Immunofluorescent protein detection in Western blotting

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Abstract: This report describes the detailed procedures for Western blot analysis using fluorescent antibodies. After electrophoresis and subsequent electroblotting, the fluorescent-labeled antibodies were visible upon ultraviolet illumination of the polyvinylidene fluoride (PVDF) membranes and could then be photographed to give an accurate record of the blots. Fluorescent labeling allows for photographic records of the blot without the need for subsequent staining steps. In an effort to evaluate this method in the study of immunogenic antigens, this study describes a study on BLV (Bovine Leukemia Virus) tumor antigens. The fluorescent labeling of antibodies for detection by immunoblotting was found to be a very sensitive and reliable alternative to the conventional methods of Western blots.

Keywords: immunofluorescence, immunoblotting, Western blotting, FITC, PVDF

Introduction

Several methods have been used to visualize antigens with the use of various antibodies. These methods include immunoprecipitation (Talbot *et al*., 1984) and electroblotting (Towbin *et al*., 1979) that were popularized subsequently in the technique of Western blotting (otherwise known as immunoblotting; Burnette, 1981). However, there are problems associated with immunoprecipitation, which include the requirement for the radio-labeling of antibodies, co-precipitation of tightly-associated macromolecules, occasional difficulty in obtaining antibodies in the precipitant, and the insolubility of some antigens.

Immunoblotting is used to identify specific antigens that are recognized by polyclonal or monoclonal antibodies (Towbin, 1979; Burnette, 1981). It involves the solubilization and electrophoretic separation of proteins, glycoproteins, or lipopolysaccarides by sodium dodecyl sulfate polyacrylamide gel electrophoresis(SDS-PAGE) or urea-PAGE, followed by quantitative transfer and irreversible binding to nitrocellulose, nylon or polyvinylidene fluoride (PVDF) membranes (De Blas & Cherwinski, 1983; Ausubel *et al*., 2002). This technique has been useful in the identification of specific antigens that were recognized by polyclonal or monoclonal antibodies. Immunoblotting has been shown to be highly sensitive (Ausubel *et al*. 2002).

This technique has important clinical applications, which include diagnostic tests for retroviral infections like HIV-1 and BLV (Bovine Leukemia Virus) (Barrie *et al*., 1996; Choi *et al*. 2002). Alternatively, the detection of antigens from viruses or bacteria with the use of monoclonal or polyclonal antibodies has been greatly facilitated by the use of immunoblotting (Balachandran *et al*., 1982; Harder *et al*. 1998; Madsen *et al*., 1987; Qiu *et al*., 1992; Doltz & Moreno, 1999; Bunger *et al*. 1996).

The aim of this study was to develop a protocol for immunoblotting that was based on the use of fluorescence to detect the antigen-antibody complexes. The procedure described herein was reasonably sensitive on a PVDF membrane with a

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minimum number of steps. In order to evaluate this method for the study of immunogenic antigens, an example was studied using BLV tumor antigens.

Materials and Methods

Preparation of immunoglobulin and conjugates

***Fluorosceinisothiocyanate (FITC)-conjugates antibodies*:**

Common chemicals were used, which included bovine immunoglobulin G (IgG), bovine serum antigen (BSA) (Merck), FITC (Fluorescein isothiocyanate, Baltimore Biological Laboratories, U.S.A), rabbit anti-bovine FITC-conjugate antibody, Bovine anti-BLV FITC-conjugate antibody, PVDF membrane (Roche, Germany).

Serum samples from healthy cows were pooled; the globulin was precipitated with 40% saturated ammonium sulfate, and then dialyzed exhaustively with the use of at least three changes of phosphate-buffered saline (PBS).

An antibody to bovine IgG was prepared by immunizing rabbit with Bovine IgG. A 200µg suspension of bovine IgG was prepared in 500µl PBS, to which an equal volume of Freund's complete adjuvant (500µl) was added and injected into rabbits via the intramuscular route. The animals were rested, given a booster dose of Freund's incomplete adjuvant at two weeks, and then were bled two weeks later. During this period, the development of antibody was monitored by performing agar gel immunodiffusion (AGID) tests. The serum globulin was precipitated, dialyzed and the FITC conjugate was prepared according to the method described by Hay and Westwood (2002).

Bovine anti-BLV sera were obtained from cattle infected with BLV. BLV infection and anti-BLV antibodies were confirmed by polymerase chain reaction (PCR) and AGID, respectively. The bovine anti-BLV FITC-conjugate antibody was prepared according to Goling (2000). Tumor BLV antigens were prepared by homogenizing the lymph nodes that were infected with BLV in PBS. BLV infection of the lymph node tumors was confirmed by PCR.

Western blotting protocol:

The bovine IgG and BLV tumor antigens (20µg/ml) were titrated into four concentrations and loaded onto separate SDS–12% PAGE Mini-PROTEIN 3 Cell gels (Bio-Rad). These were electrophoresed in Tris-glycine buffer (0.025 M Tris base, 0.192 M glycine, 0.1% SDS) at 120 V/gel for 2 h (Ausubel *et al.* 2002). The protein was then transferred onto a PVDF membrane (0.45 mm pore size) with a Mini Trans-Blot apparatus (Bio-Rad) at 50 V for 50 min (Ausubel *et al.* 2002). The membrane was blocked with 3% bovine serum albumin, 0.02 M Tris base, 0.385 M NaCl and 0.1% Tween 20 (pH 7.5) (TTBS) at room temperature for 1 h, rinsed three times with TTBS. Rabbit anti-bovine FITC-conjugate and bovine anti-BLV FITC-conjugate sera were diluted to a ratio of 1:10 with TTBS. Each membrane was incubated with their related FITC-conjugate antibodies for one night at 37°C and washed three times for 5 min with TTBS. After air-drying, the blots were scanned. Imaging System (Vilber Lourmat, France) was used to scan membranes and blots.

Results

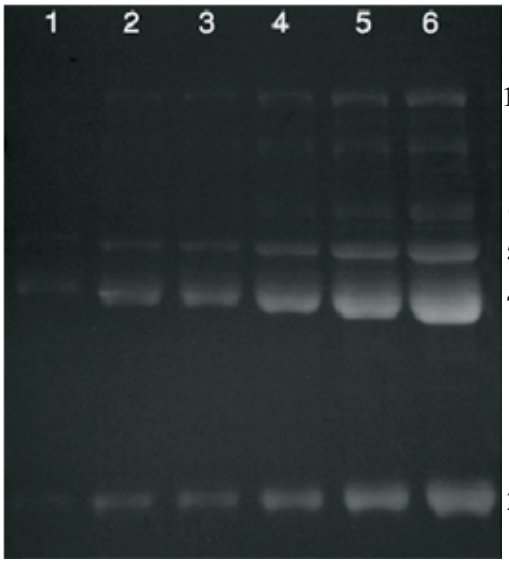
We performed a Western blot analysis to analyze FITC conjugates in order to detect proteins. When the fluorescent immunoblotting analysis was used, specific antigens were found for all of the antigens that were tested.

Rabbit anti-bovine IgG antibodies that were conjugated with FITC recognized bovine IgG with specificity and efficiency (Figure 1). The intensity of the reactions varied between the different IgG concentrations used in the assay. When the antigen was tested in serial dilutions by the FITC conjugates, different concentrations of antigen (5 µg, 10 µg, 20 µg, 40 µg, 60 µg and 80 µg) could be detected. The intensity of the reactions varied between the different dilutions of antigens.

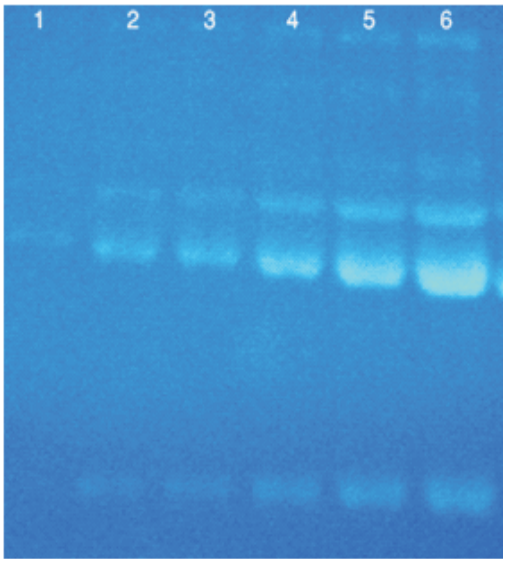
In the next experiment, we tested whether the fluorescent immunoblotting assay with bovine anti-BLV tumor antibodies conjugated with FITC could be used to test for detecting the tumor antigens.

Figure 1. Detection of bovine IgG on a PVDF blotting membrane by rabbit anti-bovine FITC- conjugate: Different concentrations of bovine IgG were used in SDS-PAGE and transferred to a PVDF membrane. 5 µg, 10 µg, 20 µg, 40 µg, 60 µg and 80 µg of IgG were loaded on the gel (left to right, line 1-6). For the Reverse image (Image A), the membrane was scanned using the Vilber Lourmat imaging system and photographed by a CCD camera (Vilber Lourmat). The membrane was also directly photographed on an ultraviolet transluminator by a digital camera (Image B).

A

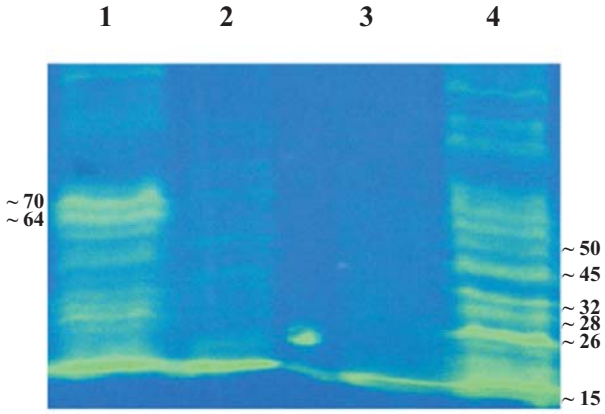


B



When this conjugate was tested by immunoblotting, eight different antigens that had a molecular weight of 70, 64, 50, 45, 32, 28, 26, and 15 kDa were

Figure 2. Detection of BLV tumor antigens on a PVDF blotting membrane:
Different samples of tumor lymph nodes from cattle infected with BLV were used to evaluate the FITC method for detecting tumor-specific antigens. The proteins were detected with anti-BLV tumor FITC-conjugate. Lines 1 and 4 represent tumor samples (lymphosarcoma), and lines 2 and 3 are the results from healthy lymph nodes. The membrane was directly photographed on an ultraviolet transluminator by a digital camera.



detected in two profiles of BLV tumor antigens in lymph nodes. No reaction was observed for non-infected lymph nodes (Fig. 2).

After electrophoresis and subsequent electro-blotting the fluorescent-labeled antibodies were visible upon ultraviolet illumination of the PVDF membranes and could be photographed to yield an accurate record of the blots. Fluorescent labeling enabled for accurate photographic records of the blot to be obtained and no subsequent staining steps were required as a consequence.

Discussion

Proteins that have undergone immunoblotting can be detected by antibody conjugates with fluorochromes, chromogenic or luminescent assays (Ausubel *et al.* 2002). Luminescent detection methods have several advantages over traditional chromogenic procedures. In general, luminescent substrates increase the sensitivity of both the horseradish peroxidase (HRPO) and phosphatase systems without the need for radioisotopes. Substrates for the latter have only been applied to protein blotting (Sandhu *et al.*, 1991; Bronstein *et al.*

1992). However, luminescent detection is laborious and can be time consuming (up to three days depending on the system used). Protocols that are based on luminescence can also be complex, since their reagents and solutions can only be purchased from specialized field test kits. It is vital to consult the instructions of the manufacturers.

The main advantage of the use of fluorescent detection in immunoblotting over chemiluminescent detection is the ability to multiplex. Multiplexing using different fluorochrome conjugates allows for the comparison of levels of several target proteins on the same blot (Gingrich *et al*., 2000). Although fluorescent Western blots are not as sensitive as chemiluminescent Western blots, they can provide better linearity and, therefore, better protein quantitation. The entire fluorescent immunoblotting procedure can also be completed in a single day, depending on antibody, antigens, transfer time and type of gel, which is substantially faster than luminescent methods.

PVDF membranes are widely used in Western blotting applications. Their durability, low non-specific binding, high signal-to- noise ratio and re-probing characteristics make them ideal for protein blotting experiments, where proteins are usually detected by chromogenic, chemiluminescent and fluorescent methods. Fluorescent detection on blotting membranes has been limited to date by high background fluorescence, which results in low sensitivity. Nonetheless, fluorescent detection in Western blotting using PVDF membranes offers several advantages, including a practically unlimited signal life, digital image acquisition and a much broader dynamic range compared to film. Moreover, imaging using fluorescent blots eliminates the need for a dark room, film and X-ray processing equipments. Digital imaging allows for the accurate quantification of band intensities.

Fluorescent protein detection in an immunoblotting system is ideal for imaging and quantitating the fluorescent samples. As an illustration of its capabilities, we demonstrated a Western blot analysis that had been hybridized with two different antibody conjugates. The technique was established with rabbit anti-bovine FITC conjugate and an example of its use in a study on BLV tumor antigens was described.

In conclusion, the fluorescent labeling of antibodies for detection by immunoblotting was found to be a very sensitive and reliable alternative to conventional methods for the detection of proteins by Western blots.

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