identity. Total protein staining of blots makes it easier to locate a particular band or spot in an array of proteins. This is especially true in the case of 2-D protein arrays or where antibodies have been used to detect a given protein type. The use of Deep Purple Total Protein Stain makes it possible to obtain this information on a single blot. This is of particular advantage where low amounts of sample are available and low levels of background are required.

A number of colorimetric and fluorescent blots stains are commercially available. The colorimetric stains most widely used are Amido Black (Napthol Blue Black), India Ink, Coomassie™ Brilliant Blue R-250, colloidal Coomassie Blue G-250, and colloidal gold. The protein detection sensitivities range from 3 ng per band for colloidal gold to 50 ng for

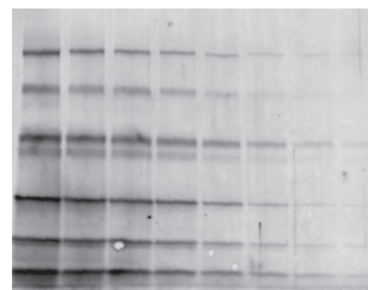

Fig 1. Low molecular weight markers two-fold diluted from approximately 128–1 ng (see Table 1), blotted to Hybond-P (PVDF) and stained with Deep Purple Total Protein Stain. The minimal level of detection was at least 1 ng of the protein concentrations listed in Table 1, referring to concentrations loaded into the gel rather than transferred to the blot. Staining of the gel after protein blotting revealed that not all protein had transferred to the membrane.

Amido Black (3). The fluorescent protein blot stains are Sypro™ Rose Plus and Sypro Ruby protein blot stains. These stains have protein detection levels of 2 ng when protein is applied directly to membranes by dot blotting (4, 5). However, if protein is quantified at the level of protein loaded to gel, the maximum sensitivity is 16 ng per band (5).

Here we demonstrate that Deep Purple Total Protein Stain is one of the most sensitive blot stains currently available. Staining with Deep Purple is rapid (30–40 min) and unlike other fluorescent stains does not require blots to be dry prior to staining. The blotting protocol is simple, robust, and disposal of the biodegradable stain is straightforward as it contains no heavy metals. Blotted proteins stained with Deep Purple Total Protein Stain can be visualized across a range of detection platforms. The fluorescent signal is stable when blots are stored dry conditions away from light. Proteins stained with Deep Purple remain amenable to subsequent *N*-terminal sequencing and other instrumental analyses. Epicocconone (2), the active molecule in Deep Purple, does not stain nucleic acid under the conditions used.

Amersham Biosciences

Products used

GE Healthcare products used:

Electrophoresis and blotting systems and consumables

LMW-SDS Marker Kit	17-0446-01
miniVE Vertical Electrophoresis Unit	80-6418-77
Hybond-P	RPN303F
Hybond ECL	RPN303D
Ettan™ IPGphor™ II IEF System	80-6505-03
Ettan IPGphor Manifold	80-6498-38
Multiphor™ II IEF System	18-1018-06
Ettan DALT*twelve* Large Vertical System	80-6466-27
Immobiline™ DryStrip Gels pH 3–10, 24 cm	17-6002-44
DeStreak™ Rehydration Solution	17-6003-19
TE 22 Mini Tank Transfer Unit	80-6204-26
PlusOne™ Tris	17-1321-01
PlusOne Glycine	17-1323-01
PlusOne SDS	17-1313-01
PlusOne ReadySol IEF 40% T, 3% C	17-1310-01
PlusOne TEMED	17-1312-01
PlusOne Ammonium Persulfate	17-1311-01
PlusOne Iodoacetamide	RPN6302
PlusOne Dithiothreitol	17-1318-01
PlusOne Glycerol	17-1325-01
Urea	17-1319-01

Imaging

Typhoon™ 9410 & ImageQuant™ TL	63-0038-55

Staining

Deep Purple Total Protein Stain	RPN6306

Other materials

Methanol
Acetic acid
Anhydrous sodium carbonate
Glycerol
Coomassie Brilliant Blue R-250
Pre-cast mini gels
Sypro Ruby Blot Stain

Methods

1-D electrophoresis and blotting

Prepare low molecular weight markers in sample loading buffer containing 50 mM dithiothreitol and heat for 10 min at 70 °C. Load Tris-Glycine 4–20% gradient mini gels prepared without SDS into a miniVE electrophoresis unit with 5 µl aliquots of protein at the quantities listed in Table 1. Run the gels with standard Tris-Glycine SDS running buffer (25 mM Tris, 192 mM Glycine, 0.1% SDS) at 110 V until the tracking dye runs off the gels.

Blot the gels to Hybond-P (PVDF) using a TE 22 Mini Tank Transfer Unit in 25 mM Tris, 192 mM Glycine and 10% methanol transfer buffer for 1 h at 400 mA.

2-D electrophoresis and blotting

Perform first dimension isoelectric focusing (IEF) using Ettan IPGphor II IEF System with 24 cm Immobiline DryStrips pH 3–10. Re-hydrate the IPG strips with 450 µl DeStreak Rehydration solution for 16 h and apply 200 µg of total cellular protein from ras-transformed NIH 3T3 cells using the cup loading method on Ettan IPGphor Manifold as described in the instructions (6). Focus the protein samples at 300 V for 3h, 1000 V for 6 h, 1000 to 8000 gradient voltage over 3 h and 8000 V for 6 h. After first dimension focusing, equilibrate the IPG strips for 15 min in SDS equilibration buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol and 1% SDS) containing 100 mM dithiothreitol followed by another 15 min equilibration in the same equilibration buffer containing 250 mM iodoacetamide. Perform the second dimension SDS-PAGE using Ettan DALT*twelve* Large Vertical System with lab-cast 8–18% gradient acrylamide Laemmli gels. Run the gels at 5 W per gel for 45 min then 17 W per gel for 4.5 h at 23 °C.

Blot the gels to Hybond ECL (nitrocellulose) overnight at 20 V in 12 mM Tris base, 96 mM Glycine and 10% methanol.

Table 1. GE Healthcare low molecular weight markers were prepared at the following concentrations (ng) per 5 µl and loaded into pre-cast 4–20% gradient mini gels.

phosphorylase b	107.3	53.6	26.8	13.4	6.7	3.4	1.7	0.8
human serum albumin	132.8	66.4	33.2	16.6	8.3	4.1	2.1	1.0
ovalbumin	235.3	117.6	58.8	29.4	14.7	7.4	3.7	1.8
carbonic anhydrase	132.8	66.4	33.2	16.6	8.3	4.1	2.1	1.0
soybean trypsin inhibitor	128	64.0	32.0	16.0	8.0	4.0	2.0	1.0
α-lactalbumin	186	93	46.5	23.2	11.6	5.8	2.9	1.5

Blot staining using Deep Purple Total Protein Stain

Hybond-P

Following electro-transfer, place the wet Hybond-P (PVDF) in 50 ml of distilled water and wash once for 5 min. Wash the blots once for 5 min in 200 mM sodium carbonate. Remove the 200 mM sodium carbonate, suspend the blots in 50 ml distilled water and add 250 µl of Deep Purple Total Protein Stain to a make a final dilution of 1/200. Stain the blots under dark conditions for 15 min. Wash the membrane once for 5 min in a solution of 10% methanol, 7.5% acetic acid. The acid creates a green background to the blot. Wash the membrane for 2–3 min with 100% methanol until the background is completely clear. Dry the blots.

Hybond ECL

Following electro-transfer, place the wet Hybond ECL (nitrocellulose) in distilled water and wash once for 5 min. Wash the blots once for 5 min in 200 mM sodium carbonate. Remove the 200 mM sodium carbonate and place the blots in 400 ml distilled water. Add Deep Purple Total Protein Stain (2 ml) to make a final dilution of 1/200 and stain the blot, away from direct light, for 15 min. Wash the blot once for 5 min in 200 mM sodium carbonate followed by two 5 min washes in distilled water. Hang the blot to dry and image. Wet nitrocellulose takes 1–2 h to fully dry, whereas the Hybond-P, because the final washes were in 100% methanol, takes only 1–2 min.

Blot staining using Sypro Ruby

Following electro-transfer and once the blot has dried, incubate face down in 10% methanol, 7% acetic acid with mild agitation for 10 min. Wash the blot four times for 5 min each in deionized water. Stain the membrane face down in Sypro Ruby Blot Stain for 15 min. Wash the membrane three more times for 1 min each in deionized water to remove excess stain and dry. Image the blot using Typhoon Variable Mode Imager according to the method described for Deep Purple stained blots.

Dot blotting

Prepare bovine serum albumin at a concentration of 3125 ng/5 µl in water and dilute four-fold to a final concentration of 190 pg/5 µl. Add 5 µl aliquots of protein to replicate strips of Hybond-P. Pre-wet each membrane area by loading 20 µl of methanol prior to loading the 5 µl protein aliquot. Fully dry both blots. Wet the upper Hybond-P panel for 30 s in methanol and stain with Deep Purple Total Protein Stain as described. Dry stain the lower panel with Sypro Ruby as previously described.

Imaging

Image the blots by placing the blot face down on the Typhoon Variable Mode Imager scanning plate. Use the following imaging conditions; 532 nm excitation laser, 100 µm resolution, and 610–30 nm BP filter for recording red emission light. Ensure the blot is pressed down during imaging to deliver even laser excitation across the membrane. To visualize the blots by eye, or to image using a CCD system, it is recommended to use UVA black light blue (BLB) lamps with transilluminators or epi-illuminators. Under these conditions the intensity of the stained protein is high and is suitable for excising fluorescent spots for subsequent analyses. UVB illumination is also acceptable.

N-terminal sequencing

In a separate experiment, separate human plasma by 2-D electrophoresis, blot to PVDF and stain as described above. Also prepare a replicate blot stained with Coomassie Brilliant Blue R-250. Stain the Coomassie Brilliant Blue R-250 blot in 0.1% Coomassie Brilliant Blue R-250 prepared in 50% methanol, 7% acetic acid for 5 min and de-stain in a solution of 50% methanol, 7% acetic acid until the background is reduced. From each blot, cut three matching spots (1 mm^2) and carry out automated Edman degradation using an Applied Biosystems 494 Procise™ Protein Sequencing System. Subject two matching protein sets to six cycles and the third set (human serum albumin) to 10 cycles.

Results and Discussion

Deep Purple staining of protein blotted to Hybond-P

Deep Purple Total Protein Stain sensitively and quantitatively stained protein on Hybond-P while exhibiting only low levels of background interference, even though the protein concentration was very low. The detection sensitivity of Deep Purple Total Protein Stain for protein blotted following 1-D electrophoresis separation was in the order of 1 ng quantities of protein (Fig 1). This compares favorably with a detection sensitivity of 16 ng for identical blots stained with Sypro Ruby Blot Stain (Fig 2). However, this underestimates the actual sensitivity of the Deep Purple blot stain as the nominal protein quantities (Table 1) are the actual amounts loaded onto gels. Indeed, the staining of gels after electro-transfer of protein to the Hybond-P membranes revealed that enough protein remained in the gel to detect with Deep Purple Total Protein Stain. Thus Deep Purple Total Protein Stain is at least as sensitive as colloidal gold (3). The Deep Purple Total Protein Stain staining protocol is simple and quick requiring 30–40 min. In addition, blots can be stained in either a wet or dry format. In a direct dot blot comparison, Deep Purple Total Protein Stain had a detection sensitivity at least 16 times greater than Sypro Ruby Blot Stain (Fig 3).

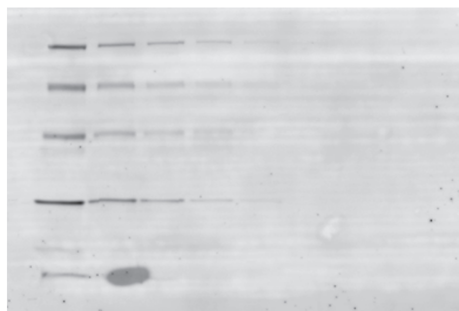

Fig 2. Low molecular weight markers two-fold diluted from approximately 128–1 ng (see table 1), blotted to Hybond-P (Fig 1) and stained with Sypro Ruby Blot Stain according to the manufacturer's instructions for staining PVDF membranes.

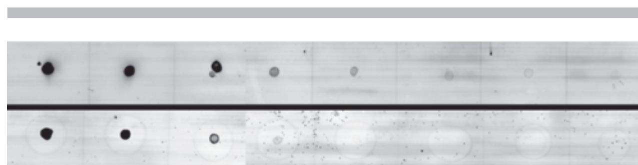

Fig 3. Dot blots stained with Deep Purple Total Protein Stain (upper image) and Sypro Ruby (lower image).

Deep Purple Total Protein Stain performed equally well for both 1-D and 2-D separated proteins blotted to Hybond-P. Hybond-P is a 0.45 µm pore-sized hydrophobic polyvinylidene difluoride (PVDF) membrane that is optimized for use in protein transfers. With higher physical strength than most other membranes, Hybond-P offers significant handling advantages and has a protein binding capacity of 125 $\mu g/cm^2$ making it ideal for use in both 1-D and 2-D electrophoresis studies, especially if further analysis or multiple cycles of handling are required. The chemical stability of the Hybond-P in a range of solvents makes it especially suited for use in subsequent studies such *N*-terminal sequencing and mass spectrometry.

Deep Purple staining of protein blotted to Hybond ECL

Deep Purple stained protein blotted to Hybond ECL (nitrocellulose) was also sensitively visualized with very low background. Figure 4 shows a large 2-D gel separation of a complex tissue sample blotted to Hybond ECL and stained with Deep Purple Total Protein Stain. Hybond ECL electro-blots stained with Deep Purple exhibit a detection sensitivity of 1 ng per band. Hybond ECL is an unsupported 100% nitrocellulose membrane that is known for delivering excellent sensitivity, resolution, and low background for labeling and detection systems. The Deep Purple staining protocols are simple, quick and deliver the ultimate sensitivity with low background.

N-terminal sequencing

The proteins were found completely amenable to automated *N*-terminal sequencing. Unambiguous sequences were obtained from all spots and proteins were positively identified. As evident from the Table 2, there were no significant differences in the repetitive yields, between Deep Purple Total Protein Stain and Coomassie Brilliant Blue R-250 stain.

Table 2. A comparison of the initial and repetitive yields in *N*-terminal sequencing for three representative proteins cut from Deep Purple and Coomassie Brilliant Blue R-250 stained blots.

	Deep Purple Total Protein Stain		**Coomassie Brilliant Blue R-250**	
Protein	Initial Yield	Repetitive Yield	Initial Yield	Repetitive Yield
Serum albumin	64 pmol	94.6%	25 pmol	96.0%
Fibrinogen	12.5 pmol	85.5%	11.5 pmol	81.6%
Haptoglobin	12.7 pmol	87.9%	13.2 pmol	87.6%

Fig 4. Electro-blot of ras transformed fibroblast protein extract stained with Deep Purple Total Protein Stain and imaged using Typhoon Variable Mode Imager.

Conclusion

Deep Purple Total Protein Stain is a widely used protein gel stain that exhibits excellent sensitivity, quantitative linearity, and is compatible with subsequent analyses such as mass spectrometry and *N*-terminal sequencing chemistry. Here we demonstrate that these characteristics are transferable to the quantification of proteins electro-blotted to polyvinylidene fluoride or nitrocellulose membranes and that Deep Purple Total Protein Stain is at least 16 times more sensitive than Sypro Ruby and Sypro Rose Plus blot stains.

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