



Deep Purple™ Gel Staining Protocol

Introduction

Deep Purple total protein stain is based on epicocconone¹, a naturally occurring biodegradable fluorescent compound extracted from a fungus. Epicocconone is a low molecular weight (410), water soluble, fluorophore that spontaneously covalently binds to primary amines (such as lysine residues in proteins) to yield an intensely red-fluorescent product² (figure 1).

This unique mechanism provides sensitive quantification of proteins across a wide variety of platforms (e.g. in *solution*, *gels*, *blots*, etc)³⁻⁶. Covalent binding of epicocconone to proteins is only stable at low pH (pH 2.4) and can be tracelessly removed by simply washing at a higher or lower pH². This pH dependent, reversible binding renders Deep Purple stained proteins particularly suitable to peptide mass fingerprinting, Edman-based sequencing, and to functional assays of non-denatured proteins.

Epicocconone is *excitable by common light sources* such as the blue (488 nm), green (543, 532 nm) and violet lasers (405 nm), enabling analysis by standard fluorescence scanners and CCD camera systems such as the Typhoon™ (GE-Healthcare), Ettan DIGE™ Scanner (GE-Healthcare), FX Molecular Imager™ (BioRad), FLA 3000 (Fuji), ChemiGenius™ (Syngene), ImageQuant™ Imager (GE-Healthcare) or similar gel documentation systems. The large Stokes' shift of epicocconone when bound to proteins (*ca* 200 nm) enables simple multiplexing with a wide range of shorter Stokes' shift fluorophores (CyDyes, fluorescein, DAPI, etc) using a single light source⁴. The spectral compatibility of Deep Purple with CyDye DIGE Fluors (Cy2, Cy3, Cy5) allows *full integration in the Ettan DIGE workflow*.

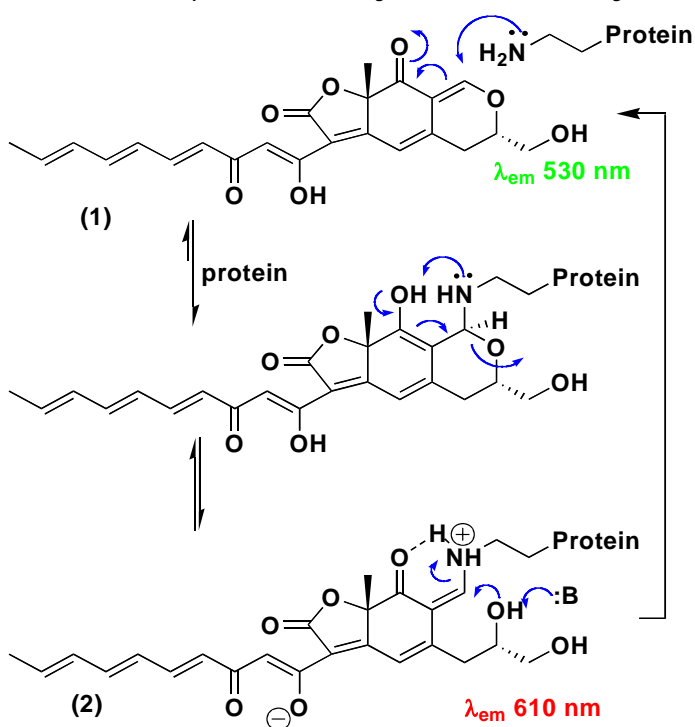


Figure 1. Spontaneous reaction of epicocconone (1) with proteins yields a fluorescent internal charge transfer complex (2) that is readily converted back to epicocconone by mild base.

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Features

- **Versatile** – Deep Purple can be used to stain 1 & 2 D gels of all gel chemistries, native and denatured proteins, nitrocellulose and PVDF blots.
- **Reversible** – stain can be removed simply by changing pH
- **MS compatible** – no cysteine peptide suppression, higher MS coverage PMT characterization.
- **Environmentally friendly** – Deep Purple is made from a biodegradable natural product that is simple and safe to dispose.
- **Sensitive** – reliably provides <50pg sensitivity when tested on 14 standard proteins of a range of molecular weights.
- **Simple and quick** – our new protocol makes Deep Purple even quicker and simpler to use.
- **Multiplex compatible** - with other fluorophores (e.g. CyDyes), other stains (eg Coomassie, ProQ Diamond™).
- **Downstream compatible** – silver with Western blotting (chemiluminescence, fluorescence detection) and Edman sequencing.
- **Clean background and no speckling** – Deep Purple does not speckle and has extremely low background fluorescence.

Background

During staining gels are basified to approximately pH 10 in order to deprotonate lysine, histidine and arginine residues. Deprotonation enables epicocconone to bind to the amines of proteins. On binding, epicocconone becomes fluorescent (figure 1) enabling ultrasensitive protein detection against a non-fluorescent background. Fluorescence is retained for up to 12-months in 1% citric or acetic acid, containing Deep Purple, because the epicocconone-protein conjugate is stable at pH 2.4. Raising the pH during tryptic digestion results in instability of the protein-fluorophore conjugate and release of unmodified protein or in the case of tryptic digestion unmodified peptides.

Improved protocol

A number of improvements in the Deep Purple staining protocol have been made resulting in a reduction in the number of steps from 7 to 4, overall hands on time from 1-2 hours to 30-40 min, total length from 4-5 to 3 hours and an increase in the robustness of the procedure. The protocol is more flexible for workflow optimization (e.g. prolonged fixation or acidification step).

1. The key to the change is the replacement of the carbonate / bicarbonate basification step prior to staining with a combined basification / staining step in borate buffer. Staining in borate buffer makes the protocol more robust than staining in water because the pH is controlled and is more resistant to carry over of acid from the fixation step. Borate (unlike carbonate or bicarbonate used previously) does not generate carbon dioxide that can accumulate on surfaces and interfere with staining. Deep Purple is also more stable in borate than carbonate and timing of the staining step is thus less critical.
2. For long-term storage of gels (up to 12-months) we recommend that gels are stored in 1% citric acid containing 1:200 dilution of Deep Purple at 4°C. The staining solution may be re-used for storage by lowering the pH with citric acid. Gels stored with the stain have considerably higher retention of their fluorescence intensity.
3. The staining no longer needs to be carried out in the dark.
4. Fixing in citric acid provides improved results compared to acetic acid.

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Protocol

In addition to the standard protocol, a superfast protocol, which provides results within 2-hours and a non-fixing protocol, designed specifically for LC-MS/MS or blotting are described.

Solutions

1. **Fixation** : 15% (v/v) ethanol; 1% (w/v) citric acid (approx pH 2.3) in water. The volume of fixation solution should be at least 20× that of the gel volume.
2. **Staining** : 100mM sodium borate, pH 10.5-10.8 in water. Dissolve 6.2g boric acid in 800 ml water and adjust pH to 10.5 with NaOH (approximately 5 ml of a 50% solution), then make to 1 liter. The volume of staining solution should be 10-20× that of the gel volume. Add 1 part Deep Purple to 200 parts borate buffer.
3. **Washing**: 15% (v/v) ethanol in water. The volume of washing solution should be at least 10-20× that of the gel volume.
4. **Acidification**: 15% (v/v) ethanol; 1% citric acid (pH 2.3) in water (same as fixation solution (1)). The volume of acidification solution should be 10-20× that of the gel volume.
5. **Storage**: Long term storage (up to 12-months) of the gels can be achieved by replacing the acidification solution (4) with 1% citric acid containing 1:200 dilution of Deep Purple. Alternatively recycled staining solution (2) may be used. The pH of the staining solution must adjusted to pH 2.4 by the addition of approximately 5% citric acid (w/v). Re-use of staining solution may produce a fluorescent precipitate that will present as speckling on the gels, thus this solution should be filtered before adding to the gel.
6. **Solution volumes**:

| Gel Dimensions | Volume of Solution Used | Volume DP concentrate added |
|---|-------------------------|-----------------------------|
| 8cm x 11 cm x 1 mm (Mini-gels) | 100 ml | 500 µl |
| 13.3 cm x 8.7 cm x 1 mm (small format 2D gels) | 150 ml | 750 µl |
| 17 cm x 17 cm x 1 mm | 500 ml | 2.5 ml |
| 17 cm x 17 cm x 1.5 mm | 500 ml | 2.5 ml |
| 15 cm x 19 cm x 1 mm | 500 ml | 2.5 ml |
| 15 cm x 19 cm x 1.5 mm | 500 ml | 2.5 ml |
| 20 cm x 25 cm x 1 mm | 800 ml | 4 ml |
| 20 cm x 25 cm x 1.5 mm | 800 ml | 4 ml |



Standard Protocol

- 1. Fixation:** Fix gels in the fixation solution (1) for a minimum of 1 hr with gentle rocking. For gels thicker than 1 mm or backed gels the fixation time should be extended to 1.5 hours. The fixation time can be extended to overnight if required for workflow or to decrease background. Additional fixation washes may also be used to decrease background levels even further though this is generally not required.
- 2. Staining:** Remove the gels from the fixation solution and place into the staining solution (2) with gentle rocking. Try to minimise carry-over of the acidic fixation solution. Stain for 1 hour for 1.0 mm thick gels. For gels 1.5 mm thick or backed gels increase the staining time to 1.5 hours. (Extending the staining time up to 4 hours will not adversely affect results. There will be some loss in fluorescence intensity if the staining time is greater than 5 hours.)
- 3. Washing:** Remove the gels from the staining solution and wash the gels by gentle rocking in the washing solution (3) for 30 minutes. (This step should be increased to 45 minutes for 1.5 mm gels or if you experience high background fluorescence.)
- 4. Acidification:** Remove the gels from the washing solution and acidify by placing them in the acidification solution (4) and rock gently for 30 min. (This step may be repeated or extended up to overnight to reduce background staining.)

Imaging:

Laser-scanning. For laser scanning based instruments we recommend the use of the green (532 nm) light source and a long pass orange filter (LP 560 nm). For exceptionally low backgrounds a red (610 nm) band pass filter may be used. For many multiplex applications (such as DIGE) scanning with the blue (457 nm) laser with a red (610 nm) band pass filter will avoid cross talk (e.g. with the Cy2 and Cy3 signal). Details of using Deep Purple with the Ettan DIGE system can be found in the GE-Healthcare Application Note *18-1177-45AA, 2003-09*.

Ettan DIGE imager. With the Ettan™ DIGE imager (GE-Healthcare) use the green (540/25 nm) light source with the orange (595/25 nm) filter. For many multiplex applications (such as DIGE) the violet excitation filter (390/20 nm) with the orange emission filter (595/25 nm) avoids cross talk (e.g. with the Cy2 and Cy3 signal).

Transilluminator-based CCD systems. We recommend that you use long wavelength UVA or black light blue lamps in combination with orange (LP 560 nm) filters and cool the gel prior to imaging.

Storage:

Gel should be stored at 4°C protected from light in the gel storage solution (5). Prior to imaging the gels should be rinsed (2 x 15 minutes) in washing solution (3).

Spot picking:

For manual spot picking we recommend the use of blacklight blue lamps with orange glasses. This provides a safer option than UV-lamps for spot cutting and is closer to the excitation maxima of epicocconone. Deep Purple stained gels can also be subsequently stained with Coomassie™ Brilliant Blue or silver to visualise spots for manual spot picking. Details of colorimetric post staining can be found in GE-Healthcare Application Note *11-0008-180AA, 2004-6*.

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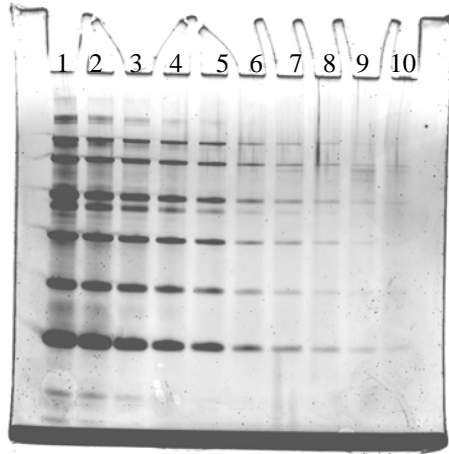


Figure 1: Staining of GE-Healthcare low molecular weight calibration standards using the borate based standard protocol on 12% Bis-Tris Minigels. Lane 10 = 32 pg of protein per band. Gel was imaged using the GE-Healthcare Typhoon 9400, green laser (532nm), with a 560 long pass filter and PMT setting of 540.

Options

Staining with Deep Purple provides for a flexible solution that can be adapted to suit your workflow. We have evaluated a number of different options that can be combined to meet all your protein staining needs.

Solutions

Fixation and acidification solution (1 & 4):

- Ethanol 15% (v/v) in water can be replaced by methanol 30% (v/v) in water
- Citric acid 1% (v/v) in water can be replaced by acetic acid 7.5% (v/v) in water
- Note: Acetic acid concentrations should not exceed 7.5%

Washing solution (3)

- Ethanol 15% (v/v) in water can be replaced by methanol 30% (v/v) in water

We recommend that you consistently use either methanol or ethanol throughout the procedure.

Superfast Protocol

The superfast method provides results within 2 hours. This protocol does not have an acid fixation step prior to staining and only minimal washing steps. The superfast method has reduced sensitivity (125 pg band) due to higher backgrounds than with the standard protocol.

- 1. Staining:** Place the gel directly into staining solution, comprising of 1:200 dilution of Deep Purple in 10% Ethanol, 100mM sodium borate (pH 10.5) for 1 hour with gentle rocking. The volume of staining solution should be 10-20x that of the gel volume. This solution **can not** be re-used for staining or storage as the proteins will diffuse over time due to the presence of ethanol.
- 2. Washing:** Remove the gels from the staining solution and wash the gels by gentle rocking in the washing solution (3) for 30 minutes. This step can be increased to up to 1 hour for thicker gels.
- 3. Acidification:** Remove the gels from the washing solution and acidify by placing them in the acidification solution (4) and rock gently for 30 min. This step may be repeated or extended up to overnight to reduce the background.
- 4. Storage:** to store gels for extended periods of time the gels should be placed into 1% citric acid containing 1:200 dilution of Deep Purple stain. Sufficient solution should be present to completely immerse the gel. The gels should be stored at 4 °C protected from light.

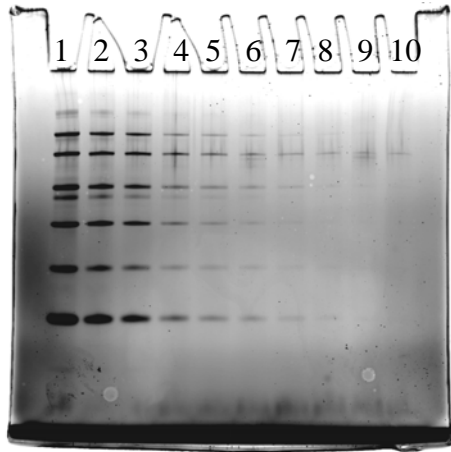


Figure 2: Superfast staining of GE-Healthcare low molecular weight calibration standards on 12% Bis-Tris Minigels. Lane 8 = 125 pg of protein per band. Gel was imaged using the GE-Healthcare Typhoon 9400 with a 560 long pass filter and PMT setting of 540.

Non-Fixing.

If LC-MS/MS or blotting is to be performed on 1DE gel bands then a non-fixing protocol can be used to rapidly stain 1DE gels. This protocol does not have an acid or alcohol fixing step in the protocol allowing greater recovery of protein from the bands after digestion. Due to the higher background and non fixing nature of the gel stain this method is not recommended for 2DE gels or for storage (though gels may be washed and acidified after bands are cut as per the standard protocol). The sensitivity of this method is reduced to 250 pg/band due to higher backgrounds.

1. **Staining:** Place the gel directly into staining solution, comprising of 1:200 dilution of Deep Purple in 100mM sodium borate (pH 10.5) for 45 min with gentle rocking. The volume of staining solution should be at least 20x that of the gel volume.
2. **Washing:** wash gel 2 x 5 minutes in 100 mM sodium borate (pH 10.5).
3. **Imaging:** Gels should be imaged using green light source (532nm) and a 610 nm BP filter (Sypro Ruby settings) to reduce background counts.

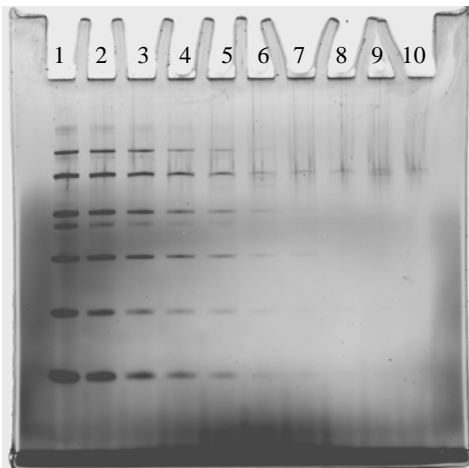


Figure 3: Non-fixing staining of GE-Healthcare low molecular weight calibration standards on 12% Bis-Tris Minigels. Lane 7 = 250 pg of protein per band. Gel was imaged using the GE-Healthcare Typhoon 9400, green laser (532nm) with a 610 band pass filter and PMT setting of 540.

Ordering

Deep Purple is available from *GE Healthcare* product code
 Product code RPN 6305 – 5 mL reconstitutes to 1 litre
 Product code RPN 6306 – 25 mL reconstitutes to 5 litres
 Store at -20°C.



Tips and troubleshooting

Preparation of solutions

- Fixing solution (1) may be prepared in advance and will store at room temperature for up to 6 months
- Boric acid / NaOH buffer may be prepared in advance and can be stored for up to 6 months. Staining solution **should be prepared fresh** before use.
- Washing solution (3) may be prepared in advance and will store at room temperature for up to 6 months.
- All solutions should be free of precipitation and protected from airborne particulate matter as this will result in speckling on the gels.
- All reagents should be of analytical grade or higher. In particular the grade of boric acid can affect the level of speckling.

Speckling

When used as recommended Deep Purple will not produce the speckles found with other fluorescent stains. Dust or particles in reagents used during staining may cause speckling. We recommend scrupulous cleanliness and the use of analytical or higher grade chemicals and reagents. If speckling is still present filtering buffers may be required. In addition plastic trays that have been previously used for Sypro products™, Coomassie or other stains may cause Deep Purple to speckle; therefore trays should be used only for Deep Purple stain or cleaned with detergent, water and methanol. The platten on laser scanners that have previously been used for Sypro products may also be source of speckling and high background.

Low signal intensity and poor sensitivity

- Ensure that the protocol has been followed accurately.
- The most common cause of low signal intensity is poor basification. Check the pH during the staining step; it should be between pH 9.5 and 10.5.
- Ensure that you use the stain at the recommended 1:200 dilution. Greater dilution will result in lower fluorescence intensity.
- Long exposure time on CCD based instruments may cause the stain to fade.
- Ensure you are using the correct filters, photomultiplier tube setting and light source on your scanner.
- Deep Purple degrades over time in high pH solutions and in bright light. Do not stain the gels for longer than the recommended time.

High background

- Ensure that the correct fixation solution was used.
- Ensure correct volumes for gel solutions have been used.
- Ensure only one gel per tray is stained. Multiple gels in one tray can result in an uneven background.
- Ensure gels are not handled or only with clean gloves to avoid contamination with dust and/or protein.
- For thicker (>1mm) or backed gels you may need to extend staining and washing times.

Boundary or negative staining

- Ensure you use a high quality SDS in the preparation and running of the gel.
- Extend the fixation time to overnight.
- Ensure you use sufficient fixation and washing solutions.
- Extend your washing time

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Reuse and dilution.

- Deep Purple slowly degrades over time in the high pH buffer. If the staining solution is to be re-used for storage it is essential that the pH be adjusted to 2.4 **immediately** after use and stored at 4 °C protected from light.
- If used immediately the staining solution may be re-used twice for gel staining.
- If sensitivity is not required beyond 1 ng per band the Deep Purple solution may be diluted up to 1:800.

Related Products

Fluorotechnics offers a range of related products including a protein quantification kit, peptide quantification kit, and a live cell imaging reagent. For details of these and our new products visit our website at <http://www.fluorotechnics.com/>.

References

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