# Improved Western Blot Assays Using BlotCycler™ Automated Wash System


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## Abstract


**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 automatic 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 automatic processing were comparable at RT, but automatic processing yielded consistently higher signal at 4°C. 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 multistep traditional manual Western blotting is an effort- and time-consuming process. The quality of results depends upon the multiple subjective and objective factors including the qualification and technical skills of the personnel and accuracy of temporal and temperature control. During manual processing, 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 all washing steps are usually performed at RT resulting in the diminishing the 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 the stable and high quality results after operating at both RT and 4°C. BlotCycler™ combines shaker and fluidic systems and delivers reagents at preset time to the membrane. BlotCycler™ uses uniquely shaped trays that ensure efficient mixing of reagents to achieve low background and minimizes amounts of antibodies required for analysis. To compare the efficiency of automatic and manual processing, we used a fluorescent based detection system, which allows simultaneous detection of the staining efficiency by up to three different primary antibodies at the RT and 4°C regimens.

## Methods

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

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

Three different samples have been used in this set of experiments: the lysate of HELA cells (HL), the recombinant protein human IL-2 (IL2), and extract of mouse pancreas (MSP).

All samples were loaded onto SDS-PAGE gels for separation. Molecular weight standard was diluted in sample buffer, and 3 µL of diluted marker was loaded. Electrophoresis was run at 150 V for 1 h using the Laemmli buffer system. Proteins were transferred from the gel onto the Nitrocellulose membrane. Membranes were blocked for 30 min at RT or for 90 min at 4°C using one of the following blocking buffers: 1% Fish Gel, 5% Goat Serum, 5% Blotto, MB-070, or 3% BSA.

The following primary antibodies have been used: rabbit anti-IL2, mouse anti-α-tubulin and rabbit anti-delta4. The blocked membranes were incubated with a cocktail of two primary antibodies for 18 hrs. at 4°C or 90 min at RT. The secondary antibodies were a cocktail of the following: DyLight™ 549 conjugated anti-mouse IgG, and DyLight™ 649 conjugated Anti-rabbit IgG. The secondary antibodies were diluted in blocking buffer and incubated for 30 min at RT or 90 min at 4°C. Fluorescent images were collected in the ChemiDoc MP™ (BioRad ) molecular imaging system. Data for each DyLight™ fluorophore were collected independently using bandpass filter at the following excitation/emission wavelengths: 530nm/605nm for the DyLight™ 549 and 625nm/695nm for DyLight™ 649. The fluorescent intensity between different blots was normalized using MW standard.

## 1. Incubation with different blocking buffers

In order to obtain high quality results using Western blotting, it is important to select the efficient blocking buffer. A good blocking buffer maximizes 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 condition of incubation. We have compared the efficiency of five commonly used blocking buffers to prevent nonspecific binding of the fluorescently labeled secondary antibody during automated processing (Fig. 1).

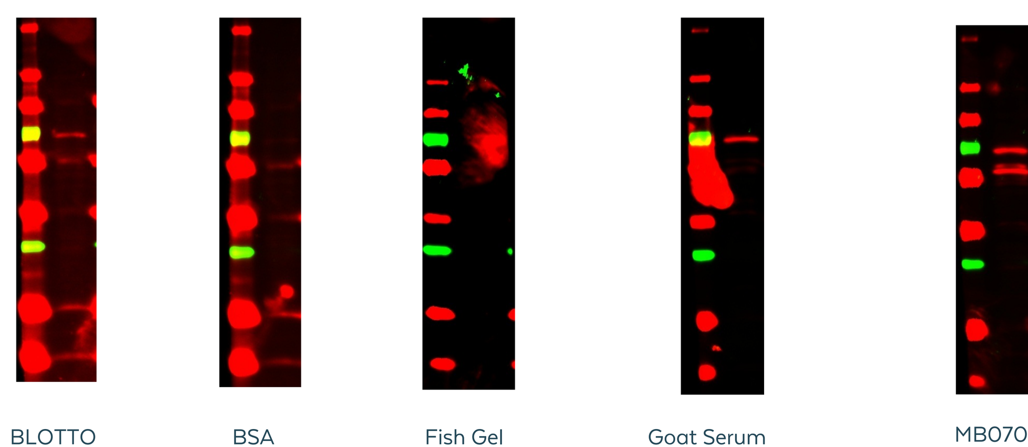



Fig. 1 The gel was loaded with an extract of mouse pancreas in all lanes. Electrophoresis was run as described. Proteins were transferred from the gel onto the Nitrocellulose membrane. Membranes were blocked for 90 min at 4°C using different blocking buffers listed above. Rabbit-a-Delta-4 (1:1000) were incubated overnight at 4°C. DyLight™ 649 conjugated Anti-rabbit IgG secondary antibody was used. The secondary antibody was diluted 1:20,000 and incubated for 90 min at 4°C. Data was collected independently using bandpass filter at 625nm/695nm for DyLight™ 649.

## 2. The comparison of automated and manual blot

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 all steps at 4°C using BlotCycler™ yields higher signal intensity and lower background than manual processing with primary antibody incubation at 4°C and all other step 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 or RT (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.

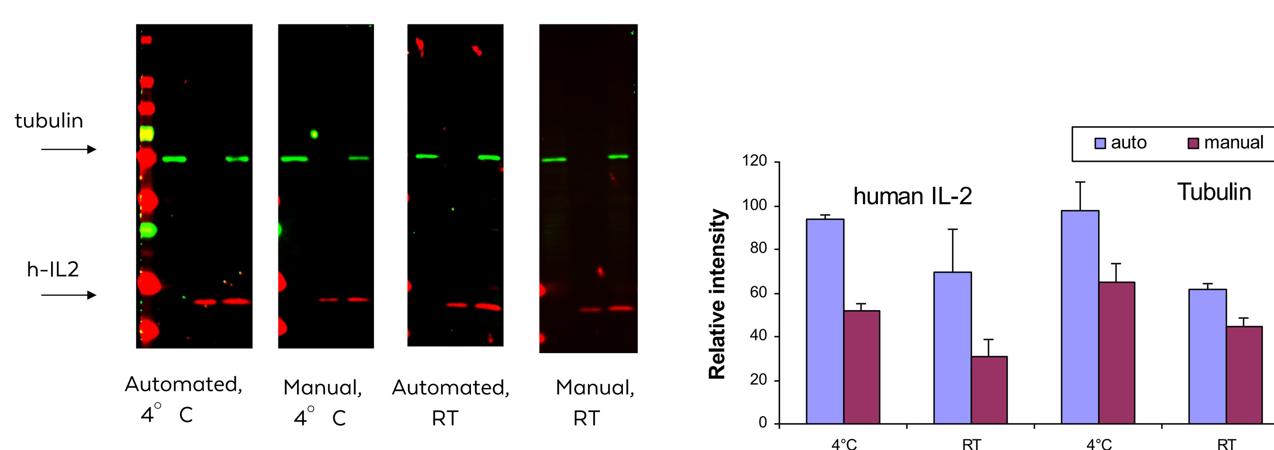
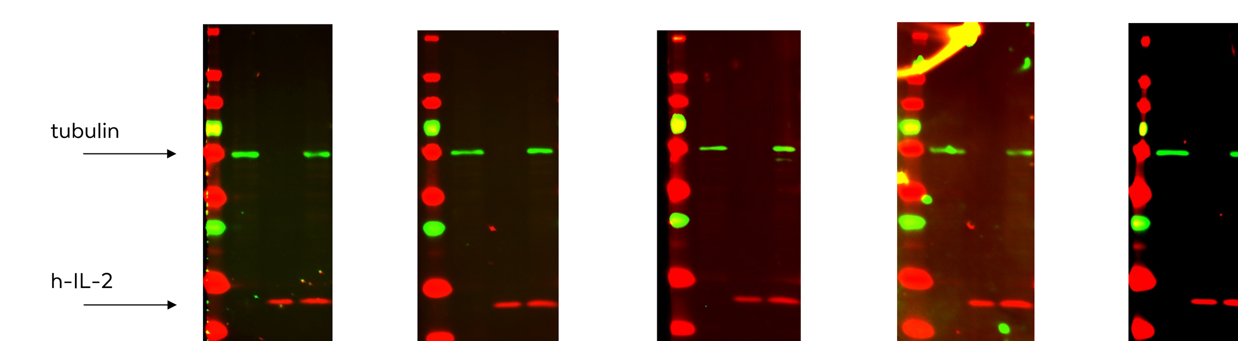



Fig. 2 Lane 1 HeLa whole cell lysate, Lane 2 recombinant Human IL-2, Lane 3 mixture of HeLa whole cell lysate and h-IL2. Electrophoresis was run as described. Proteins were transferred from the gel onto the Nitrocellulose membrane. Membranes were blocked for 30 min at RT or for 90 min at 4°C using MB-070. Rabbit anti-IL2 and mouse anti-α-tubulin primary antibody have been used. Primary antibodies were incubated for 90 min at RT for 18 hours at 4°C. DyLight™ 549 conjugated anti-mouse IgG and DyLight™ 649 conjugated Anti-rabbit IgG secondary antibodies were used. The secondary antibodies were incubated for 30 min at RT and 90 min at 4°C .Data for each DyLight™ fluorophore were collected independently at excitation/emission wavelengths: 530nm/605nm for the DyLight™ 549 and 625nm/695nm for DyLight™ 649. The fluorescent intensity between different blots was normalized using MW standard.

## 3. Reproducibility of automated blot processing

One of the problems of manual processing is the variability of results between blots. In this experiment we process several identical blots using BlotCycler™. In addition to MW standard each blot contained three lanes: HeLa whole cell lysate; the recombinant protein Human IL-2, and mixture of HeLa whole cell lysate and h-IL-2. The five identical blots were incubated with a mix of rabbit anti-IL2 and mouse anti-α-tubulin and then probed with corresponding secondary antibodies: DyLight™ 549 conjugated anti-mouse IgG, and DyLight™ 649 conjugated anti-rabbit IgG. The automated processing showed excellent reproducibility (Fig. 3) that could be results of the precise timing and consistency of solution changes, efficient washing and elimination of operator caused errors and ensures the standardization of the results.




Reproducibility of automated processing

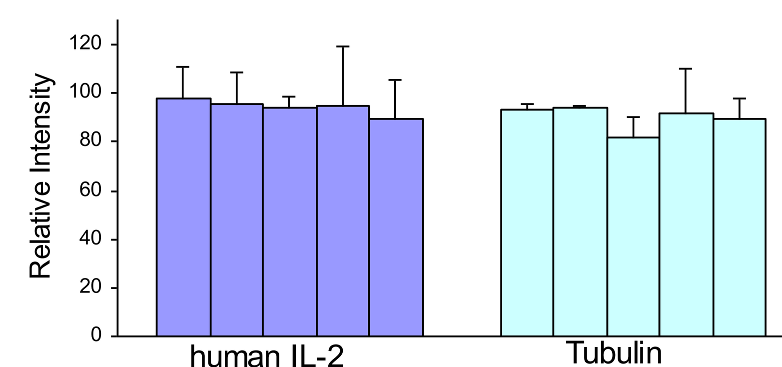



Fig. 3 (Top) First lane contains a HeLa whole cell lysate, the second lane contains a recombinant Human IL-2, and the third lane mixture of HeLa whole cell lysate and h-IL2. Electrophoresis was run as described. Proteins were transferred from the gel onto the Nitrocellulose membrane. Membranes were blocked for 90 min at 4°C using MB-070. Rabbit anti-IL2 and mouse anti-α-tubulin primary antibody have been used. Primary antibodies were incubated for 1080 min at 4°C. DyLight™ 549 conjugated anti-mouse IgG, and DyLight™ 649 conjugated Anti-rabbit IgG secondary antibodies were used. The secondary antibodies were incubated for 90 min at 4°C. Data for each DyLight™ fluorophore were collected independently at excitation/emission wavelengths: 530nm/605nm for the DyLight™ 549 and 625nm/695nm for DyLight™ 649. (Bottom) The calculated fluorescent intensity between different blots was normalized using MW standard.

## 4. Optimization of the antibody concentration

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

In this experiment we prepare 10 identical strips each contains two lanes: MW markers and extract of mouse pancreas. The blots were probed with rabbit anti-Delta4 primary antibody and DyLight™ 649 conjugated anti-rabbit 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.

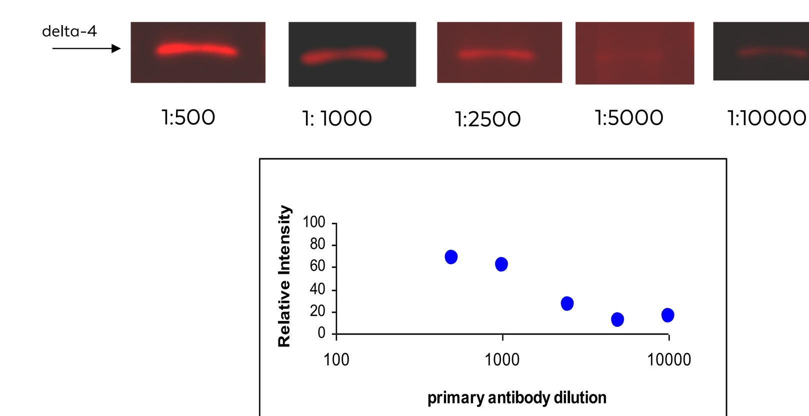



Fig. 4a The gel was loaded with an extract of mouse pancreas in all lanes. Electrophoresis was run as described. Proteins were transferred from the gel onto the Nitrocellulose membrane. Membranes were blocked for 90 min at 4°C using MB-070. Rabbit-a-Delta-4 was incubated for 1080 min at 4°C at different dilutions. DyLight™ 649 conjugated Anti-rabbit IgG secondary antibody was used. The secondary antibody was diluted 1:20,000 and incubated for 90 min at 4°C. Data was collected independently using bandpass filter at 625nm/695nm for DyLight™ 649. The fluorescent intensity between different blots was normalized using MW standard.

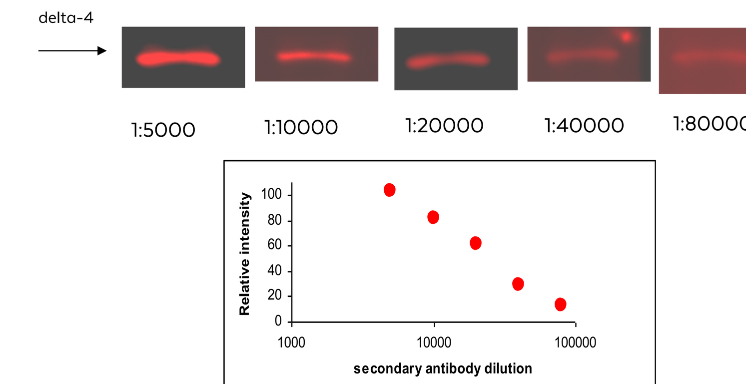



Fig. 4b The extract of mouse pancreas was loaded in all lanes. Electrophoresis was run as described. Proteins were transferred from the gel onto the Nitrocellulose membrane. Membranes were blocked for 90 min at 4°C using MB-070. Rabbit-a-Delta-4 was incubated for 18 hrs at 4°C at dilutio. DyLight™ 649 conjugated Anti-rabbit IgG secondary antibody was incubated for 90 min at 4°C at different dilutions. Data was collected independently at 625nm/695nm for DyLight™ 649. The fluorescent intensity between different blots was normalized using MW standard.

## Summary

We have used fluorescent labeled secondary antibodies to compare manual and automated processing. The multi-color visualization presented here 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 probably related to minimal variability in washing and incubation times. BlotCycler™ can be used to optimize the concentrations of primary and secondary antibodies potentially providing huge saving by decreasing the amount of antibody used.

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

**Products**

| Product | Part number | Product | Part number |
|---|---|---|---|
| HeLa Whole Cell Lysate | W09-000-364 | Anti-Mouse IgG (H&L) DyLight™ 549 | 610-142-121 |
| DELTA-4 Antibody | 600-401-696 | Anti-Rabbit IgG (H&L) DyLight™ 649 | 611-143-122 |
| IL-2 Human Recombinant Protein | 009-001-B95 | NORMAL GOAT SERUM (NGS) | B304 |
| IL-2 Antibody | 209-401-B95 | Blocking Buffer for Fluorescent Western Blotting | MB-070 |
| Alpha-Tubulin Antibody | 200-301-880 | Blotto Immunoanalytical Grade (Non-Fat Dry Milk) | B501-0500 |

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