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iBlot® Western Detection Kits

Immunodetection in 30 minutes

Introduction

Immunodetection of proteins following gel electrophoresis is one of the most widely used techniques in life science research. Traditionally, this procedure consists of multiple steps, requires skilled labor, and takes up to two days to complete. While this method is essential for specific protein identification, it is also a tedious process and can hinder the pace of research.

The iBlot® Gel Transfer Device (Cat. No. IB1001), a recent innovation from Life Technologies, has drastically shortened the time needed to transfer proteins from a gel to a membrane. Instead of the typical 1–8 hour process, the transfer can frequently be completed in just 7 minutes. Here we will review the use of the iBlot® Gel Transfer Device with the new iBlot® Western Detection Kits. These kits can reduce the immunodetection assay time from as long as 2 days to less than 30 minutes, using a simple and reliable protocol.

Reducing the time for immunodetection

Traditional immunodetection methods require blocking of the blotting membrane to prevent nonspecific interactions, followed by incubation with a primary antibody for several hours to overnight. After incubation with the primary antibody, several washes are performed before the blot is incubated with a secondary antibody for at least an hour. The obvious drawbacks to such methods include the time required to complete the protocol, the inconsistency of results, and the difficulty of processing large numbers of blots.

The iBlot® Western Detection Kits include a unique fabric matrix that efficiently and quickly absorbs liquid reagents, such as antibody solutions, but readily releases these reagents under the influence of an electric field. Using the iBlot® Gel Transfer Device and its programmable power supply, the transfer of the membrane-blocking buffer and the primary and secondary antibodies can be completely controlled.

iBlot® Western Detection Kits offer:

- Rapid immunodetection—complete detection of a blot typically in less than 30 minutes
- High throughput—analyze multiple blots in one day
- Excellent sensitivity—comparable to current methods
- Reliable reproducibility—high-quality reagents and simple protocols enable publication-quality results in a day

The iBlot® Western Detection Kits are used with the iBlot® Gel Transfer Device using setting P9, which has been preprogrammed with three substeps. Once a substep is completed, the device is automatically paused until the matrix has been removed and replaced. The current substep is indicated by the number of horizontal lines adjacent to "P9" on the display (Figure 1).

Figure 1. LCD display of the iBlot® Gel Transfer Device during operation of the P9 program. The P9 program has three substeps; the active substep is indicated on the display by the number of horizontal lines.

This application note focuses on the optimization of secondary antibody concentrations by using different antibody–antigen pairs to help new users quickly obtain publication-quality results with the iBlot® Western Detection Kits. The iBlot® Western Detection Kit workflow is summarized below for the benefit of new users; a detailed workflow can be found in the manuals included with the kits, and online at www.invitrogen.com/iblotwd. All steps of the iBlot® western detection protocol are performed after gel electrophoresis and transfer of the proteins to a blotting membrane.

1. While antibody and wash solutions were being prepared, the membrane was briefly rinsed with deionized water and the blocking solution was applied to the first matrix.
2. The transfer sandwich, consisting of the transfer matrix soaked in blocking solution, the membrane, and the top and bottom iBlot® Western Detection Stacks, was assembled and run for 2 min using program P9, substep 1, of the iBlot® Gel Transfer Device.
3. The blocking solution–soaked matrix in the stack was replaced with a second matrix soaked with the primary antibody solution. The reassembled sandwich was run for 3 min using program P9, substep 2.
4. The primary antibody matrix was replaced with a third matrix soaked with the secondary antibody solution. The reassembled sandwich was run for 3 min using program P9, substep 3.
5. The membrane was removed from the sandwich, rinsed three times in wash solution, exposed to the appropriate substrate, and visualized according to the manufacturer's instructions.

Materials and methods

Protein electrophoresis

GST-tagged EGFR protein (Cat. No. PV3872) and Positope™ control protein (Cat. No. 46-0172) were prepared in NuPAGE® LDS Sample Buffer (Cat. No. NP0008) with 50 mM DTT and heated at 70°C for 10 min. The samples were loaded onto a NuPAGE® 4–12% 1.0 mm Bis-Tris Gel (Cat. No. NP0321BOX) and run with 1X MES SDS buffer (Cat. No. NP0002). Three replicates of each sample set were run on a single gel to allow testing of multiple antibody conditions for immunodetection with the iBlot® Western Detection Kits. Gels were run for 37 min at 200 V.

iBlot® dry transfer

Gels were transferred using the iBlot® Transfer Stack, Regular (Nitrocellulose) (Cat. No. IB3010-01) or the iBlot® Transfer Stack, PVDF Regular (Cat. No. IB4010-01) on the iBlot® Gel Transfer Device. The recommended P3 program (20 V for 7 min) was used for all transfers.

Antibodies and detection reagents

Mouse anti-GST (Cat. No. G1160, Sigma) or mouse anti-c-myc (Cat. No. R950-25) was used as primary antibody. For the traditional (control) blots the primary antibody was detected using the WesternBreeze® Chemiluminescent or Chromogenic Anti-Mouse Kit (Cat. No. WB7104 and WB7103, respectively). All reagents for detection were prepared as specified in the WesternBreeze® manual. Blots processed with the iBlot® Western Detection Kits were detected with the supplied reagents.

Immunodetection

Traditional (control) blot processing

Traditional blots were processed according to the WesternBreeze® protocol. Briefly, the membrane was blocked for 30 min using the WesternBreeze® blocking solution, incubated with the primary antibody at a dilution of 1:5,000 for 1 hr, washed, and then incubated for 30 min with the supplied prediluted secondary antibody before the final washes and exposure to the WesternBreeze® substrate. All washes were carried out according to the protocol using the supplied buffers.

iBlot® Western Detection Kits

For detection using the iBlot® Western Detection Chemiluminescent or Chromogenic (anti-mouse) Kit (Cat. No. IB7110-01 and IB7310-01, respectively), the instructions provided were followed, with the changes indicated in this application note made to the secondary antibody concentrations. For all experiments, the blocking solution included in the kits was applied to a matrix and transferred to the blot using the iBlot® Gel Transfer Device for 2 min at setting P9, substep 1. The primary antibody, at a dilution of 1:2,500, was applied to a second matrix and transferred to the blot for 3 min at setting P9, substep 2.

Determining the optimal secondary antibody concentration

For one-step, rapid determination of the optimal secondary antibody concentration, two aliquots of the secondary antibody, which is provided at a concentration of 1:10, were diluted to final concentrations of 1:2,500 and 1:5,000 for chemiluminescent detection on nitrocellulose, 1:5,000 and 1:10,000 for chemiluminescent detection on PVDF, and 1:1,000 and 1:2,500 for chromogenic detection. These secondary antibody solutions were applied to a matrix and transferred to the blot using the iBlot® Gel Transfer Device for 3 min at setting P9, substep 3. The blot was rinsed in the wash solution and incubated with the supplied detection reagents.

To test multiple secondary antibody conditions, one gel was run with replicate samples. To prepare for the detection, the matrix and blotting membrane were cut into three pieces and the different antibody concentrations were individually applied to each matrix. The cut matrix sections were then placed on the corresponding blotting membrane sections. Prior to application of the antibody-soaked matrices, reusable spacers provided with the iBlot® Western Detection Kits were placed between the membranes (Figure 2). The spacers prevent cross-contamination between different antibody solutions while allowing simultaneous detection using different conditions. Using spacers also reduces the number of gels and the volume of reagents required for optimization of a secondary antibody. The use of the spacers also allows combination of different reagents (such as different primary antibodies) with one stack set.

Results

Primary antibody concentration

The concentration of the primary antibody can affect both detection sensitivity and background when using the iBlot® Western Detection Kits. A very low antibody concentration can result in weak or no signal, and an overly high antibody concentration can cause high background. As a starting point, we recommend using a primary antibody concentration twice that used for traditional immunodetection (e.g., if a 1:5,000 dilution of the primary antibody is normally used, a dilution of 1:2,500 would be used with the iBlot® Western Detection Kits). We typically find that no additional optimization of the primary antibody is required (data not shown). It is important to note that because the volume of primary antibody solution used with the iBlot® Western Detection Kits is half that of the traditional method, the total amount of the primary antibody used per blot is the same as in the traditional method.

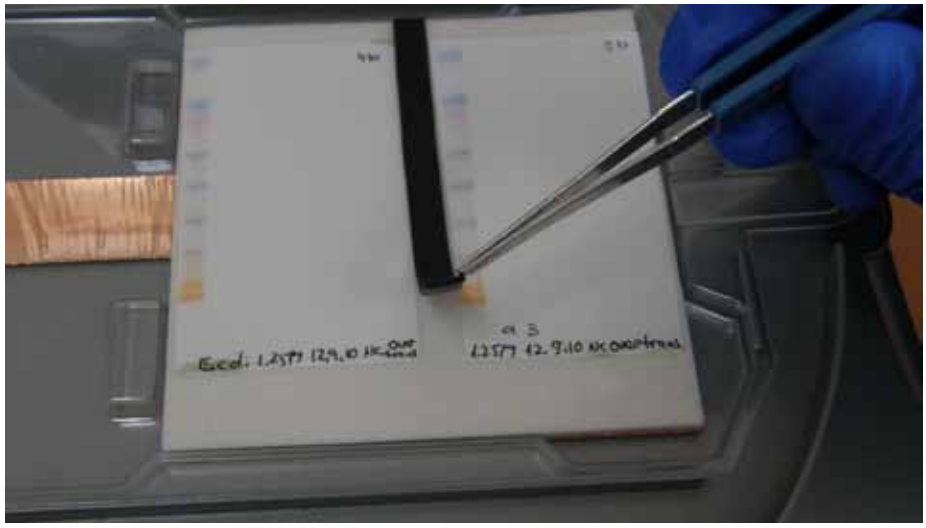

Figure 2. Spacers enable testing of multiple antibody concentrations. Spacers were placed between membranes prior to transfer of the secondary antibody, to enable antigen detection using different secondary antibody concentrations.

Optimization of iBlot® Western Detection Kit results

Increased secondary antibody concentrations provide greater sensitivity using the iBlot® Western Detection Kits

To verify the optimal secondary antibody concentration needed for detection, two secondary antibody concentrations were tested for each antibody–antigen pair. Detection was performed using either chemiluminescent or chromogenic substrates (see materials and methods). Higher concentrations of secondary antibody were used with the chromogenic substrate than with the chemiluminescent substrate to account for the different detection limits of these systems.

Increased secondary antibody concentrations provide greater sensitivity with chemiluminescent detection reagents

Immunodetection of antigens on a nitrocellulose membrane was compared using traditional methods and the iBlot® Western Detection Kit. GST-tagged EGFR protein was detected with a mouse anti-GST antibody. Two concentrations of the secondary antibody were tested with the iBlot® Western Detection Kit. The antigen–antibody complexes were visualized as described in the materials and methods (chemiluminescent detection). In all three blots, as little as 0.375 ng of antigen was detected; a higher concentration of secondary antibody provided improved detection across the range of protein amounts when the iBlot® Western Detection Kit was used, without any increase in background signal (Figure 3).

Increased secondary antibody concentrations provide greater sensitivity with chromogenic detection reagents

The ability to detect antigens on a nitrocellulose membrane was further analyzed using chromogenic detection of the antibody–antigen complexes. GST-tagged EGFR protein was detected with a mouse anti-GST antibody. Two concentrations of the secondary antibody were tested with the iBlot® Western Detection Kit, and detection was performed as described in the materials and methods section. In all three blots, 1.5 ng of antigen was detected (Figure 4). However, increasing the secondary antibody concentration for the iBlot® western detection method enabled detection of 0.375 ng of protein, producing results comparable to the traditional method (Figure 4A and 4C).

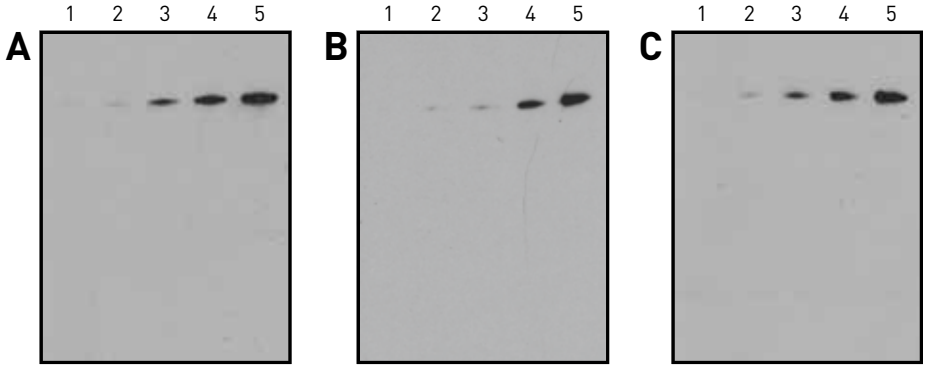

Figure 3. Increased sensitivity of chemiluminescent detection with higher secondary antibody concentrations. GST-tagged EGFR protein on nitrocellulose membranes was detected (**A**) by traditional methods using a WesternBreeze® kit and (**B**, **C**) using the iBlot® Western Detection Kit. The amounts of EGFR protein loaded were: (1) 0.187 ng; (2) 0.375 ng; (3) 0.75 ng; (4) 1.5 ng; (5) 3 ng. The prediluted secondary antibody supplied with the WesternBreeze® kit was used for the control blot. The secondary antibody final dilutions used for the iBlot® Western Detection Kit were (**B**) 1:5,000 and (**C**) 1:2,500.

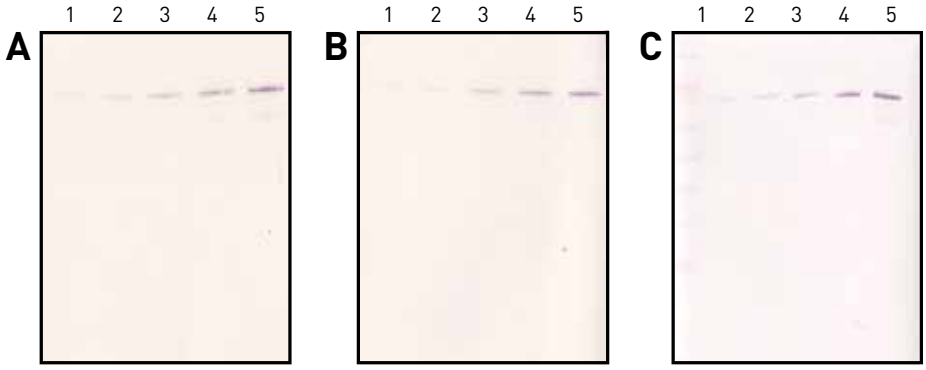

Figure 4. Increased sensitivity of chromogenic detection with higher secondary antibody concentrations. Chromogenic detection of GST-tagged EGFR protein on nitrocellulose membranes by (**A**) traditional methods using a WesternBreeze® kit was compared to (**B**, **C**) the iBlot® Western Detection Kit. The amounts of EGFR protein loaded were: (1) 0.187 ng; (2) 0.375 ng; (3) 0.75 ng; (4) 1.5 ng; (5) 3 ng. The prediluted secondary antibody supplied with the WesternBreeze® kit was used for the control blot. The secondary antibody final dilutions used for the iBlot® Western Detection Kit were (**B**) 1:2,500 and (**C**) 1:1,000.

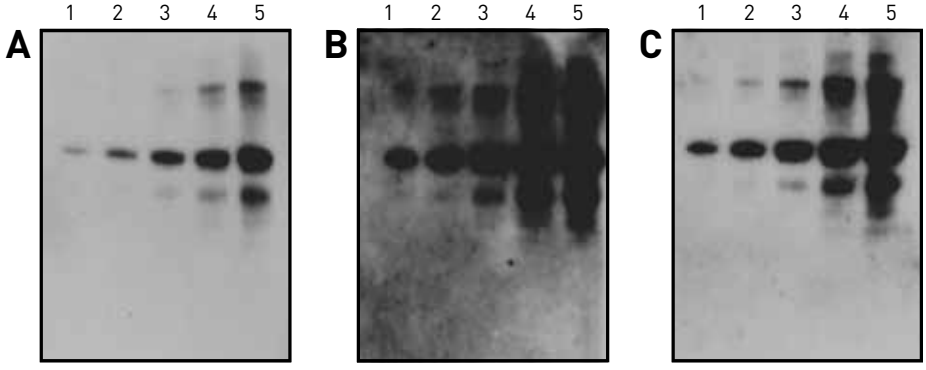

Figure 5. Lower concentrations of secondary antibody reduce background on PVDF membranes. Positope™ protein was detected (**A**) by traditional methods using a WesternBreeze® kit and (**B**, **C**) using the iBlot® Western Detection Kit. The amounts of Positope™ protein loaded were: (1) 15.6 ng; (2) 31.2 ng; (3) 62.5 ng; (4) 125 ng; (5) 250 ng. The prediluted secondary antibody supplied with the WesternBreeze® kit was used for the control blot. The secondary antibody final dilutions used for the iBlot® Western Detection Kit were (**B**) 1:5,000 and (**C**) 1:10,000.

Lower secondary antibody concentrations can reduce nonspecific background

To examine the performance of the iBlot® Western Detection Kits with PVDF membranes, the results of traditional immunodetection were compared to the results of two membranes probed with different dilutions of secondary antibody using the iBlot® Western Detection Chemiluminescent Kit. Lower concentrations of the secondary antibody were tested because the background characteristics of PVDF membranes are different from those of nitrocellulose membranes. Positope™ protein, a recombinant c-myc–tagged protein, was detected with a mouse anti–c-myc antibody. As little as 15.6 ng of protein could be detected in all three blots. However, at a 1:5,000 dilution of secondary antibody, significant background signal was observed with the iBlot® Western Detection Kit compared to the traditional method (Figures 5A and 5B). This background was eliminated when the secondary antibody concentration was decreased to 1:10,000 (Figure 5C).

Conclusion

In this application note, we have demonstrated that simple optimization of the secondary antibody concentration can improve immunodetection sensitivity and minimize background when using the iBlot® Western Detection Kits. The conditions for several antigen–antibody pairs can be optimized quickly and efficiently by using the reusable spacers provided with the kit to separate blots and probe them with multiple secondary antibody concentrations simultaneously. As we observed, a higher secondary antibody concentration may provide a stronger signal, but there is also the potential of increased background. If high background is observed, decreasing the secondary antibody concentration may reduce the background. In addition to the secondary antibody concentration, the type of membrane, nitrocellulose or PVDF, can also influence the outcome of immunodetection, and optimization is recommended for each type of membrane. The iBlot® Western Detection Kit manuals provide additional optimization procedures that can be performed to improve results if the initial testing of secondary antibody concentrations is not satisfactory.

The iBlot® Western Detection Kits enable the completion of immunodetection in less than 30 minutes, without compromising sensitivity and quality. When combined with the iBlot® Western Blotting System, the entire blotting and immunodetection process can now typically be completed in less than 1 hour.

Ordering information

iBlot® Western Detection Kits		
Product	**Quantity**	**Cat. No.**
iBlot® Western Detection, Chemiluminescent Kit (Anti-Mouse) — Regular, 10-Pak	10 reactions	IB7110-01
iBlot® Western Detection, Chemiluminescent Kit (Anti-Mouse) — Mini, 10-Pak	10 reactions	IB7110-02
iBlot® Western Detection, Chemiluminescent Kit (Anti-Rabbit) — Regular, 10-Pak	10 reactions	IB7210-01
iBlot® Western Detection, Chemiluminescent Kit (Anti-Rabbit) — Mini, 10-Pak	10 reactions	IB7210-02
iBlot® Western Detection, Chromogenic Kit (Anti-Mouse) — Regular, 10-Pak	10 reactions	IB7310-01
iBlot® Western Detection, Chromogenic Kit (Anti-Mouse) — Mini, 10-Pak	10 reactions	IB7310-02
iBlot® Western Detection, Chromogenic Kit (Anti-Rabbit) — Regular, 10-Pak	10 reactions	IB7410-01
iBlot® Western Detection, Chromogenic Kit (Anti-Rabbit) — Mini, 10-Pak	10 reactions	IB7410-02
IBlot® Western Detection Stacks (Regular), 10-Pak	10 reactions	IB7010-01
iBlot® Western Detection Stacks (Mini), 10-Pak	10 reactions	IB7010-02
Additional products		
Product	**Quantity**	**Cat. No.**
NuPAGE® Novex® 4–12% Bis-Tris Gel, 1.0 mm, 12 well	Box of 10	NP0322BOX
NuPAGE® MES SDS Running Buffer (20X)	500 mL	NP0002
iBlot® Gel Transfer Device	1 unit	IB1001
iBlot® Transfer Stack, Regular (Nitrocellulose)	10 sets/box	IB3010-01
iBlot® Transfer Stack, Mini (Nitrocellulose)	10 sets/box	IB3010-02
iBlot® Transfer Stack, PVDF Regular	10 sets/box	IB4010-01
iBlot® Transfer Stack, PVDF Mini	10 sets/box	IB4010-02

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