Pure Protein and Total Lysate Chemiluminescence Detection via Peltier-Cooled CCD

Aim: To visualise and evaluate the performance of chemiluminescent immunoblots using Wealtec's Dolphin-Chemi plus image system

INTRODUCTION

Luminescence: Luminescence is the generation of light without the production of heat. Several types of luminescence are known, among them bioluminescence, which takes place within many living organisms and makes e.g. fireflies and certain jellyfish glow in the dark. The type of luminescence is determined by the source of energy that results in the light emission. A *chemi*luminescent reaction occurs when a chemiluminescent compound, e.g. luminol or lucigenin, becomes excited due to a chemical reaction. As the chemiluminescent compound decays to its ground energy level, it emits energy in the form of detectable chemiluminescent light.

Chemiliminescent enzyme immunoassay: The chemiluminescence phenomenon is widely used, not least in molecular biology, where it serves as a highly sensitive detection system for visualising e.g. specific protein- or nucleic acid samples. When used for immunoassays, an enzyme catalysing the chemiluminescent reaction, e.g. horseradish peroxidase (HRP) or alkaline phosphatase (AP), is covalently bound to an antibody. The enzyme-conjugated antibody binds either directly, as a primary antibody, to the antigen of interest blotted onto a membrane, or as a secondary antibody, to another non-conjugated primary antibody already bound to the antigen of interest. When using a system with a primary and a secondary antibody, several secondary antibodies can bind to the primary antibodies, and the signal thus becomes amplified. Prior to detection, chemiluminescent reagents are added to the blot with the bound enzyme-conjugated antibodies. The enzyme, e.g. HRP or AP, promotes the oxidation of the chemiluminescent compound which becomes excited and emits light as it decays to its ground state. A schematic illustration of a chemiluminescence-blot is provided in figure 1.

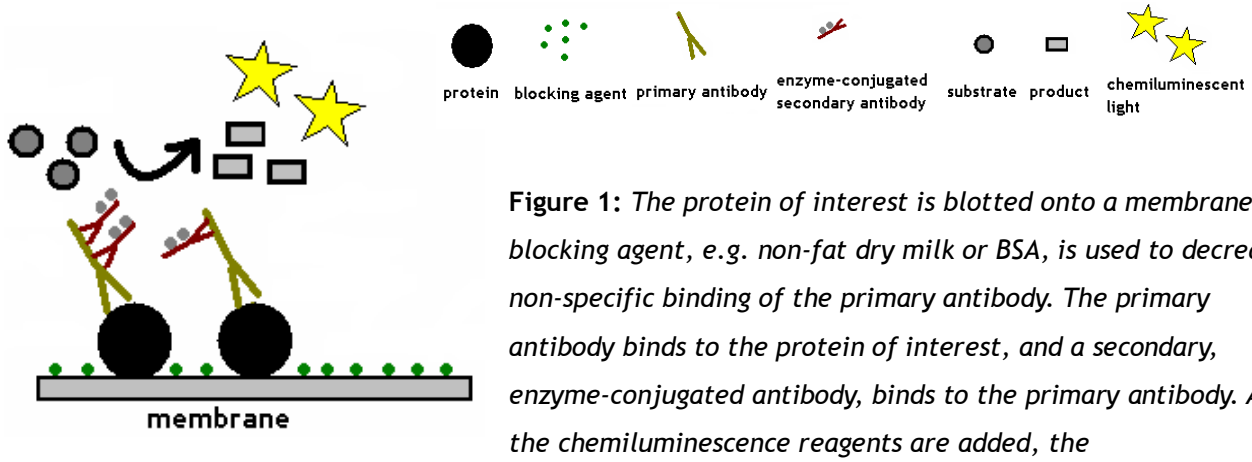


Figure 1: *The protein of interest is blotted onto a membrane. A blocking agent, e.g. non-fat dry milk or BSA, is used to decrease non-specific binding of the primary antibody. The primary antibody binds to the protein of interest, and a secondary, enzyme-conjugated antibody, binds to the primary antibody. As the chemiluminescence reagents are added, the chemiluminescent substrate becomes oxidised by the enzyme. As the substrate returns from a chemically excited state to its ground energy state, it emits chemiluminescent light.*

Chemiluminescence detection: Traditionally, chemiluminescent detection of immunoblots has been performed with x-ray followed by scanning of the film for densitometric analysis. Handling of X-ray films require a darkroom and chemicals for developing the films and is, taken together, a rather inconvenient and time-consuming process. The development of CCD-cameras has made chemiluminescent detection and analysis of immunoblots much more flexible and manageable. CCD-camera detection requires no darkroom or chemicals for X-ray film development, it is highly sensitive and very convenient to use.

Dolphin-Chemi Plus Image system is equipped with a 12-bit CCD-camera. Peltier-cooling down to -40°C reduces noise due to high temperatures often generated by long exposure times, frequently used in chemiluminescence. When connected to the Dolphin-1D analysis software, chemiluminescence results obtained from the CCD-camera can be instantly processed and analysed. Image enhancement by filtering and background subtraction improves the quality of the resultant image.

MATERIAL AND METHODS

Western blotting; The protein concentration of lysates of the mouse macrophage cell line J774 was determined according to Lowry (Lowry *et al.*, 1951) and spectrophotometrically read using the protein function of SpectroArt 200 spectrophotometer (Wealtec Bioscience Co., Ltd. Taipei, Taiwan). A mixture of bromophenol blue and DTT was added to each sample to a final concentration of 0.05 % and 0.5 % respectively. The protein samples were boiled for 5 minutes at 95°C on a block heater (Wealtec Bioscience Co., Ltd.). 30 µg samples were the loaded on to 12 % lab-made SDS- polyacrylamide gels. After electrophoretic separation (40 V, 16 mA prerun, 200 V, 60 mA/gel run) in Tris-Glycine Buffer (25 mM Tris-HCl, 192 mM glycine, 3.5 mM SDS), the proteins were transferred onto a nitrocellulose membrane (Millipore Corporation, Billerica, MA, U.S.A.) by western blotting (100 V, 235 mA in blotting buffer (25 mM Tris-HCl, 192 mM glycine and 20 % methanol)). After transfer, the membranes were blocked in TBS containing Tween-20 (TBST) supplemented with 5 % non-fat dry milk. The membranes were then washed in TBST and incubated at 4°C over night with primary antibodies diluted in TBST supplemented with 0.1 % dry milk. The primary antibody used was mouse monoclonal ß-actin (1:1000; Santa Cruz Biotechnology). The membranes were washed in TBST and then incubated with a secondary goat anti-mouse antibody conjugated with HRP (1:1000; Santa Cruz Biotechnology) diluted in TBST supplemented with 0.1 % dry milk. The chemiluminescent substrate (Immobilon Western Chemiluminescent HRP Substrate, Millipore Corporation) was mixed according to the manufacturers instructions and added to the membranes before the samples were visualised in Dolphin-Chemi Plus (Wealtec Bioscience Co., Ltd.).

Dot Blot; Recombinant ß-actin (0.047 µg/µl, Abnova Corporation, Taipei, Taiwan), was serially twofold diluted down to 8.5 pg in ddH_20. 3 µl samples were blotted onto a dry nitrocellulose membrane. The membrane was left to dry on filter paper and then blocked for 90 minutes in TBST supplemented with 5 % non-fat dry milk. The membrane was then incubated for 1 hour at RT on a rocker with a primary monoclonal ß-actin antibody (1:1000; Santa Cruz Biotechnology), diluted in TBST supplemented with 0.1 % dry milk. The incubation step was followed by washing in TBST and incubation at RT with a secondary HRP-conjugated antibody (1:1000; Santa Cruz Biotechnology) diluted in TBST supplemented with 0.1 % dry milk. After washing in TBST and finally ddH_20, the chemiluminescent substrate (Immobilon Western Chemiluminescent HRP Substrate,

Millipore Corporation) was added and the blot was visualised in Dolphin-Chemi Plus (Wealtec Bioscience Co., Ltd.).

Visualisation; The sample tray was driven to the highest possible position and the blots, wrapped in cling film, were put on the tray. The Epi-illumination was turned on for positioning the blot and focusing the obtained image. The iris was then 85-90 % opened, and the blots were exposed to the CCD-camera for 60-300 seconds. To obtain a clearer image, an extra close up 2-filter was positioned in the filter wheel position covering the camera lens. All adjustments in image appearance, filter wheel positioning and exposure times were performed using the Dolphin-1D software (Wealtec Bioscience Co., Ltd.).

RESULTS

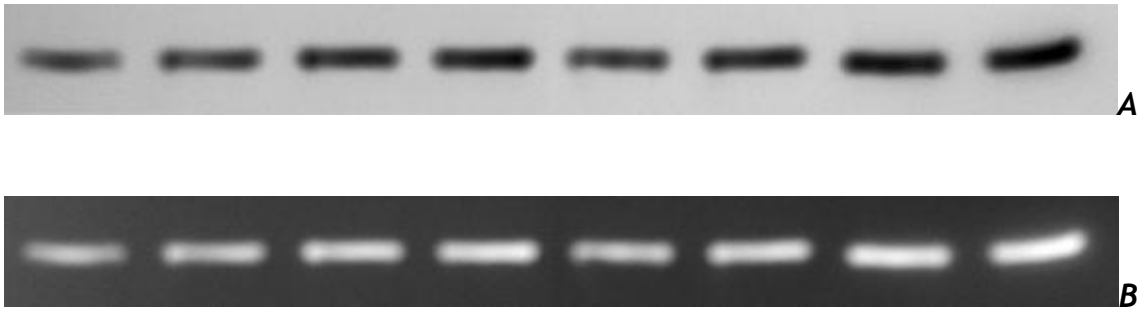
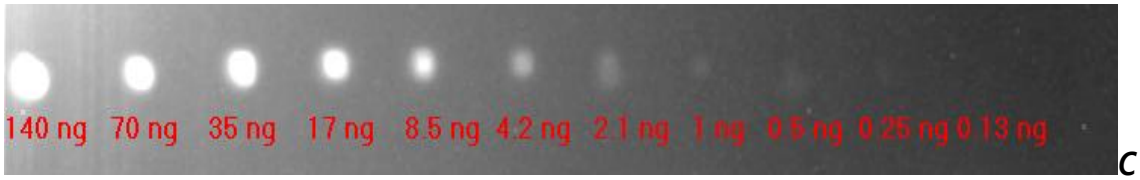


Figure 2: *A and B; Chemiluminescent visualisation of J774 mouse macrophage β-actin. 30 µg samples were loaded onto 12 % SDS-PAGE gels. Proteins in the gel were blotted onto a NC-membrane. After blocking non-specific binding sites with TBST supplemented with non-fat dry milk, the membrane was incubated with a primary mouse anti-β-actin antibody followed by a HRP-conjugated goat anti-mouse secondary antibody. The blot was subjected to chemiluminescence reagents and then visualised with Dolphin-Chemi Plus. The blot was exposed for 60 sec. Image A is a reversed colour version of the image generated by the image system in B.*

A

B



C

Figure 3: *A-C; Chemiluminescent visualisation of recombinant β-actin, serially twofold diluted and dot-blotted onto a dry NC-membrane. Following blocking in TBST supplemented with non-fat dry milk, the blot was incubated with a primary mouse anti β-actin antibody, and then a secondary goat anti-mouse HRP-conjugated antibody. The blot was subjected to chemiluminescence reagents and visualised in Dolphin-Chemi Plus image system. The blot was exposed for 30 seconds in (A), 100 seconds in (B) and 300 seconds in C).*

CONCLUSION

Generating an optimal image of a chemiluminescent blot is mainly a question of trial and error and may be time-consuming. CCD-cameras make the chemiluminescent optimisation process, which may include establishing the best antibody concentrations and exposure times, much less tedious. With x-ray film, only one setting can be tested a given time point. Once exposed to light, the film can not be reused. With a CCD-camera, the flexibility is much greater, which makes it easy to play around with exposure times and light throughput.

Dolphin-Chemi Plus together with the Dolphin-1D software offers an excellent system for capture and analysis of high quality chemiluminescent blot images. Motorisation of focusing, zooming, light input as well as the positioning of the sample tray makes it easy to test different settings in order optimise the quality of the generated image. A sharp image of ß-actin in a whole cell lysate blot was generated within 60 seconds (*fig. 2)*, with the distance between the blot and the CCD-camera kept as short as possible where a sharp focus was still possible. Detection of low abundance proteins generally requires more optimisation. *Fig. 3* shows a 2-fold dilution series of recombinant ß-actin dotted onto a NC-membrane. The lowest detectable limit of ß-actin with current settings, antibody and chemiluminescent substrate was 0.25 ng, which is considerably lower than detected with most commercial proteins dyes such as Coomassie brilliant blue. As seen in *fig. A-C*, longer exposure times enhance sensitivity.

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