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Ultra-High-Speed Western Blot using Immunoreaction Enhancing Technology

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Abstract

A western blot (also known as an immunoblot) is a canonical method for biomedical research. It is commonly used to determine the relative size and abundance of specific proteins as well as post-translational protein modifications. This technique has a rich history and remains in widespread use due to its simplicity. However, the western blotting procedure famously takes hours, even days, to complete, with a critical bottleneck being the long incubation times that limit its throughput. These incubation steps are required due to the slow diffusion of antibodies from the bulk solution to the immobilized antigens on the membrane: the antibody concentration near the membrane is much lower than the bulk concentration. Here, we present an innovation that dramatically reduces these incubation intervals by improving antigen binding via cyclic draining and replenishing (CDR) of the antibody solution. We also utilized an immunoreaction enhancing technology to preserve the sensitivity of the assay. A combination of the CDR method with a commercial immunoreaction enhancing agent boosted the output signal and substantially reduced the antibody incubation time. The resulting ultra-high-speed western blot can be accomplished in 20 minutes without any loss in sensitivity. This method can be applied to western blots using both chemiluminescent and fluorescent detection. This simple protocol allows researchers to better explore the analysis of protein expression in many samples.

Introduction

A western blot (also known as an immunoblot) is a powerful and fundamental technique across a broad range of scientific and clinical disciplines. This technique is used to examine the presence, relative abundance, relative molecular mass, and post-translational modifications of proteins[1]. Combined with digital image analysis, this method can reliably analyze the abundance of proteins and protein modifications[2]. Although western blot is performed routinely, it is a time-consuming and labor intensive method. Long incubations of the antibody with membranes are required. Here, we describe a modification of

the incubation method that overcomes this limitation without sacrificing sensitivity.

During incubation of the membrane, antibodies float in solution while antigens are immobilized on the membrane. Because of their high affinity, the rate of antibody binding to the antigen is faster than the diffusion of antibodies from the bulk solution to the membrane. This creates a low concentration “depletion layer” (**Figure 1**). It may take hours for more distant antibodies to reach the membrane via passive diffusion, which is the main factor responsible for long incubation times in this technique. This effect is called the mass transport limitation (MTL)[3]. It has been proposed that repetitive draining and replenishing the antibody-containing solution may disrupt the depletion layer and overcome MTL[4]. Here, we have devised a unique technique that implements this cyclic draining and replenishing (CDR) concept to diminish the effect of MTL in a traditional western blotting protocol and remarkedly shorten the required incubation period.

In order to maintain the detection sensitivity with a short incubation time, we made use of an immunoreaction enhancing technology that accelerates the antigen-antibody reaction, thereby improving the signal-to-noise ratio[5]. Many commercially available immunoreaction enhancing agents (IRE) consist of 2 components (Solution 1 and 2, see **Table of Materials**). The proprietary composition or mechanism of action of IRE has not been disclosed, but we have previously found that IRE decreased the dissociation constant between the antigen and antibody in solid phase binding assays, indicating increased affinity is, at least in part, responsible for the enhancing effect of IRE[6]. The combination of CDR with IRE yields an ultra-high-speed western blotting protocol that reduces the entire procedure time without sacrificing sensitivity.

Protocol

1. SDS-PAGE and transfer to a PVDF membrane

1. Perform SDS-PAGE to separate proteins based on relative size.
   NOTE: Any commercial or home-made gel system is fine. Please follow the manufacturer’s protocol.
2. Transfer separated proteins from a gel onto a PVDF membrane.
   NOTE: Common transfer methods (semi-dry or wet transfer) are suitable. Please follow the manufacturer’s protocol.

2. Blocking of PVDF membranes

1. Incubate the membranes in appropriate blocking buffers for 1 h with agitation at room temperature.
   NOTE: Depending on the primary antibody and detection system, an appropriate blocking buffer should be selected. For example, the most typical blockers are BSA, nonfat dry milk, casein, and commercial synthetic polymers. PBS and/or TBS with 0.1% Tween20 are the most commonly used buffers. This blocking step may be done overnight at 4 ˚C.

3. Incubation with primary antibody

1. Prepare 10% immunoreaction enhancing agent-1 solution (IRE-1) with distilled water. Vortex well.
2. Prepare the diluted primary antibody with 10% IRE-1. Mix it gently and thoroughly.

3. If using a larger membrane (4 cm x 8 cm – 8 cm x 8 cm), add 8 mL of the antibody solution into 50 mL conical centrifuge tubes. If using a smaller membrane (2 cm x 8 cm – 4 cm x 8 cm), add 3 mL of the antibody solution into 14 mL round-bottom polypropylene tube (**Table of Materials**).
4. Pick up the PVDF membrane with tweezers and drain the blocking solution briefly. Insert the PVDF membrane into this tube with gloved fingers and ensure that the entire membrane adheres to the wall of the tube. When the PVDF membrane is inserted into a tube, face the "protein side" of the membrane that was originally in contact with the gel inward. Close the cap tightly.
5. Insert the 50 mL tube into a glass bottle for the hybridization oven.
   NOTE: When 14 mL tubes are used for this incubation, insert a 14 mL tube into the 50 mL tube, using a pair of plastic ring holders to keep the inner tube in the center of the 50 mL tube.
6. Turn on the hybridization oven.
7. Incubate the membrane with 6 rpm rotation for at least 5 min.
   NOTE: Make sure that the membrane adheres to the wall at all times and that the (inner) tube is horizontal to cover the membrane with the antibody solution evenly. Calibrate the dilution of the antibody and incubation time prior to the actual experiment.

4. Membrane wash

1. Stop the rotation.
2. Carefully remove the PVDF membrane from the tube with tweezers, and place the membrane into a container with 50 mL of PBS-T.
3. Rinse the membrane briefly with PBS-T.
4. Rinse the membrane in the container with distilled water until no bubbles appear. This is to remove the majority of the antibody.
5. Transfer the PVDF membrane from the container to the salad spinner that contains 250 mL of PBS-T.
6. Place the strainer basket in the spinner. Ensure that the lid has been put on securely.
7. Activate the spinner and run it for about 20-30 s.
8. Discard the solution and briefly rinse the inside of the spinner with distilled water.
9. Add 250 mL of PBS-T into the spinner again.
10. Repeat steps 4.6 and 4.7.

5. Incubation with the secondary antibody

1. Prepare 10% immunoreaction enhancing agent-2 solution (IRE-2) with distilled water. Vortex well.
2. Prepare the diluted secondary antibody with 10% IRE-2. Mix it gently and thoroughly.
3. Select the volume of the antibody solution and tube as stated in step 3.3.
4. Pick up the PVDF membrane with tweezers and drain the solution briefly. Insert the PVDF membrane into the tube as stated in step 3.4.
5. Insert the 50 mL tube into a glass bottle for the hybridization oven.
6. Turn on the hybridization oven.
7. Incubate the membrane with 6 rpm rotation for at least 5 min. Make sure that the membrane adheres to the wall at all times and that the (inner) tube is horizontal to evenly cover the membrane with the antibody solution.

NOTE: Calibrate the incubation time prior to the actual experiment.

1. When the secondary antibody is fluorescently conjugated, perform the incubation in the dark.

6. Membrane wash

1. Follow steps 4.1 to 4.10.

7. Image acquisition

1. Chemiluminescent detection
    1. Place a piece of semi-transplant flexible film (10 cm x 15 cm) on a flat surface.
    2. Mix 2 components of the chemiluminescent substrate in a 3 mL tube.
    3. Immediately transfer 1.5 mL of the mixed substrate to the semi-transplant flexible film and place the PVDF membrane upon it. Then, transfer the rest of the substrate solution onto the membrane.
    4. Incubate the PVDF membrane with the mixed substrate on a semi-transparent flexible film for 1 min.
    5. Pick up the PVDF membrane with tweezers and drain the substrate solution briefly.
    6. Sandwich the membrane between a pair of transparency films.
    7. Acquire the image under chemiluminescent mode.
2. Fluorescent detection
    1. Pick up the PVDF membrane with tweezers and drain the substrate solution briefly.
    2. Sandwich the membrane between a pair of transparency films.
    3. Acquire the image under fluorescent mode.

NOTE: Steps 7.1 and 7.2 should be performed in close proximity to the imaging machine to ensure maximum sensitivity.

4. When the background signal is high, perform a washing step in a container with 80-100 mL of PBS-T: 10 min rinse 3 times for the primary antibody and 5 min rinse 6 times for the secondary antibody.

NOTE: Both primary and secondary antibodies diluted with 10% IRE solutions can be used up to 8-10 times without loss of sensitivity[6]. The solution should be kept at 4 °C for up to 1 month.

Representative Results

This example illustrates the effectiveness of the CDR method together with immunoenhancing technology on western blot. Static incubation was performed on a semi-transparent flexible film where PVDF membranes were incubated with the antibody solution, whereas CDR incubation was in a hybridization oven by rotating tubes that contained the membranes and the antibody solution (Movie). **Figure 2** showed quantities of antigen and antibody, respectively, necessary for chemiluminescent detection on western blots. In both static and CDR incubations, usage of IRE solution increased the sensitivity: IRE reduced the lowest quantity of cell lysates (antigen) needed to detect ß-actin from 2.2 µg to 1.1 µg under static conditions and further reduced from 1.1 µg to 0.275 µg using CDR (**Figure 2A**). Similarly, the minimum concentration of anti-6x His tag antibody was lowered from 100 ng/mL to 50 ng/mL under static conditions and from 100 ng/mL to 12.5 ng/mL under CDR condition (**Figure 2B**). CDR incubation for either 5 min or 10 min dramatically lowered the detection limit compared to static incubation (60 min with primary and 30 min with secondary antibodies). Finally, the combination of CDR with IRE revealed superior sensitivity on

the western blot (0.275 µg of cell lysates for ß-actin detection and 12.5 ng/mL of anti-6x His tag antibody) even within this extremely short incubation period.

The second example demonstrates the successful application of this method to a western blot with fluorescent detection (**Figure 3**). Fluorescent detection, in contrast to chemiluminescent detection, has the unique ability to detect multiple targets on the same blot at the same time without stripping/re-probing antibodies. $(His)_6$ tagged AP and ß-actin in the cell lysates were simultaneously detected using a two-color fluorophore. CDR incubation with IRE not only accelerated the detection but also increased the sensitivity, resulting in unmasking the degradation of $(His)_6$ tagged AP (**Figure 3A**).

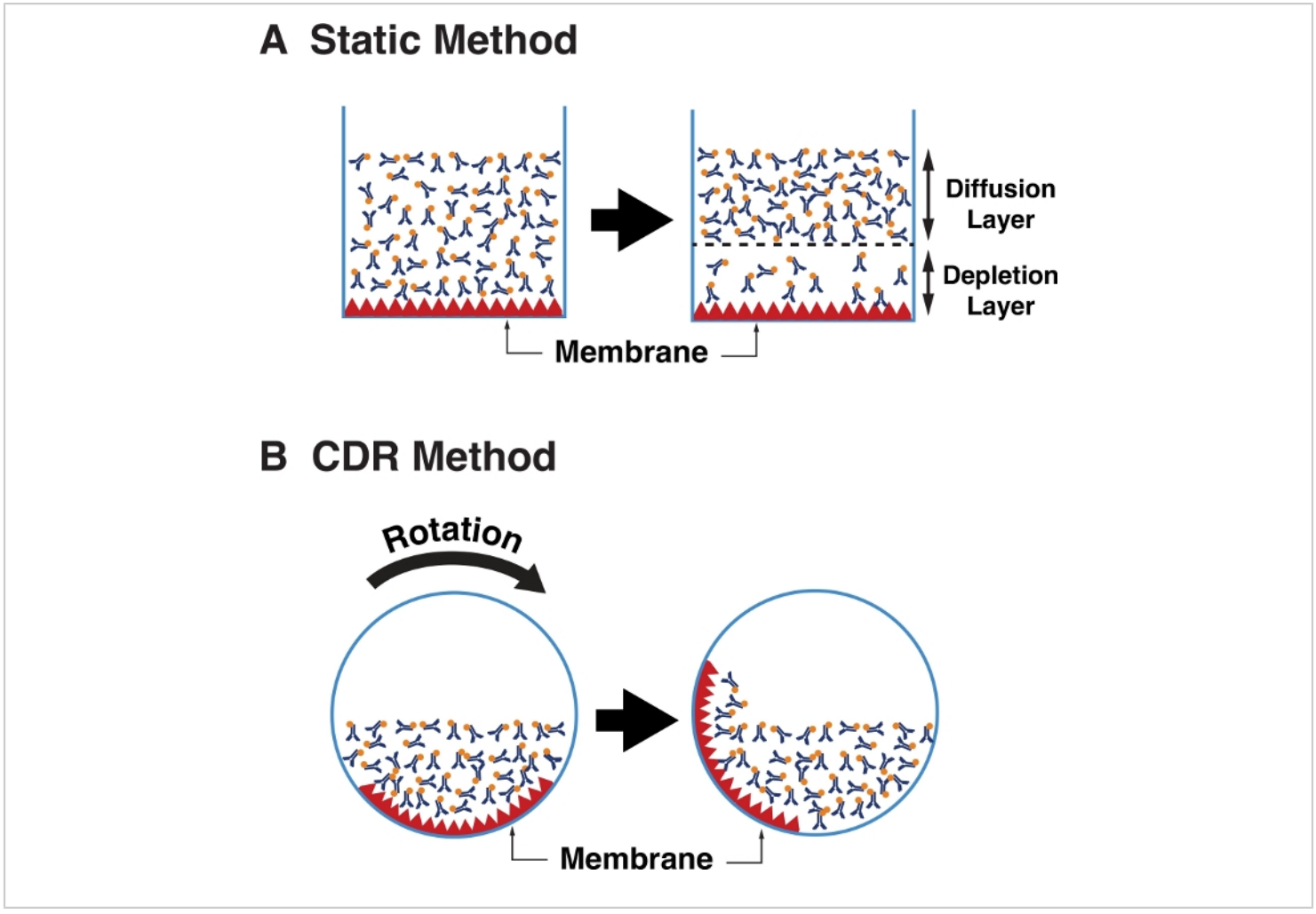

Figure 1: Schematic illustration of the cyclic draining-replenishing (CDR) method.

(**A**) A depletion layer near the membrane rapidly forms under static conditions when a probe has a high affinity for the antigen but a limited ability to diffuse through the solution. Thus, travel of the probe to the membrane becomes a rate limiting step and long incubation times compensate for mass transfer limitation. (**B**) CDR method by rotating the tube while the membrane adheres to the wall eliminates the depletion layer and replenishes the same probe solution to keep the probe concentration near the membrane constant. This figure has been modified from Higashi et al.[6] . Please click here to view a larger version of this figure.

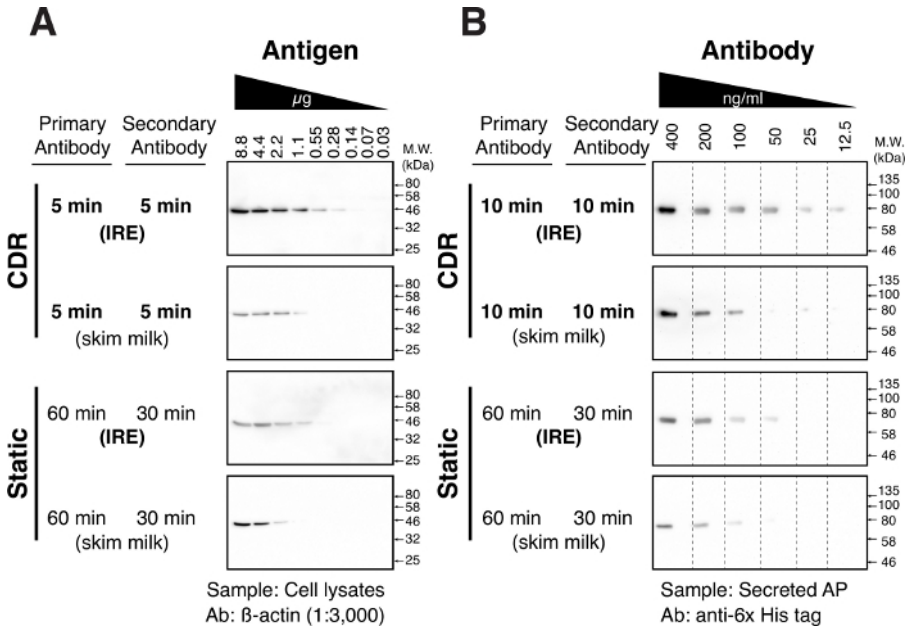

Figure 2: (**A**) Different amounts of 293 cell lysates (1:2 serial dilutions from 8.8 µg/lane) were separated by SDS-PAGE, followed by transfer to a PVDF membrane and blocking with 5% skim milk in PBS-T. The blot was probed with mouse anti-ß-actin antibody (1:3,000 dilution). (**B**) After separation of conditioned media derived from transfected 293 cells with pAPTAG5 (GenHunter) containing the secreted $(His)_6$ tagged alkaline phosphatase (AP, 8 x 10^{-14} mol/lane), proteins were transferred to PVDF membranes. Each membrane was subjected to western blot with different concentrations of anti-6x His tag antibody (1:2 serial dilutions from 400 ng/mL). The membranes were imaged as a single image and dotted lines indicate the border of individual membranes. This figure has been modified from Higashi et al.[6] . Please click here to view a larger version of this figure.

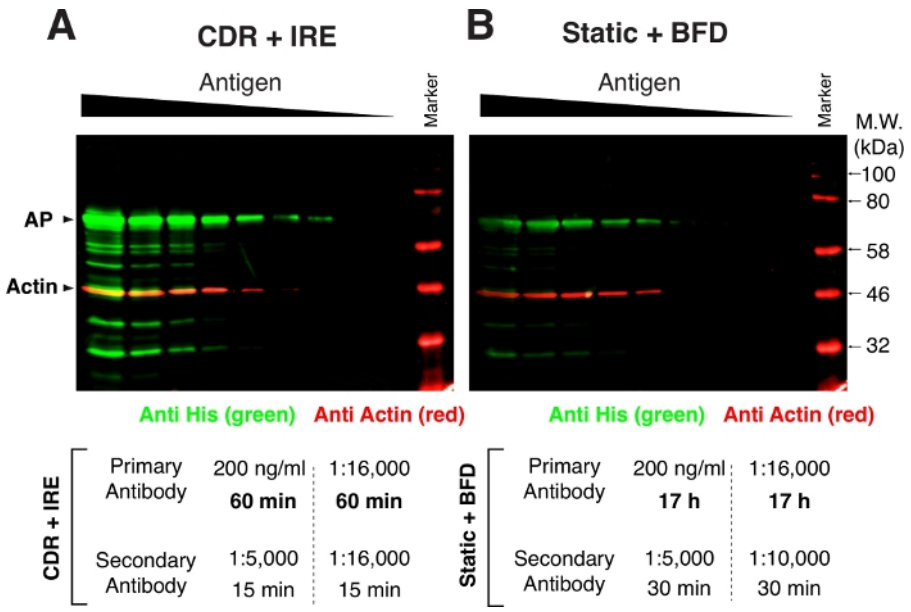

Figure 3: Simultaneous detection of multiple targets on a western blot using fluorescent detection and CDR in conjunction with IRE.

Different amounts of lysates (1:2 serial dilutions from 10 µg/lane) from 293 cells transfected with pAPTAG5 were separated and transferred to a PVDF membrane. After blocking with the Blocking Buffer for fluorescent detection (BFD) for 1 h, the blot was probed with anti-6X His tag and anti ß-actin antibodies, followed by IRDye 800CW goat anti-rabbit IgG and IRDye 680RD goat anti-mouse IgG. A: antibodies diluted with 10% IRE solution under CDR condition, B: antibodies diluted with BFD containing 0.1% Tween20 under static condition. Because of higher sensitivity with CDR and IRE combination, the degradation of $(His)_6$ tagged AP was seen. This figure has been modified from Higashi et al.[6] . Please click here to view a larger version of this figure.

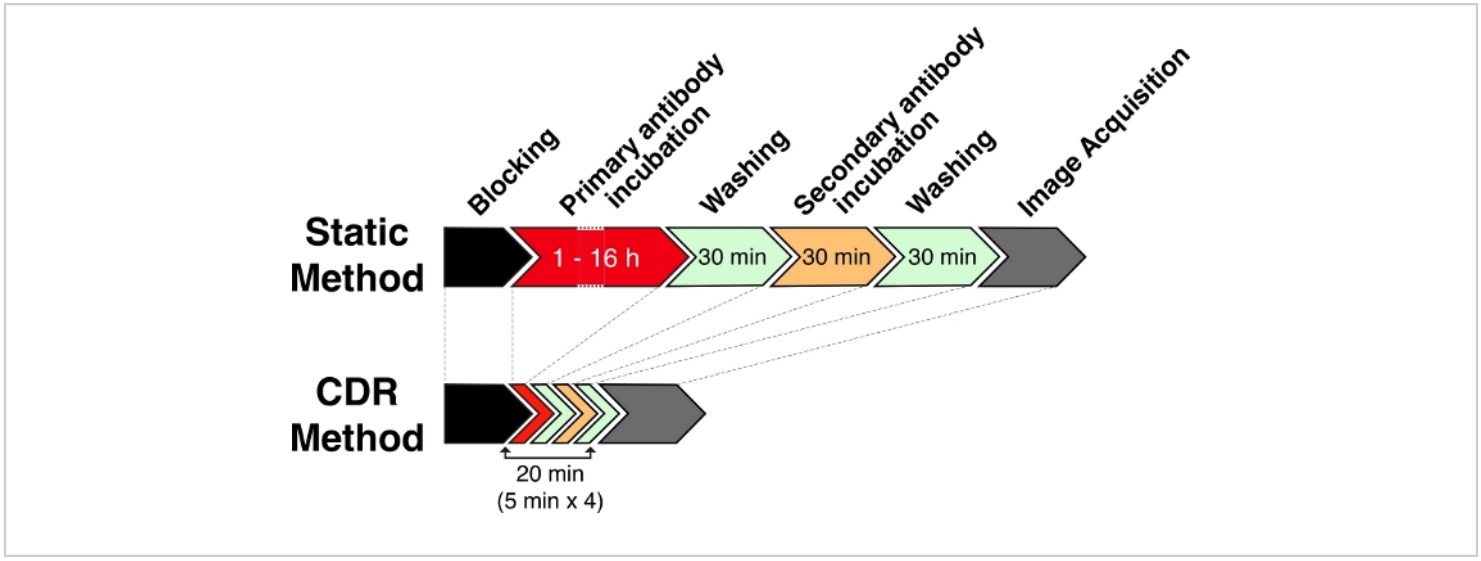

Figure 4: Schematic illustration of an enhanced and ultra-high speed CDR western blot compared to a traditional static method.

This figure has been modified from Higashi et al.[6]. Please click here to view a larger version of this figure.

Discussion

Western blot is a widely used analytical technique to detect specific proteins that was developed ~40 years ago[7,8]. Since then, the technique has continued to evolve, with subsequent innovations improving the sensitivity, speed, and quantitation of the technique[2,9,10,11,12,13]. The enhanced ultra-high-speed western blotting protocol presented here makes several substantial enhancements to the existing technique. We reduce incubation time without sacrificing sensitivity by lowering the detection threshold with IRE. No special or expensive equipment is needed: rotating a tube in a hybridization oven is sufficient to overcome MTL. The key innovation is that CDR effectively eliminates the depletion layer in the assay. Although a roller-culture apparatus is commonly employed to reduce the volume of solution, the potential of this method to overcome MTL and reduce incubation times has not been illustrated. Because of the drastic reduction of the overall time for the entire procedure (**Figure 4**), much higher quantities of western blot data per day can be obtained when the blocked membranes are in one's hand, which is almost impossible to do so with a traditional western blotting protocol. It should be noted that incubation of the membrane on a rocking platform shaker is equivalent to static incubation[4,6]. Rinsing PVDF membranes in a household commercial salad spinner with a large volume of washing solution also decreases the duration of the procedure (**Figure 4**).

The successful use of this protocol relies on the optimization of the antibody dilution with IRE solution and incubation time. A wide range of antibody titration is recommended at the beginning of the assays since CDR in conjunction with IRE increases the sensitivity substantially (**Figure 2**). Incubation time is another factor to be considered. When one has good western blot results with 1 h incubation with primary antibodies under static conditions, incubation time could be reduced to 5-10 min under CDR conditions in

general. When overnight incubation is needed with primary antibodies for example, a slightly longer CDR incubation time (30 min - 6 h) should be evaluated. Dilution and incubation time with secondary antibodies are also critical. Incubations greater than 30 min under CDR conditions usually give higher background. Thus, careful titration of secondary antibodies is required with up to 30 min of CDR incubation. When the background signal is high after careful titration of antibodies, extensive washing steps (10 min rinse 3 times for the primary antibody and 5 min rinse 6 times for the secondary antibody in a container with 80-100 mL of washing solution) should be performed.

The quality of the western blot data often depends on the antibodies used. When we had a difficult antibody used for western blot, protein markers sometimes showed substantial signals even after careful antibody titration and extensive washing. Since we noticed that the CDR method with IRE tends to increase the non-specific binding, addition of blocking reagents (such as skim milk and others) into antibody diluents may improve the signal-to-noise ratio. It is a time-consuming screening step, but it is worth trying.

The western blot has become important for quantitative applications[2]. A semi-quantitation can be performed with chemiluminescent detection. The procedure presented here is applicable to stripping and re-probing to this end[6]. Because of a wider dynamic range, fluorescent detection is the best option. With this protocol, fluorescent detection provides a linear dynamic range for quantitative analysis[6]. It is worth mentioning that CDR incubation time with fluorescent detection seems longer than that with chemiluminescent detection.

The western blot has a wide array of applications and remains a universal method to study protein abundance, protein-protein interactions, and post-translational modifications. For instance, anti-carbohydrate antibodies can be easily used for western blot with this protocol. Since a variety of carbohydrate moieties expressed on the outer surface of viral, bacterial, and fugus are pathogen-specific, they are invaluable targets for pathogen recognition and diagnosis of infectious diseases. Thus, the application of this simple protocol could impact many areas of investigation, shaving hours of waiting time not only for experiments but also clinical immunodiagnostics that rely on western blotting technology.

Disclosures

The authors have declared no competing interests directly relevant to the contents of this article.

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