Fluorescent Western Blotting

Typhoon Variable Mode Imager

Key words: fluorescence, chemifluorescence, Western blotting, imaging, Typhoon, CyDyes, ECL Plus, ECF, Fluorescein

Western immunodetection of proteins using chemiluminescence reagents, such as ECL™ and ECL Plus™, has gained wide acceptance as a safe, sensitive, and simple technique. Sensitive and quantitative non-radioactive Western analysis can also be accomplished using fluorescence detection. Convenient one-step fluorescence imaging does not require lengthy film exposures or development processes and generally offers better signal stability and a wider linear dynamic range than chemiluminescence methods (1) allowing more accurate quantitation.

Fluorescent Western detection employs either a direct or an enzyme-amplified (chemifluorescence) method (1). The direct fluorescence method uses antibodies labelled with fluorescent dyes, such as Cy™3 or Cy5. The chemifluorescence method uses enzyme-conjugated antibodies that react with a fluorogenic substrate to yield a highly fluorescent product. Figure 1 demonstrates the basic principle of the chemifluorescence method using the ECF™ Western Blotting Kit as an example.

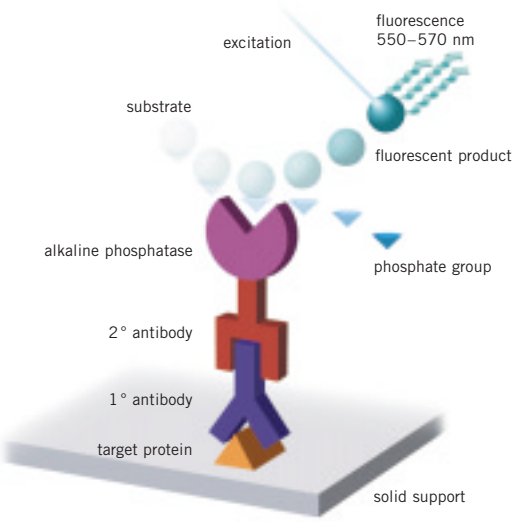

Fig 1. Schematic diagram of the ECF Western blot detection. Proteins are detected by chemifluorescence using alkaline phosphatase-labelled secondary antibody. Alkaline phosphatase cleaves a phosphate group from the ECF substrate to yield a highly fluorescent product.

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
EPS 301 Power Supply	18-1130-01
Amersham Biosciences miniVE Vertical Electrophoresis System	80-6418-77
Amersham Biosciences Blot Modul	80-6418-96
Hybond-P	RPN2020F
Fluorescein-linked anti-mouse Ig	N1031
Cy3-linked anti-rabbit IgG	PA43004
Cy5-linked anti-mouse IgG	PA45002
ECF Western Blotting Kit	RPN5780
ECL Plus Western Blotting Detection Reagents	RPN2132
HRP-linked anti-mouse IgG	NA 931
ECL Blocking Agent	RPN 2125

PlusOne™ reagents

Acrylamide IEF	17-1300-01
Bromophenol Blue (BPB)	17-1329-01
Dithiothreitol (DTT)	17-1318-01
Glycerol (87% w/w)	17-1325-01
Glycine	17-1323-01
Methylenebisacrylamide	17-1304-01
SDS	17-1313-01
Tris	17-1321-01
Tween™ 20	17-1316-01

Other materials required

- Tubulin (ICN)
- Actin (Sigma)
- Monoclonal anti-β-tubulin antibody (Sigma)
- Rabbit anti-actin antibody (Sigma)
- DDAO phosphate (Molecular Probes)
- Tris buffered saline buffer with Tween 20 (TBS-T)
  - 20 mM Tris-Cl, pH 7.6
  - 137 mM sodium chloride
  - 0.1% Tween 20
- Tris buffer with magnesium chloride (Tris/$MgCl_2$)
  - 10 mM Tris-Cl, pH 9.5
  - 1 mM magnesium chloride
- Treatment buffer (TB)
  - 62.5 mM Tris-Cl, pH 6.8
  - 2% SDS
  - 10% glycerol
  - 0.01% Bromophenol Blue

Amersham Biosciences

This application note describes the use of both direct fluorescence and chemifluorescence detection in Western blot analysis. Fluorescein-, Cy3-, and Cy5-conjugated antibodies were used for direct fluorescence detection, while ECL Plus, ECF, and DDAO phosphate were used as fluorogenic substrates for chemifluorescence detection (Table 1). Note that ECL Plus substrate can be used for both chemifluorescence and chemiluminescence detection. Storm chemifluorescence imaging of Western blots developed with ECL Plus results in detection limits that match those obtained with 5 minutes of film-based chemiluminescence detection and offers advantages of two-fold wider linear dynamic range (2). In this application note, we focus on the chemifluorescence property of ECL Plus substrate, which is also compatible with fluorescence imaging using the Typhoon™ 9400 series of variable mode imagers.

Table 1. Chemifluorescence substrates, excitation and emission maxima of the resulting fluorescent products, and enzymes used for chemifluorescent Western detection.

Chemifluorescence substrate	Excitation (nm)	Emission (nm)	Enzyme
ECL Plus*	430	503	Horseradish Peroxidase (HRP)
ECF	440	560	Alkaline Phosphatase (AP)
DDAO phosphate	646	660	Alkaline Phosphatase (AP)

*ECL Plus substrate can be used for both chemifluorescence and chemiluminescence detection.

Typhoon 8600, 9200 and 9210 have two excitation sources for fluorescence imaging, a green Nd-YAG (532 nm) and a red He-Ne (633 nm) laser. Typhoon 9400 and 9410 have an additional blue Argon-ion laser with two excitation lines (457 nm and 488 nm). The laser lines are compatible with the excitation spectra of various fluorochrome-conjugated antibodies and fluorescent products from common chemifluorescent Western blot imaging.

Protocol

❶ Preparing the sample

1.1. Prepare two-fold serial dilutions of protein sample in TB/DTT (0.1 M) buffer.

❷ Electrophoresing the sample

2.1. Prepare 1-mm-thick gels with 4% stacking gel and 10% resolving gel using Amersham Biosciences™ miniVE System.

2.2. Load the prepared samples onto the gel.

2.3. Perform electrophoresis using EPS 301 Power Supply for 2 h at 12.5 mA constant current per gel.

❸ Western blotting

3.1. Following electrophoresis, transfer the samples from the gel to a piece of Hybond™-P membrane using standard Western blotting protocols.

3.2. Incubate the blot in blocking solution (5% ECL Blocking Agent in TBS-T solution) for 1 h at room temperature with gentle shaking.

3.3. Dilute the primary antibody in blocking solution (Table 2).

3.4. Incubate the blot with the diluted primary antibody for 1 h at room temperature with gentle shaking.

3.5. Wash the blot in 100–150 ml of TBS-T: two 5-min washes, one 15-min wash followed by two additional 5-min washes.

3.6. During the last wash, dilute the fluorochrome-conjugated secondary antibody or enzyme-conjugated secondary antibody in blocking solution (Table 2).

3.7. Incubate the blot with the diluted fluorochrome-conjugated secondary antibody or enzyme-conjugated secondary antibody solution prepared in Step 3.6 for 1 h at room temperature with gentle shaking.

3.8. Wash again as in step 3.5. At this point, the fluorescent blots are ready for imaging (Step 5). For chemifluorescent blots, continue with chemifluorescence substrate incubation procedures (Step 4).

Note: Concentrations of both primary and secondary antibodies should be optimized for each new Western protocol or an established protocol using a new batch of antibody (3).

Table 2. Summary of antibody dilutions described in this application note.

Chemifluorescence method

Protein target	Substrate	Primary antibody dilution	Secondary antibody dilution
β-tubulin	ECL Plus	Monoclonal anti-β-tubulin 1:1250	HRP-linked anti-mouse IgG 1:5000
β-tubulin	ECF	Monoclonal anti-β-tubulin 1:200	AP-linked anti-mouse IgG 1:2500
β-tubulin	DDAO phosphate	Monoclonal anti-β-tubulin 1:200	AP-linked anti-mouse IgG 1:5000

Direct fluorescence method

Protein target	Fluorochrome	Primary antibody dilution	Secondary antibody dilution
β-tubulin	Fluorescein	Monoclonal anti-β-tubulin 1:200	Fluorescein-linked anti-mouse Ig 1:1000
β-tubulin	Cy5	Monoclonal anti-β-tubulin 1:200	Cy5-linked anti-mouse IgG 1:2000
Actin	Cy3	Rabbit anti-actin 1:100	Cy3-linked anti-rabbit IgG 1:1000

❹ Chemifluorescence substrate incubation

4.1. ECL Plus substrate

4.1.1. Place the washed blot, protein-side up, on a clean plastic sheet on a flat surface.

4.1.2. Prepare ECL Plus substrate (40:1 mixture of component A and B) as instructed by the product manual. Apply ECL Plus substrate at 100 µl/cm^2 of membrane, so that the blot is completely covered. Incubate at room temperature for 5 min.

4.1.3. Carefully pick up the blot with clean forceps. Briefly drain the excess reagent and place the wet blot in an open sheet protector (low fluorescence). Avoid bubble formation between the blot and the sheet protector.

4.1.4. Keep the blot in the dark, at room temperature for 30 min before imaging. The chemifluorescence signal intensity typically increases exponentially during the first 30 min of incubation and then begins to plateau.

4.2. ECF substrate

4.2.1. Place the washed blot, protein-side up, on an open sheet protector (this must be a low fluorescence material or high backgrounds may occur).

4.2.2. Apply ECF substrate at 5 µl/cm^2 of membrane. Close the sheet protector slowly so the blot is covered with the substrate completely. Work out any bubbles along with excess substrate using a laboratory wipe, and incubate at room temperature for 1 to 5 min.

4.2.3. The incubation time for optimal sensitivity depends on the target concentration on the blot and should be experimentally determined for each new Western application.

4.3. DDAO phosphate

4.3.1. Dissolve DDAO phosphate powder in deionized water to make a stock solution at a concentration of 1.25 mg/ml.

4.3.2. Calculate the volume of the final substrate solution needed for incubation (100 µl needed per cm^2 of membrane). The final substrate solution is a 1:1 000 dilution of stock solution in Tris/$MgCl_2$ buffer.

4.3.3. Place the washed blot, protein-side up, on a clean plastic sheet on a flat surface. Apply the diluted DDAO phosphate solution onto the blot, so that the blot is completely covered. Incubate at room temperature for 5 min. The incubation time for optimal sensitivity depends on the target concentration on the blot and should be experimentally determined for each new Western application.

4.3.4. Carefully pick up the blot with clean forceps. Briefly drain the excess reagent and place the wet blot in an open sheet protector (low fluorescence). Avoid bubble formation between the blot and the sheet protector.

❺ Imaging

Position the blot, protein-side down, onto the clean glass platen. *Blots can be air dried in the dark before imaging. Dry blots were found to give slightly better signal-to-noise ratio for the procedures described in this application note.* If a wet blot sample is used for imaging, keep the blot in the low fluorescence sheet protector. Ensure there are no air bubbles trapped between the protein side of the blot and the protector sheet.

5.1. Choose the instrument settings as shown in Table 3 and then select **"Press Sample"** choice. In addition, choose the following parameters and appropriate PMT voltage setting (the recommended range is 450–800 V):

Focal Plane – Platen

Pixel Size – 200 µ

Sensitivity – Normal

Table 3. Instrument settings for imaging fluorescent Western blots.

Chemifluorescence method		
Substrate (excitation, emission maxima)	**Laser**	**Emission filter**
ECL Plus (430 nm, 503 nm)	457 nm*	520BP40*
ECF (440 nm, 560 nm)	532 nm	526SP†
DDAO phosphate (646 nm, 660 nm)	633 nm	670BP30
Direct fluorescence method		
Fluorochrome (excitation, emission maxima)	**Laser**	**Emission filter**
Fluorescein (495 nm, 520 nm)	532 nm	526SP†
Cy3 (550 nm, 570 nm)	532 nm	580BP30
Cy5 (649 nm, 670 nm)	633 nm	670BP30

*Only available on Typhoon 9400 and 9410 models.
†For explanation on this filter selection, please see reference 4.

The optimal excitation and emission set-ups (Table 3) for imaging fluorescent Western blots were experimentally determined. It is important to note that in Table 3 the excitation wavelength and emission filter selection for optimal sensitivity do not necessarily match the excitation and emission maxima of the fluorochrome of interest. For membrane applications, substantial background fluorescence primarily due to non-specific antibody binding may occur. Shifting the excitation and emission wavelengths slightly away from the fluorochromes' spectral maxima wavelengths sometimes greatly reduces the noise and improves the overall signal-to-noise ratio. For the most sensitive results, we recommend that the optimal set-up should be experimentally determined for imaging each of the fluorescent Western blot applications.

For more details about Typhoon emission filter selection and other practical aspects of using Typhoon scanners, please refer to Typhoon Instrument Guide as well as *Fluorescence Imaging: principles and methods* (1).

Results

Two examples of fluorescent Western blots imaged on Typhoon are shown in Figure 2a and Figure 2b.

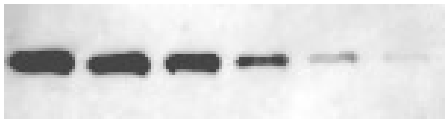

Fig 2a. Typhoon image of chemifluorescent Western blot of serial two-fold dilutions of tubulin using ECL Plus substrate. Lanes from left to right contain 16, 8, 4, 2, 1 and 0.5 ng of β-tubulin.

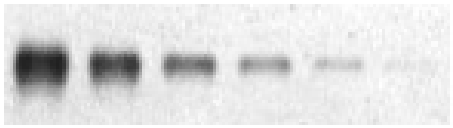

Fig 2b. Typhoon image of fluorescein labelled fluorescent Western blot of serial two-fold dilutions of tubulin. Lanes from left to right contain 32, 16, 8, 4, 2 and 1 ng of β-tubulin.

The detection limits and linear ranges for β-tubulin detection using some of the fluorescent Western blots are summarized in Table 4. Typhoon imaging of Western blots developed with ECL Plus results in detection limits that match those obtained with 8 min of film-based chemiluminescence detection and offers advantages of four-fold wider linear dynamic range over film-based detection.

Table 4. Summary of Typhoon limit of detection and linear range for β-tubulin detection by chemifluorescence and direct fluorescence methods.

Chemifluorescence method		
Substrate	**Limit of detection* (β-tubulin)**	**Linear range**
ECL Plus	0.5 ng	30 fold
ECF	2–4 ng	10–20 fold
DDAO phosphate	1–2 ng	10–20 fold
Direct fluorescence method		
Fluorochrome	**Limit of detection* (β-tubulin)**	**Linear range**
Fluorescein	1 ng	130 fold

*The limit of detection is the threshold at which the background-corrected signal-to-noise ratio is at least three.

It is important to note that Typhoon has a wide, linear dynamic range of five orders of magnitude. The linear ranges shown in Table 4 are limited by experimental factors in transferring the protein from gel to membrane and subsequent treatment of the blot. Both sensitivities and linear ranges for Western blots are highly dependent on multiple experimental factors including the type of protein target, antibody purity and affinity for the protein target. For each new Western detection protocol, the concentrations of both primary and secondary antibodies should be optimized. When using a new batch of antibody or changing the supplier of antibodies, the protocol should be re-examined to make sure the optimal results are obtained. The results presented here were obtained using the products, materials, and optimized protocols described in this application note.

The ability to detect more than one protein target simultaneously on the same Western blot is demonstrated in Figure 3. A mixture of tubulin and actin were first separated by SDS-polyacrylamide electrophoresis before it was transferred to Hybond-P membrane. The primary antibody used was a mixture of monoclonal anti-β-tubulin antibody and rabbit anti-actin antibody. The secondary antibody was a mixture of Cy5-linked anti-mouse IgG and Cy3-linked anti-rabbit IgG. Direct fluorescence detection using both Cy3 (actin) and Cy5 (tubulin) antibody conjugates at the same time improves the speed and throughput of analysis.

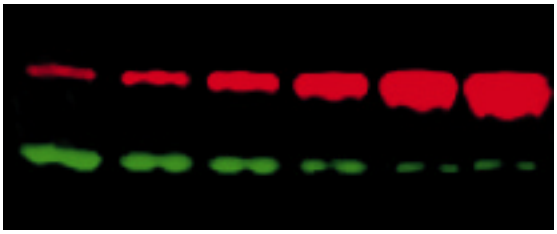

Fig 3. Typhoon image of a dual target Western blot obtained using settings from Table 3. Both actin and tubulin, resolved in serial two-fold dilution, are detected. Tubulin (red) was detected using anti-β-tubulin monoclonal antibody and Cy5-linked anti-mouse IgG. From left to right, lanes 1 to 6 contain 15.5 ng, 31 ng, 62 ng, 125 ng, 250 ng, 500 ng of β-tubulin. Actin (green) is detected with rabbit anti-actin antibody and Cy3-linked anti-rabbit IgG. From left to right, lanes 1 to 6 contain 640 ng, 320 ng, 160 ng, 80 ng, 40 ng, and 20 ng of actin.

Conclusions

Typhoon is a versatile and sensitive tool for the detection and analysis of fluorescent Western blots using a wide variety of commonly available fluorochromes and fluorogenic chemifluorescence substrates. Chemifluorescence detection of Western blots combines the advantages of signal stability and wider linear range from fluorescence and signal amplification from chemiluminescence. It is potentially a more sensitive method than direct fluorescence due to signal amplification. Chemifluorescence can be used in place of any standard chemiluminescence method using alkaline phosphatase (AP)- and horseradish peroxidase (HRP)-conjugated antibodies with minimal protocol modification. Direct fluorescence is a more simple and rapid method for Western blot detection. Recent developments in dye chemistry and instrumentation such as Typhoon laser-based scanning systems have significantly improved the sensitivity of Western detection by direct fluorescence. In addition, direct fluorescence imaging is particularly convenient for simultaneous detection of more than one protein target.

References*

1. *Fluorescence Imaging: principles and methods*, Amersham Biosciences, code number 63-0035-28, (2000).
2. Application Note #60: Storm Image Analysis of Horseradish Peroxidase (HRP)-based Western Blots using Amersham Biosciences ECL Plus Substrate, Amersham Biosciences, code number 63-0028-71, (1999).
3. Technical Note #59: Optimization of Amersham Biosciences ECL Plus Detection of Western Blots for Storm Image Analysis, Amersham Biosciences, code number 63-0028-81, (1999).
4. *Fluorescence Imaging: principles and methods*, Amersham Biosciences, code number 63-0035-28, p. 115 (2000).

*References cited in this application note can also be found at: http://www.mdyn.com.

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