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Ultra-High-Speed Western Blot using Immunoreaction Enhancing Technology

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TITLE:

Ultra-High-Speed Western Blot using Immunoreaction Enhancing Technology

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KEYWORDS:

Western blot, Immunoblot, Chemiluminescence, Fluorescence, Immunoreaction enhancing agent, Mass transport limitation

SUMMARY:

An ultra-high-speed western blotting technique is developed by improving the kinetics of antigen-antibody binding through cyclic draining and replenishing (CDR) technology in conjunction with an immunoreaction enhancing agent.

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

1.1. 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.

1.2. 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

2.1. 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

3.1. Prepare 10% immunoreaction enhancing agent-1 solution (IRE-1) with distilled water. Vortex well.

3.2. Prepare the diluted primary antibody with 10% IRE-1. Mix it gently and thoroughly.

3.3. 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**).

3.4. 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.

3.5. 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.

3.6. Turn on the hybridization oven.

3.7. 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

4.1. Stop the rotation.

4.2. Carefully remove the PVDF membrane from the tube with tweezers, and place the membrane into a container with 50 mL of PBS-T.

4.3. Rinse the membrane briefly with PBS-T.

4.4. Rinse the membrane in the container with distilled water until no bubbles appear. This is to remove the majority of the antibody.

4.5. Transfer the PVDF membrane from the container to the salad spinner that contains 250 mL of PBS-T.

4.6. Place the strainer basket in the spinner. Ensure that the lid has been put on securely.

4.7. Activate the spinner and run it for about 20-30 s.

4.8. Discard the solution and briefly rinse the inside of the spinner with distilled water.

4.9. Add 250 mL of PBS-T into the spinner again.

4.10. Repeat steps 4.6 and 4.7.

5. Incubation with the secondary antibody

5.1. Prepare 10% immunoreaction enhancing agent-2 solution (IRE-2) with distilled water. Vortex well.

5.2. Prepare the diluted secondary antibody with 10% IRE-2. Mix it gently and thoroughly.

5.3. Select the volume of the antibody solution and tube as stated in step 3.3.

5.4. 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.

5.5. Insert the 50 mL tube into a glass bottle for the hybridization oven.

5.6. Turn on the hybridization oven.

5.7. 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.

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

6. Membrane wash

6.1. Follow steps 4.1 to 4.10.

7. Image acquisition

7.1. Chemiluminescent detection

7.1.1. Place a piece of semi-transplant flexible film (10 cm x 15 cm) on a flat surface.

7.1.2. Mix 2 components of the chemiluminescent substrate in a 3 mL tube.

7.1.3. 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.

7.1.4. Incubate the PVDF membrane with the mixed substrate on a semi-transparent flexible film for 1 min.

7.1.5. Pick up the PVDF membrane with tweezers and drain the substrate solution briefly.

7.1.6. Sandwich the membrane between a pair of transparency films.

7.1.7. Acquire the image under chemiluminescent mode.

7.2. Fluorescent detection

7.2.1. Pick up the PVDF membrane with tweezers and drain the substrate solution briefly.

7.2.2. Sandwich the membrane between a pair of transparency films.

7.2.3. 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.

7.2.4. 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 AND TABLE LEGENDS:

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].

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, 8×10^{-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].

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].

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].

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[2,9-13]. 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.

ACKNOWLEDGMENTS:

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DISCLOSURES:

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

REFERENCES:

1 MacPhee, D. J. Methodological considerations for improving Western blot analysis.

Journal of Pharmacol Toxicology Methods. **61** (2), 171-177 (2010).
2 Janes, K. A. An analysis of critical factors for quantitative immunoblotting. *Science Signaling.* **8** (371), rs2 (2015).
3 Schuck, P., Zhao, H. The role of mass transport limitation and surface heterogeneity in the biophysical characterization of macromolecular binding processes by SPR biosensing. *Methods in Molecular Biology.* **627**, 15-54 (2010).
4 Li, J., Zrazhevskiy, P., Gao, X. Eliminating Size-Associated Diffusion Constraints for Rapid On-Surface Bioassays with Nanoparticle Probes. *Small.* **12** (8), 1035-1043 (2016).
5 Toyobo. Immunoreaction enhancing: technology. http://www.toyobousa.com/lifescience-immunoreaction-enhancing.html (2020).
6 Higashi, S. L. et al. Old but not Obsolete: An Enhanced High Speed Immunoblot. *Journal of Biochemistry.* (2020).
7 Towbin, H., Staehelin, T., Gordon, J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proceedings of the National Academy of Sciences of the United States of America.* **76**, 4350-4354 (1979).
8 Burnette, W. N. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate--polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Analytical Biochemistry.* **112** (2), 195-203 (1981).
9 Mishra, M., Tiwari, S., Gomes, A. V. Protein purification and analysis: next generation Western blotting techniques. *Expert Review of Proteomics.* **14** (11), 1037-1053 (2017).
10 Ciaccio, M. F., Wagner, J. P., Chuu, C. P., Lauffenburger, D. A., Jones, R. B. Systems analysis of EGF receptor signaling dynamics with microwestern arrays. *Nature Methods.* **7** (2), 148-155 (2010).
11 Treindl, F. et al. A bead-based western for high-throughput cellular signal transduction analyses. *Nature Communications.* **7**, 12852 (2016).
12 Sajjad, S., Do, M. T., Shin, H. S., Yoon, T. S., Kang, S. Rapid and efficient western blot assay by rotational cyclic draining and replenishing procedure. *Electrophoresis.* **39** (23), 2974-2978 (2018).
13 Kurien, B. T., Scofield, R. H. A brief review of other notable protein detection methods on blots. *Methods in Molecular Biology.* **536**, 557-571 (2009).

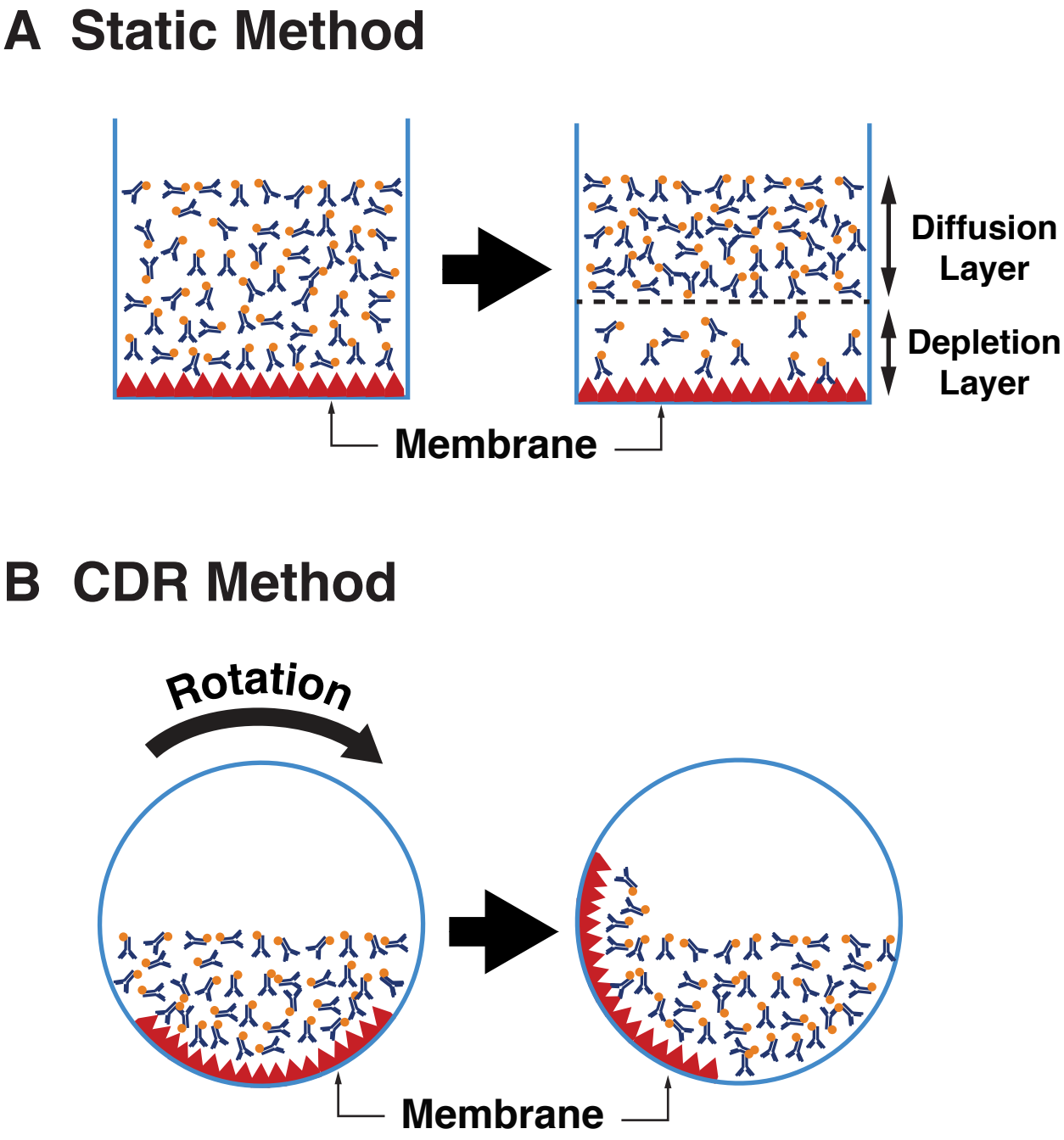
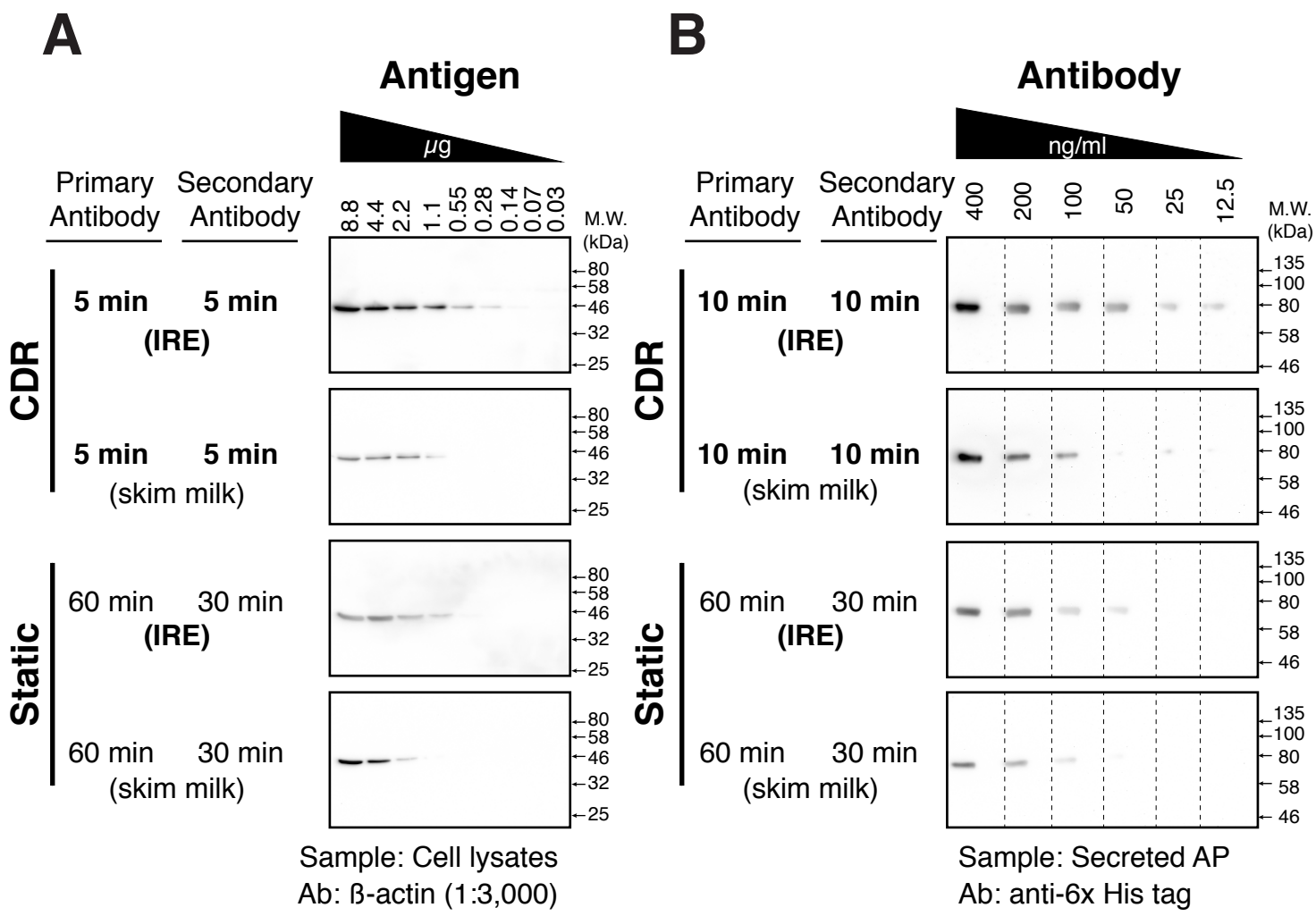
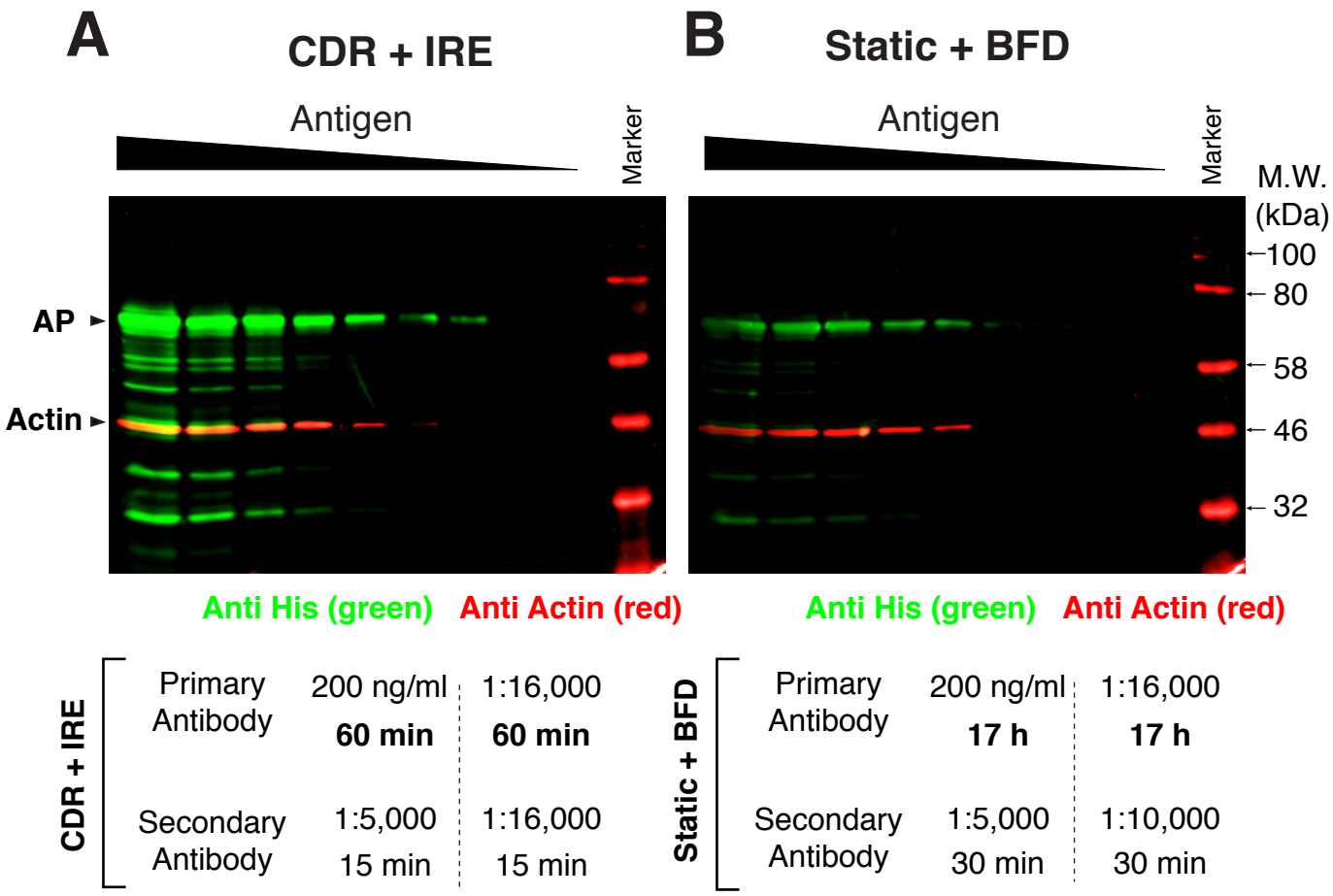


Figure 2

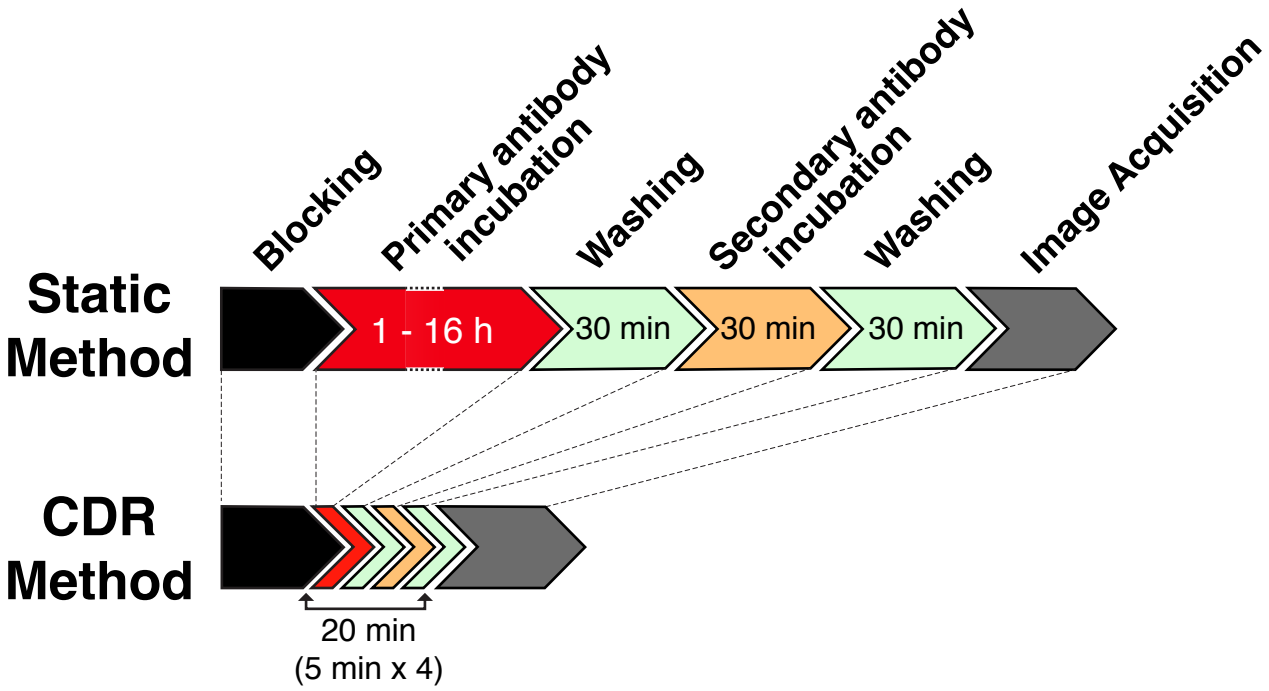




CDR + IRE			
Primary Antibody	200 ng/ml **60 min**	1:16,000 **60 min**
Secondary Antibody	1:5,000 15 min	1:16,000 15 min

Static + BFD			
Primary Antibody	200 ng/ml **17 h**	1:16,000 **17 h**
Secondary Antibody	1:5,000 30 min	1:10,000 30 min

Figure 4



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
14 mL Round Bottom High Clarity PP Test Tube, Graduated, with Snap Cap	Falcon	352059	
Apollo Transparency Film for Laser Printers	N/A	N/A	Any kinds of Laser Printer Transparency Film are fine.
Azure Imaging System 600	Azure Biosystems	Azure 600	Any kind of Image Acquiring Sytem is fine for chemiluminescent and/or fluorescent detection.
Can Get Signal Immunoreaction Enhancer Solution	TOYOBO	NKB-101	
CARNATION Instant Nonfat Dry Milk	Carnation	N/A	
ECL anti-mouse IgG, Horseradish peroxidase linked F(ab')2	GE Healthcare	NA9310V	
ECL anti-rabbit IgG, Horseradish peroxidase linked F(ab')2 fragment from donkey	GE Healthcare	NA9340V	
HYBAID Micro-4	N/A	N/A	Any hybridization oven is fine as long as the tubes are rotated evenly at a horizontal position.
Immobilon-P PVDF Membrane, 0.45 µm pore size	Millipore Sigma	IPVH304F0	
IRDye 680RD Goat anti-Mouse IgG Secondary Antibody	LICOR	926-68070	
IRDye 800CW Goat anti-Rabbit IgG Secondary Antibody	LICOR	926-32211	
KPL LumiGLO Reserve Chemiluminescent Substrate	seracare	5430-0049	
Mouse anti-ß actin antibody	Millipore Sigma	A5316	
Odyssey Blocking Buffer (PBS)	LICOR	927-40100	Blocking Buffer for fluorescent detection
OXO Salad Spinner	OXO	32480V2B	Any salad spinner is fine as long as the PVDF membranes are rinsed vigorously without tear.

Parafilm Sealing Film	Bemis	Parafilm M PM996	semi-transparent flexible film
Polyethylene Flat-Top Screw Caps for 50 mL Conical Bottom Centrifuge Tubes	Falcon	352070	
Rings to hold 14 ml tube in the center of 50 ml tube	N/A	N/A	Prepared in a machine shop
Rabbit anti-6X His tag antibody	Abcam	ab9108	
Tween20	Millipore Sigma	P2287	

Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

We performed spelling and grammar checking with Word and have personally proofread the manuscript ourselves.

• Protocol Language: Please ensure that ALL text in the protocol section is written in the imperative voice/tense as if you are telling someone how to do the technique (i.e. "Do this", "Measure that" etc.) Any text that cannot be written in the imperative tense may be added as a "Note", however, notes should be used sparingly and actions should be described in the imperative tense wherever possible.
1) Examples NOT in the imperative: 1.1, 1.2, 2.1

We changed the protocol 1.1, 1.2, and 2.1.

• Protocol Detail: Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Some examples:
1) 1.1: how is SDS PAGE performed. Mention specifics.
2) 1.2: How? Please cite appropriate references for steps you are not describing.

There are many ways to perform SDS-PAGE such as commercial precast gels and home-made gels. Even among commercial precast gels, many different buffer systems are available such as Tris-Glycine and Tris-Bis. We hope our expression in this protocol 1.1 is OK.

There are also many ways to perform transfer of proteins from a gel to a membrane such as wet types, semi-dry types, and commercial systems (iBlot2 Western Blotting system). We hope our expression in this protocol 1.2 is OK.

• Discussion: JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

We added one paragraph mentioning modifications and limitations.

• **Figures: Remove text "Figure #" from all figures.**

We removed figure numbers.

• **References: Please spell out journal names.**

We spelled out journal names using Endnote.

• **Commercial Language: JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are Can Get Signal® (CGS), MilliQ, Falcon, Falcon, SeraCare LumiGLO, parafilm, Odyssey.**

1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.

We replaced all commercial product names with plain words.

2) Please remove the registered trademark symbols TM/R from the table of reagents/materials.

We remove them from the table.

3) Please check figures as well.

We remove them from the figures.

• **Table of Materials: Please sort this alphabetically.**

We sorted alphabetically.

• **If your figures and tables are original and not published previously or you have**

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A copy of email is attached at the submission.

Response to the reviewers

Reviewer #1:

1. The true comparison for the CDR method would be horizontal motion-based incubation rather than static incubation. Traditional Western blot is not based on static incubation of reagents and therefore, the comparative study needs to be re-designed. The authors have pointed out in their discussion that both static and rocking shaker are comparable as others have found. However, the authors need to provide evidence of the same in this study.

We appreciate this reviewer's comment. We agree that there are many ways to incubate membranes with antibodies. For example, membrane and antibody are (1) in a container with or without rocking, (2) in a sealed plastic bag, (3) on a parafilm, and (4) in a roller bottle. Different incubation methods are selected by individual labs, but the volume of diluted antibody is one of the factors considered. Roller-culture apparatus is commonly employed to reduce the volume of solution, but the potential of this technique to overcome MTL and shorten incubation times has not been well characterized.

In this study, we compared 2 methods (incubation on a parafilm vs. incubation in a roller bottle using a hybridization oven). It is clear that use of a roller bottle (CDR method) decreased the required amount of antigen (Fig. 2A) as well as the concentration of antibody (Fig. 2B).

In our previous publication in *Journal of Biochemistry*, we have shown that there was no difference in the sensitivity between Rocking and Static conditions as supplementary Figure S3, which was cited as reference 6 in our discussion. For your convenience, we provide it as **Reviewer's Figure 1**.

2. Authors mention CGS reagent improves/maintains sensitivity of the signal, however, no quantitation has been provided to + CGS and - CGS blots.

Western blot has been used for quantitation purposes, but it is limited to a semi-quantitation with chemiluminescent detection. We hesitated to show the quantitation of individual bands with chemiluminescence. This is the reason why we loaded different amounts of cell lysates in each lane (Figure 2A) to show the threshold of detection. Presence of CGS lowered the detection limit in both static and CDR conditions. Further, we showed the increased sensitivity by CGS using slot blot in our previous publication in *Journal Biochemistry* as Supplementary Figure S2. In this figure, antibody concentration and incubation time were the same to study the effects of CGS and CDR. We provide this as **Reviewer's Figure 2**.

Minor Concerns:

1. Line 136; step 4.2 Why not perform the washes within the tube rather than removing the membrane and placing it in a container with 50 ml PBST?

We appreciate this comment. We have already tried several different ways to wash the membrane. As suggested, one of them was rinsing the membrane in the same 50 ml tube with rotation. We also tried a vortex method where the membrane was transferred to a new 50 ml tube and the tube was vortexed with PBS-T several times for 20 – 30 seconds each. When a larger membrane was used and rinsed with these alternative methods, we had a huge background at the final images. To decrease this background, we had to repeat these washing steps many times. It turned out that this took much longer than a traditional washing method using a container with agitation. In contrast, washing with a much larger volume of PBS-T in a salad spinner was more efficient to reduce the time. However, we mentioned the possibility of using traditional washing steps in the discussion in the case of high background.

2. Figure 2: All the blot data is highly adjusted in terms of background noise. Please provide data that displays a blot background along with raw data image files.

The data for Figure 2A and 2B were acquired with the same exposure times (15 s for 2A and 30 s for 2B) on the same day at the same time. The images were cropped but not otherwise altered or adjusted.

Reviewer #2:

-The authors describe the step-by-step protocol that they recently disclosed in Higashi S. et al. 2020 (J. Biochem.) but it looks like that the cited publication already contains exemplificative videos and the figures are not modified at all, but just copy/pated from the same recent publication (Figure 2 same as Figure 2, Figure 3 same as Figure 6 and Figure 4 same as Figure 7 of Higashi S., 2020, respectively).
Even though this may not be a concern for JoVE papers, I would recommend the authors to provide additional representative examples.

When we were invited to submit a paper by JoVE's editor, we were told that no additional data were necessary. (Please see our response below.)

-The author state "When overnight incubation is needed with primary antibodies for example, a slightly longer CDR incubation time (30 min - 6 h) should be evaluated." (line 310). Indeed, more than half of the experimental time for a Western blot experiment is dedicated to sample preparation (preferably fresh), gel preparation and loading, transfer to membrane and blocking.

We agree with this reviewer's comment. The purpose of this manuscript is to show a comprehensive method to shorten the time for western blotting itself. Figure 4 shows

the comparison between a traditional method and our CDR method, where all membranes are already blocked. To avoid such confusion, we added a phrase in the discussion.

Moreover, since one of the hottest and explored reasearch field is based on analysis of post-translationally modified proteins, e.g. phospho-proteins, which are inherently less abundant than housekeeping genes such as actin, the incubation times with primary antibodies may turn out to be too long, even with the CDR+CGS protocol, thereby preventing anyways the use of CDR+CGS in a single day experiment.
Did the authors think about showing as additional results a representative quantification of a phosphorylated protein different from pERKs (which is a very abundant one)? It would be a compelling evidence to show a case where the sensitivity limit of traditional protocols is overcome by the CDR+CGS without sacrifycing the time, in the context of a low-abundance antigen.

We totally agree with this comment. PTM is one of the hottest areas in biological research. In our previous publication in *Journal Biochemistry* as Figure 4, we showed the autophosphorylation of exogenous v-src, where overnight incubation with the traditional method was reduced to 4 h with CDR+CGS. This figure is provided as **Reviewer's Figure 3**.

One well-known PTM is glycosylation. We are working on chondroitin sulfate (CS) and heparan sulfate (HS) proteoglycans. Glycosaminoglycan (GAG) chains are linear polysaccharide with repetitive disaccharides with different sulfations, resulting in the huge heterogeneity. Although monoclonal antibodies (CS-56 for CS and 10E4 for HS, both IgM) have been used for histochemistry for a long time, these antibodies have gained infamy for their ambiguous signals and lack of reliable controls on western blot. At least 18-hour incubation with CS-56 was required without the aid of CGS. We applied this methodology to these antibodies and representative data are provided as **Reviewer's Figure 4**. We analyzed minimum amounts of antigen and proved the specificity of signals. The manuscript with these data is now under a second revision.

-When discussing Figure 2, the authors state "In both static and CDR incubations, usage of CGS solution increased the sensitivity:" (line 230). While this holds true when comparing intra-condition (CDR or Static), counterintuitively the use of skim milk (60min-30min) under static conditions in Figure 2A, apparently resulted in a much brighter signal of the first bands as compared to its parallel under the CDR conditions. Indeed, although the static conditions with skim milk resulted in a way brighter initial signal, there does not appear to be a dilution curve at all. Conversely the CDR condition with skim milk has dimmer initial bands, reaches lower antigen amounts but, strangely, without a sequential fold-decrease curve as in its parallel of Figure 2B.
How do the authors explain this? Why didn't the authors provide band quantification histograms to show that the dilution curve is accurate? Since Western blotting is used for relative quantification of samples, it is of paramount importance that any methodology

aimed at reaching lower limits of sensitivity is also able to keep the relative differences over a wide dynamic range so as not to result in a misinterpreation of results.

We appreciate this comment and it is a good point. As mentioned above in the response to Reviewer 1, the data for Figure 2A and 2B were acquired with the same exposure times (15 s for 2A and 30 s for 2B) on the same day at the same time. The images were cropped but not adjusted. While we do not have a straightforward and precise explanation of this pattern of results, we are confident that the careful planning and execution of these experiments has produced reliable data; transfer from a gel to a membrane was performed individually (no multiple transfer simultaneously) and membranes were blocked in separate containers. There could be many reasons such as uneven transfer from a gel to a membrane and altered activity of chemiluminescent substrate. This is another reason why we loaded different amounts of antigens in each lane (Figure 2A) and used different concentrations of antibody in individual membranes (Figure 2B). To have a better solution, we showed fluorescent detection with slot blot in our previous publication. Because of fluorescent detection, we can obtain a wider dynamic range compared to chemiluminescent detection. Because of slot blot, uneven transfer is likely to be avoided. We provide this data as **Reviewer's Figure 5**.

Minor Concerns:

-Please provide a vendor and a catalog number for the plastic ring holders required for 14mL tube fixation inside the 50mL Falcon tube during rotatory incubation.

We appreciate this comment. We created these rings in a machine shop. A sentence was added to the list.

As exemplified by the authors and as shown in the video in Higashi et al. 2020, the membranes are washed by placing them in NOT inside the salad spinner's strainer basket but rather directly inside the outer container. Why do the authors perform such a procedure?

As mentioned above, we attempted many techniques for washing the membrane and have used the one that was most effective. When membranes were placed inside the salad spinner's strainer basket, we observed that the membrane's exposure to dynamic PBS-T solution was actually fairly low, with the membrane exhibiting little movement. This technique thus yielded higher background. When membranes were placed outside the strainer basket at the margins of the device, its exposure to the PBS-T solution was greatly enhanced, and the membrane was more vigorously washed with a large volume of PBS-T. This yielded lower background, and therefore this is the technique we used for our experiments.

Did the author ever tried nitrocellulose instead? Many labs use nitrocellulose instead of PVDF membranes, the latter being more resistant. Is nitrocellulose going to be damaged by this procedure?

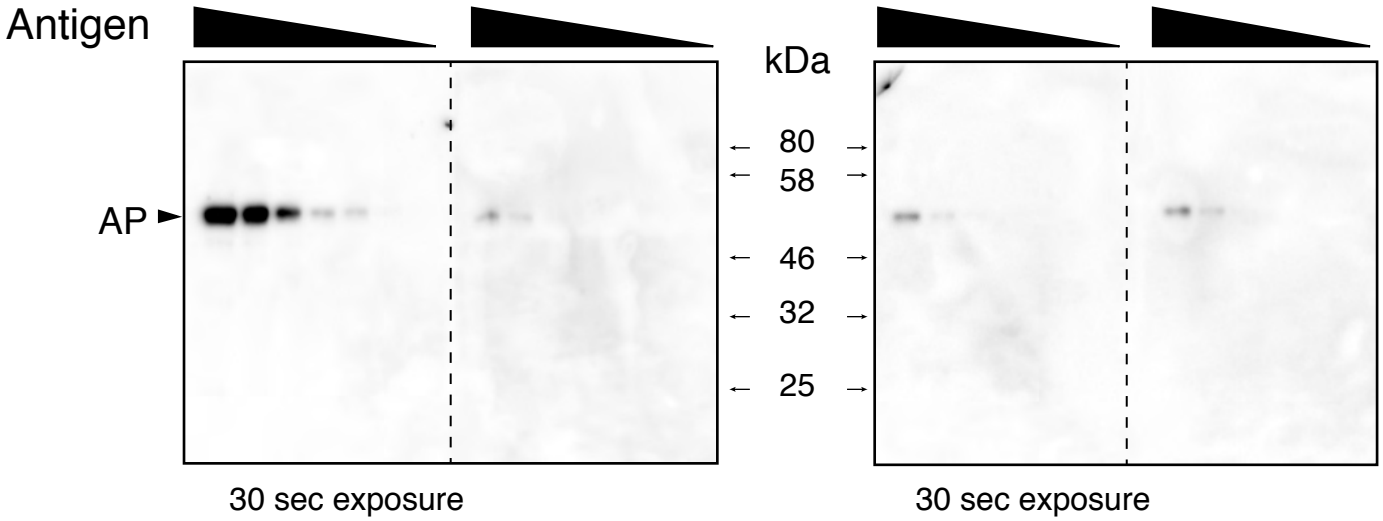
We have tried this method with nitrocellulose membranes. As long as the nitrocellulose membrane attaches to the wall of the tube during the incubation with the antibodies, it worked. Washing membrane in a salad spinner should be carefully performed, otherwise the nitrocellulose membrane could be damaged easily.

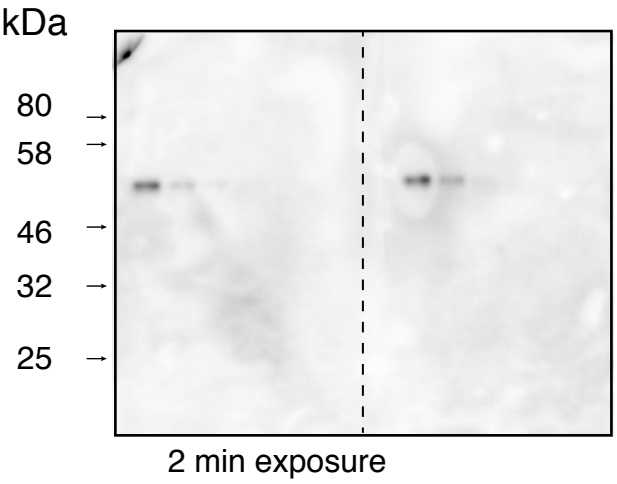
-Please use actual numbers to indicate antigen and antibody amounts, respectively in Figure 2A and B,rather than using an arrow.

Figure 2 was changed accordingly.

Reviewer's Figure 1

	5 min **CDR**	5 min **CDR**	5 min **Rocking**	5 min **Static**
Primary	1:15,000 CGS-1	1:5,000 Skim milk	1:15,000 CGS-1	1:15,000 CGS-1
Secondary	1:15,000 CGS-2	1:5,000 Skim milk	1:15,000 CGS-2	1:15,000 CGS-2

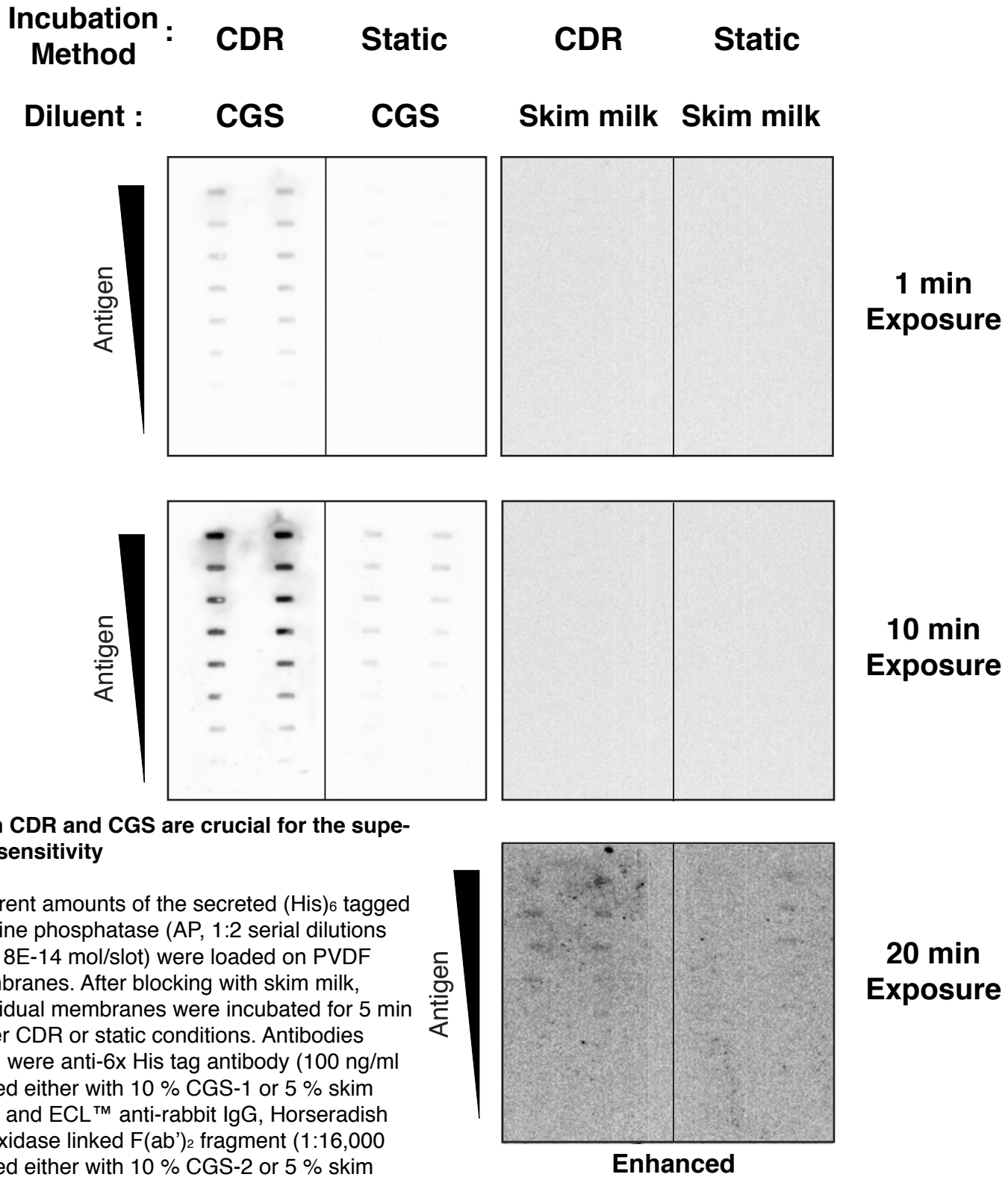




Comparison between CDR method, Static, and Rocking incubation on immunoblot

Different amounts of the secreted $(His)_6$ tagged alkaline phosphatase (AP, 1:2 serial dilutions from 8E-14 mol/lane) were separated, followed by transfer to PVDF membranes. Individual membranes were subjected to different incubation methods with anti-6x His tag antibody for 5 min. Chemiluminescence detection was performed and the membranes were imaged as a single image and dotted lines indicate the border of individual membranes.

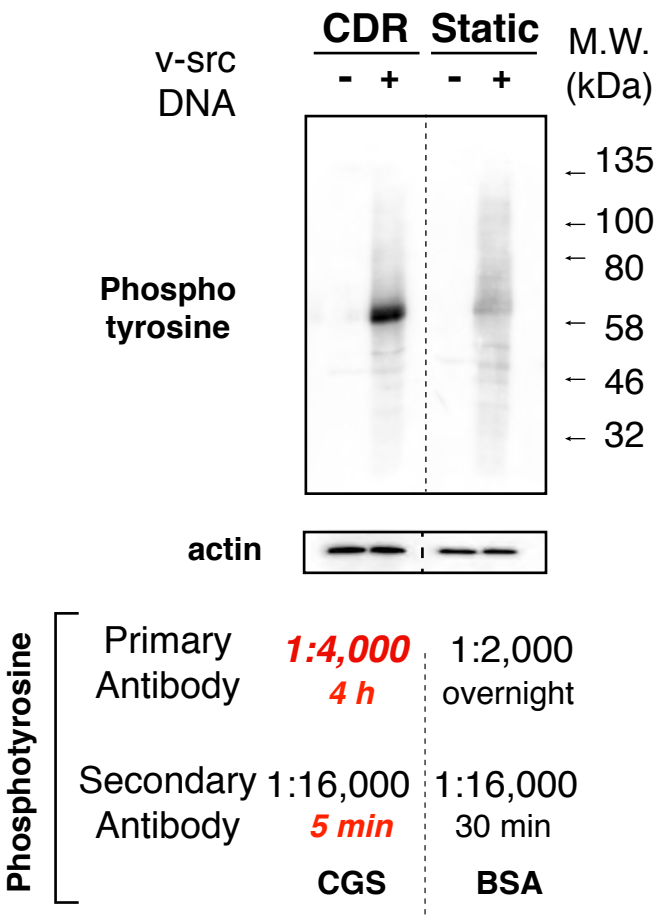
Reviewer's Figure 2



Both CDR and CGS are crucial for the superior sensitivity

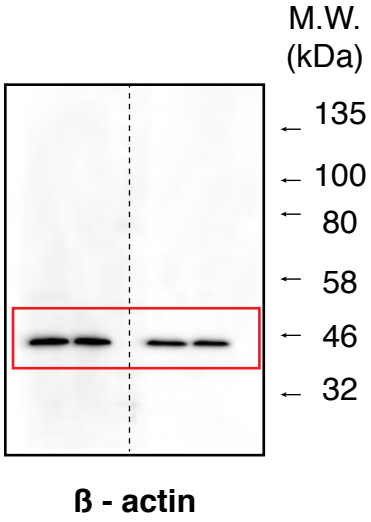
Different amounts of the secreted $(His)_6$ tagged alkaline phosphatase (AP, 1:2 serial dilutions from 8E-14 mol/slot) were loaded on PVDF membranes. After blocking with skim milk, individual membranes were incubated for 5 min under CDR or static conditions. Antibodies used were anti-6x His tag antibody (100 ng/ml diluted either with 10 % CGS-1 or 5 % skim milk) and ECL™ anti-rabbit IgG, Horseradish peroxidase linked $F(ab')_2$ fragment (1:16,000 diluted either with 10 % CGS-2 or 5 % skim milk). Regular washing of the membranes was utilized. Chemiluminescence detection was performed after 1 min, 10 min, and 20 min exposure.

Reviewer's Figure 3



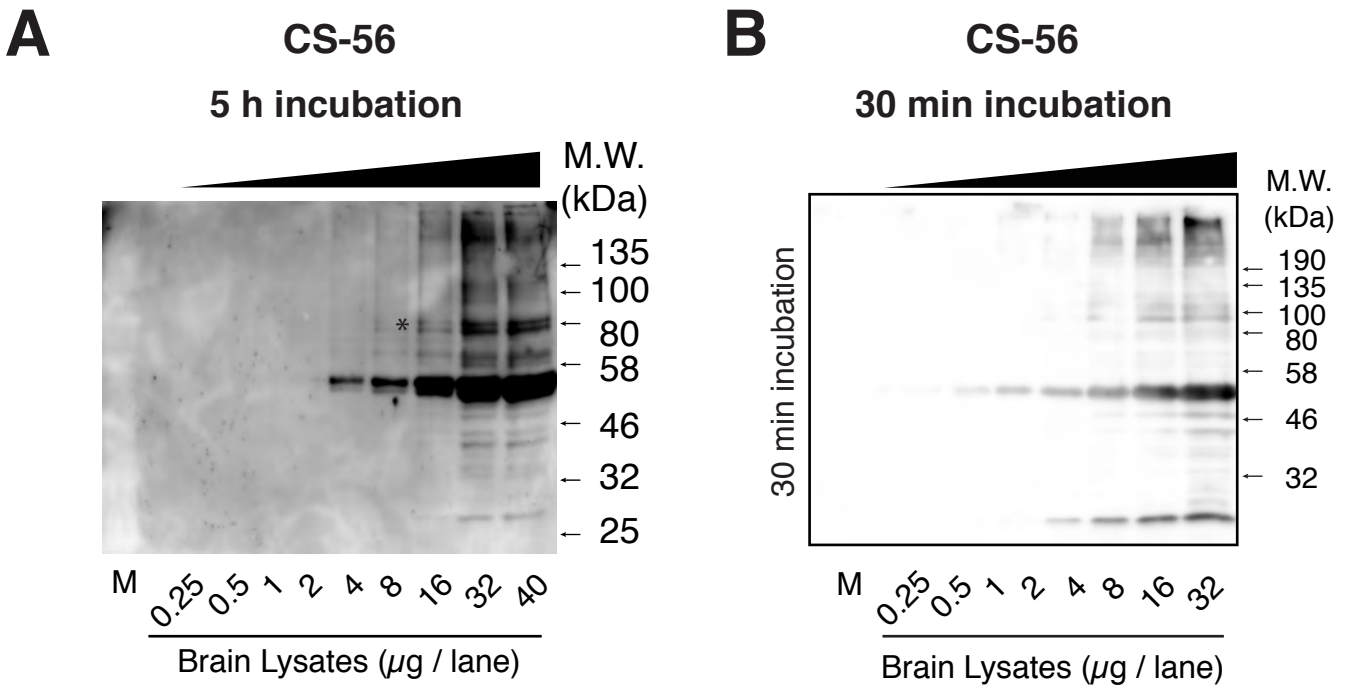
Detection of tyrosine phosphorylation induced by exogenous expression of chicken v-src.

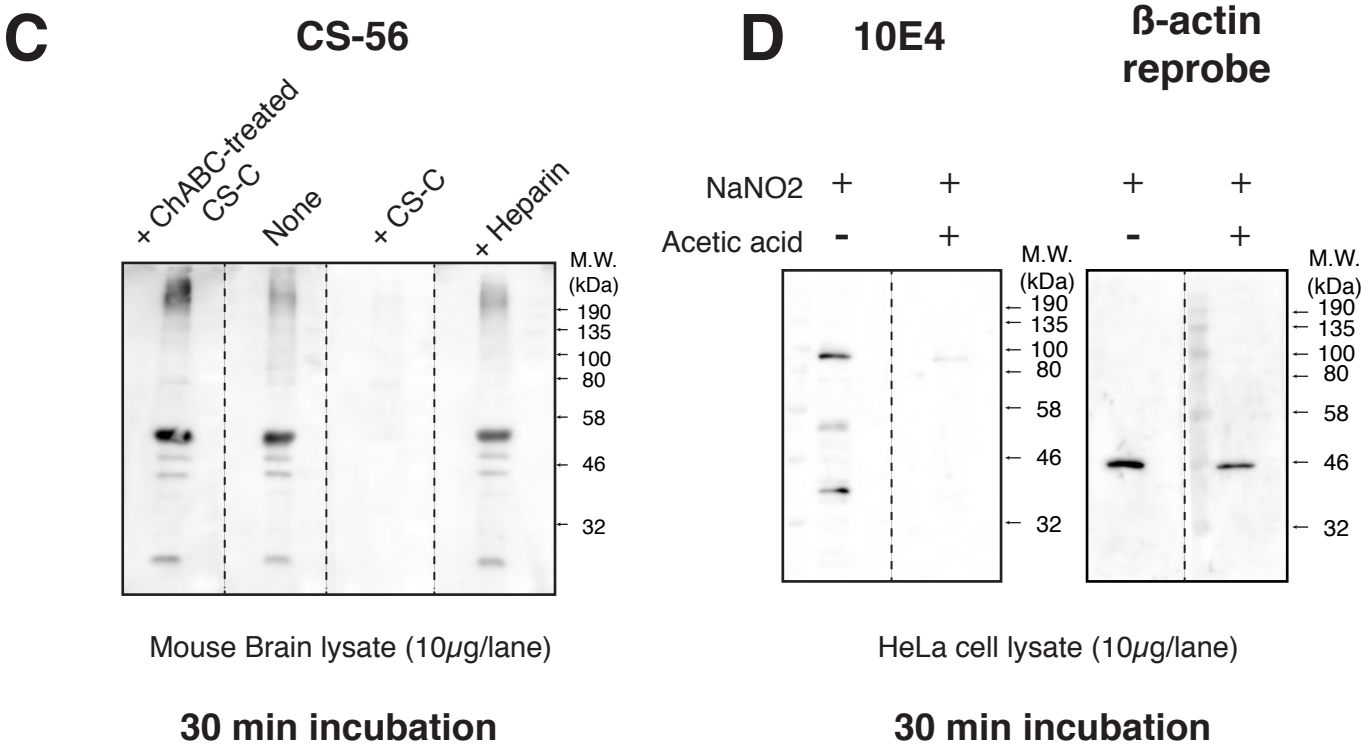
Cell lysates (20 μg/lane) from 293 cells transfected with pLNCX-v-src were separated by SDS-PAGE, followed by immunoblot with anti-phospho tyrosine antibody. The blots were imaged as a single image and dotted line indicates the border of individual membranes. After stripping the bound antibody, the membranes were re-probed with mouse anti-ß-actin antibody.



The original image of immunoblot data reprobed with anti-ß actin antibody. The red squares highlight the portion shown in the figure.

Reviewer's Figure 4





Reviewer's Figure 4

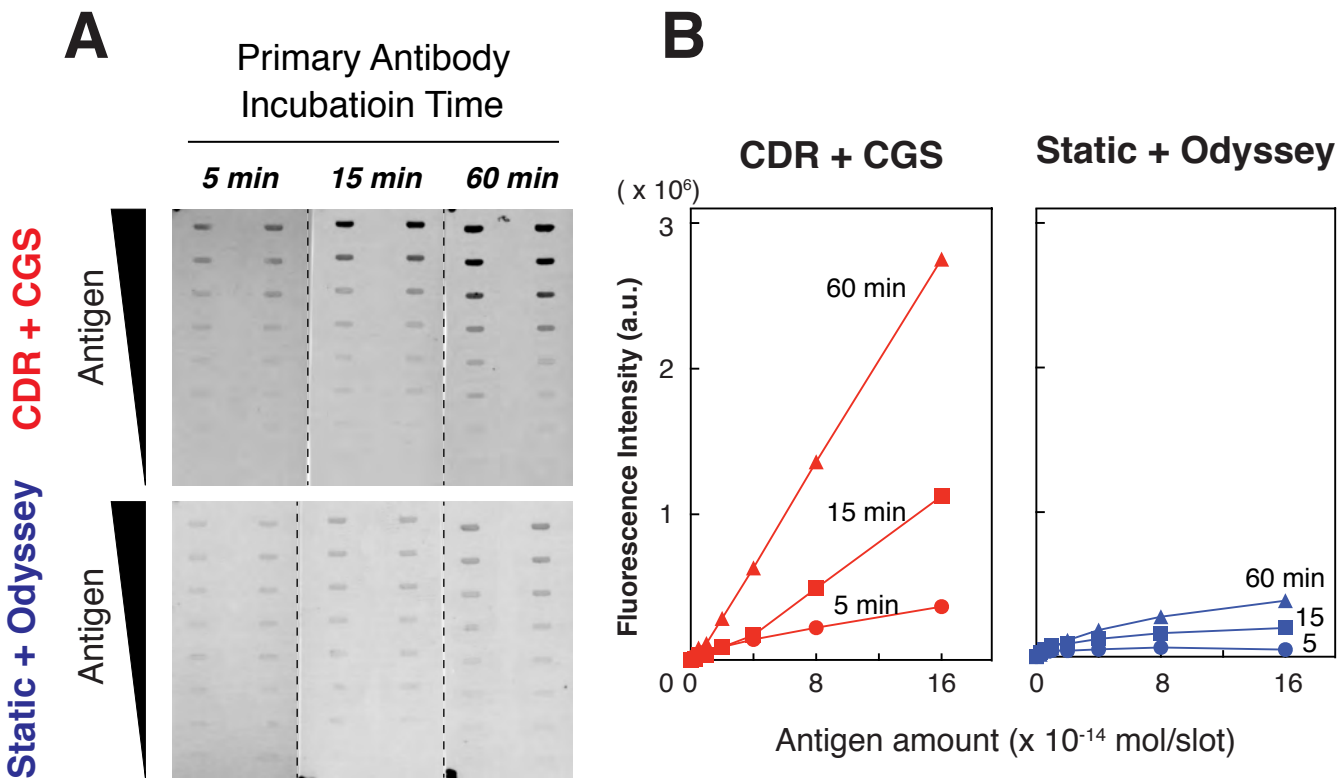
A. Optimization of Immunoblot with CS-56 (anti CS GAG chain) with a traditional method
Different amounts of brain lysates obtained from 7-week-old mice prepared in SDS were separated and transferred to a PVDF membrane, followed by **5 h incubation** with CS-56 (1:10,000 dilution with 10% CGS-1) under static condition. Asterisk indicates non-specific signal detected by anti-mouse IgM antibody since it was detected in the absence of CS-56 (not shown in this figure). While at least 18 h incubation with CS-56 was required without the aid of CGS, 5 h incubation was enough to obtain this blot in the presence of CGS. We found that loading more than 16 μg/lane resulted in the appearance of additional bands with higher molecular weights.

B. Optimization of Immunoblot with CS-56 with CDR method
PVDF membrane was prepared as (A). CS-56 was diluted (1:5,000) with 10% CGS-1 containing skim milk solution and incubated with PVDF membrane for **30 min** with CDR method. Proteomics analysis revealed that 50kDa bands contained brevican. Loading more than 8 μg/lane of mouse brain lysates resulted in the appearance of additional bands with higher molecular weights.

C. Proof of specific signals of CS-56
Brain lysates (10 μg/lane) prepared in SDS were separated and transferred to a PVDF membrane. CS-56 diluted (1:5,000) with 10% CGS-1 containing skim milk solution was pre-incubated at 22˚C for 30 min with either 500 ng/ml CS-C, heparin, or CS-C pretreated with chondroitinase ABC (ChABC). Immunoblot was performed as described in (B). The membranes were imaged as a single image and dotted lines indicate the border of individual membranes. CS-C is an antigenic GAG chain for CS-56 while heparin is a non-related GAG chain. ChABC is an enzyme to degrade CS GAG chains. Pre-incubation with the antigenic CS-C GAG chain eliminated CS-56 signals on immunoblot, demonstrating the specificity of these signals.

D. Proof of specific signals of 10E4 (anti-HS GAG chain)
HeLa cell lysates (10 μg/lane) was separated and transferred to a PVDF membrane. The membranes were treated with $NaNO_2$ alone or $NaNO_2$ together with acetic acid, followed by blocking. 10E4 was diluted (1:3,000) with 10% CGS-1 containing gelatin from cold water fish, and the incubation was performed with CDR method for 30 min. After acquiring the image, the bound antibody was stripped, and the membrane was reprobed by anti-ß actin antibody. The membranes were imaged as a single image and dotted lines indicate the border of individual membranes. The epitope for 10E4 is known to sensitive to chemical treatment with $NaNO_2$ and acetic acid. Chemical treatment of PVDF membrane reduced 10E4 signals on immunoblots, demonstrating the specificity of these signals.

Reviewer's Figure 5



CDR in conjunction with CGS in fluorescence immunoblot exhibits great dynamic range with reduced incubation time

(A) Different amounts of the secreted $(His)_6$ tagged AP (1:2 serial dilutions from 16E-14 mol/slot in duplicate) were loaded and the membranes were blocked at 4˚C for 60 min with Odyssey blocking buffer (ODS). The membranes were incubated for indicated times with 200 ng/ml of anti-6X His tag antibody diluted either in 10 % CGS-1 under CDR condition (top) or ODS containing 0.1 % Tween20 (ODS-T) under static condition (bottom). After rapid washing, the membranes were incubated for 15 min with goat anti-rabbit IgG-IRDye 680RD (1:5000 dilution) either in 10 % CGS-2 under CDR condition (top) or ODS-T under static condition (bottom).
(B) Quantitation of raw images was implemented and the average of fluorescent intensity at each condition was plotted.

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Dear Hiro Katagiri,

RE. **4 figures (with modification) - Sayuri L Higashi, Kazuya Yagyu, Haruna Nagase, et al. Old but not obsolete: an enhanced high-speed immunoblot, The Journal of Biochemistry 2020;0(0):1–8 doi:10.1093/jb/mvaa016**

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To Whom It May Concern:

I am a corresponding author of the article entitled “Old but not obsolete: an enhanced high-speed immunoblot” published in Journal of Biochemistry (https://doi.org/10.1093/jb/mvaa016). We got an invitation from JOVE to submit an article and would like to reuse our own data (4 figures) with modification. I would greatly appreciate if you could provide how to proceed.

I am looking forward to hearing from you soon.

Sincerely yours,

Hiro Katagiri

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