Immunodetection of proteins is often performed manually and involves multiple steps that easily introduce bias and errors. We have compared the traditional manual Western blotting and a novel type of Automated Western blotting by using BlotCycler™ processor developed by Precision Biosystems, and DyLight™ fluorochrome conjugated secondary antibodies developed by Rockland Immunochemicals. The results of manual and automated processing were comparable at RT, but automatic processing yielded considerably higher signal at 4°C. Automatic processing using BlotCycler™ provides excellent reproducibility and can efficiently used for optimization of primary and secondary antibodies concentration.

Abstract

It is a common practice to incubate primary antibody at 4°C and perform all other steps at RT. However, this eliminates advantages of incubation at low temperature on background and signal intensity. In preliminary experiments we noticed that performing antibody incubation using BlotCycler™ yields higher signal intensity and lower background than manual processing with primary antibody incubation at 4°C and all other steps at RT. Therefore we evaluated the role of automation in increased sensitivity and performed all steps both manual and automated processing entirely at 4°C (Fig. 2).

Whereas automation increases signal to improve the quality of Western blot both at 4°C or RT the effect is much more pronounced at 4°C. It may be explained by difficulty of manual processing at 4°C, since it requires more intensive washing.

2. The comparison of automated and manual blot processing

Optimal primary and secondary antibody concentrations depend on the antibody’s specific activity. Optimization is essential when one or more of the experimental variables such as the antigen, primary antibody, or secondary antibody are changed. However the optimization of antibody concentration can be time consuming and it is difficult to compare multiple blots processed in slightly different conditions. BlotCycler™ allows simultaneous processing of up to six different blots with six different primary or secondary antibodies at the same time with high reproducibility.

In this experiment we prepared 10 identical strips each containing two lanes: MW markers and extract of mouse pancreas. The blots were probed with rabbit-anti-Delta-4 primary antibody and DyLight™ 649 conjugated anti-rabbit IgG secondary antibody. In the first experiment we used 5 different dilutions of primary antibody (Fig. 4A) and a single dilution of secondary antibody (1:20,000). In the second experiment we selected a single dilution of primary antibody (1:1000) and six different dilutions of the secondary antibody (Fig. 4B). Using BlotCycler™ we were able to construct the titration curve for both primary and secondary antibodies that can be used to select optimal antibodies concentration depending on the experiment objective.

3. Reproducibility of automated blot processing

One of the problems of manual processing is the variability of results between blots. In this experiment we processed several identical blots using BlotCycler™. In addition to MW standard each blot contained three lanes: HeLa white cell lysate, the recombinant protein Human IL-2, and mixture of HeLa white cell lysate and recombinant protein Human IL-2. As described, Proteins were transferred from the gel onto the Nitrocellulose membranes. Membranes were blocked for 30 min at RT or for 30 min at 4°C using MB-073.

Rabbit anti-Delta-4 and mouse anti-IL2 primary antibodies were used. Primary antibodies were incubated for 60 min at RT or for 90 min at 4°C. DyLight™ 649 conjugated anti-rabbit IgG secondary antibody was used. The secondary antibodies were incubated for 30 min at RT or 30 min at 4°C. For data each DyLight™ fluorophore were collected independently at wavelength wavelengths.

4. Optimization of the antibody concentration

Western blot (Towbin et al,1979) provides important information about levels of protein expression in biological samples. The multipurpose standard Western blotting is an effort- and time-consuming process. The quality of results depends upon multiple subjective and objective factors including the qualification and technical skills of the personnel and accuracy of temporal and temperature control. During the blotting procedure: incubation with primary antibody is often done at 4°C to reduce background and to increase the intensity of specific signals. Contrary to this, the incubation with secondary antibodies and washing steps are usually performed at RT resulting in the best quality of the entire process. To circumvent these problems, standardize the procedure and improve the quality of Western blotting, Precision Biosystems has developed a device, BlotCycler™ that reduces manual labor during membrane processing and provides stable and high quality results after moving at both RT and 4ºC. BlotCycler™ combines shorter and rapid systems and delivers results in a preset time to the membranes. BlotCycler™ uses uniquely shaped trays that ensure efficient mixing of reagents to achieve low background and minimizes the amount of antibodies required for analysis. To compare the efficiency of automatic and manual processing, we used a fluoroscent based detection system, which allows simultaneous detection of the staining efficiency by up to three different primary antibodies at the RT and 4°C regimes.

Methods

The standard protocol for Western blotting has been used in these sets of experiments both for manual and automated processing:

- SDS-PAGE and western blot transfer
- Block Nitrocellulose membrane and probe with Rockland’s unique primary antibodies
- Detect with DyLight™ conjugated secondary antibodies

Tubulin and human IL-2

Fig. 2 Lane 1 & 4 are Caco-2 cells, Lane 2 & 5 are MDA-MB-231 cells, Lane 3 & 6 are HepG2 cells. Western blot results were as described. Proteins were transferred from the gel onto the Nitrocellulose membranes. Membranes were blocked for 30 min at RT or 30 min at 4°C using MB-073.

Rabbit anti-Delta-4 and mouse anti-IL2 primary antibodies were used. Primary antibodies were incubated for 60 min at RT or for 90 min at 4°C. DyLight™ 649 conjugated anti-rabbit IgG and DyLight™ 649 conjugated anti-rabbit IgG secondary antibody were used. The secondary antibodies were incubated for 30 min at RT or 30 min at 4°C. For each DyLight™ fluorophore were collected independently at wavelength wavelengths.

Manual, Automated, RT

Automated, 4ºC

Automatic processing were comparable at RT, but automatic processing yielded consistently higher signal at 4ºC. Automatic processing using BlotCycler™ showed excellent reproducibility and can be efficiently used for optimization of primary and secondary antibodies concentration.

Introduction

Western blot (Towbin et al,1979) provides important information about levels of protein expression in biological samples. The multipurpose standard Western blotting is an effort- and time-consuming process. The quality of results depends upon multiple subjective and objective factors including the qualification and technical skills of the personnel and accuracy of temporal and temperature control. During the blotting procedure: incubation with primary antibody is often done at 4°C to reduce background and to increase the intensity of specific signals. Contrary to this, the incubation with secondary antibodies and washing steps are usually performed at RT resulting in the best quality of the entire process. To circumvent these problems, standardize the procedure and improve the quality of Western blotting, Precision Biosystems has developed a device, BlotCycler™ that reduces manual labor during membrane processing and provides stable and high quality results after moving at both RT and 4ºC. BlotCycler™ combines shorter and rapid systems and delivers results in a preset time to the membranes. BlotCycler™ uses uniquely shaped trays that ensure efficient mixing of reagents to achieve low background and minimizes the amount of antibodies required for analysis. To compare the efficiency of automatic and manual processing, we used a fluoroscent based detection system, which allows simultaneous detection of the staining efficiency by up to three different primary antibodies at the RT and 4°C regimes.

Incubation with different blocking buffers

In order to obtain high quality results using Western blotting, it is important to select an efficient blocking buffer. A good blocking buffer maintains the signal-to-noise ratio and does not react with the targeted protein or primary or secondary antibodies. The efficiency of blocking depends on the conditions of incubation. We compared the efficiency of five commonly used blocking buffers to prevent nonspecific binding of the fluorescently labeled secondary antibody during automated processing (Fig. 1).

Fig. 1 The gel was loaded with an extract of mouse pancreas at all lanes. Electrophoresis was run as described. Proteins were transferred from the gel onto the Nitrocellulose membrane. Membranes were blocked for 40 min at 4°C, and different blocking buffers listed above. BSA (1% BSA) was used as negative control. The primary antibody was incubated for 1:100 dilution and incubated for 90 min at 4°C. Data was collected independently using Bandscan software at 480nm/666nm for DyLight™ 649.

Fig. 3 (Top) First lane contains a HeLa whole cell lysate, the second lane contains a recombinant Human IL-2, and the third lane contains an extract of mouse whole cell lysate and HU. Electrophoresis was run as described. Proteins were transferred from the gel onto the Nitrocellulose membranes. Membranes were blocked for 30 min at RT or 30 min at 4°C using MB-073. Rabbit anti-Delta-4 and mouse anti-IL2 primary antibodies were used. Primary antibodies were incubated for 30 min at RT or 30 min at 4°C. DyLight™ 649 conjugated anti-rabbit IgG secondary antibody was used. The secondary antibodies were incubated for 30 min at RT or 30 min at 4°C. Bandpass filter at 625nm/695nm for DyLight™ 649. The fluorescent intensity between different blots was normalized using MW standard.

Fig. 4a The gel was loaded with an extract of mouse pancreas in all lanes. Electrophoresis was run as described. Proteins were transferred from the gel onto the Nitrocellulose membranes. Membranes were blocked for 60 min at 4ºC using MB-073. Rabbit anti-Delta-4 was incubated for 18 hrs at 4°C at dilution. DyLight™ 649 conjugated anti-rabbit IgG secondary antibody was used. The secondary antibody was diluted 1:20,000 and incubated for 60 min at 4°C. Data was collected independently without bandpass filter at 625nm/666nm for DyLight™ 649. The fluorescent intensity between different blots was normalized using MW standard.

Fig. 4b The extract of mouse pancreas was loaded in all lanes. Electrophoresis was run as described. Proteins were transferred from the gel onto the Nitrocellulose membranes. Membranes were blocked for 60 min at 4ºC using MB-073. Rabbit anti-Delta-4 was incubated for 18 hrs at 4°C at dilution. DyLight™ 649 conjugated anti-rabbit IgG secondary antibody was used. The secondary antibody was diluted 1:20,000 and incubated for 60 min at 4°C. Data was collected independently without bandpass filter at 625nm/666nm for DyLight™ 649. The fluorescent intensity between different blots was normalized using MW standard.

Summary

We used fluorescent labeled secondary antibodies to compare manual and automated processing. This multi-color visualization presented herein demonstrates that BlotCycler™ is able to produce clear and high quality results, allowing a straightforward process for the identification of individual protein.

Using two identical protocols we showed that BlotCycler™ increases the signal to noise ratio by reducing background and increasing signal intensity. BlotCycler™ provides extremely good reproducibility that is crucially needed to monitor variability in staining and incubation times. BlotCycler™ optimizes the concentrations of primary and secondary antibodies potentially providing huge saving by decreasing the amount of antibody used.

Thus BlotCycler™ offers a time-saving alternative to manual processing of fluorescent western blot by providing very low background, high signal-to-noise ratio, ability to multiplex detection and excellent reproducibility. BlotCycler™ provides opportunities for easy optimization of western blotting and significantly improves the quality of the procedure.