Old but not obsolete: an enhanced high-speed immunoblot

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The immunoblotting technique (also known as western blotting) is an essential tool used in biomedical research to determine the relative size and abundance of specific proteins and protein modifications. However, long incubation times severely limit its throughput. We have devised a system that improves antigen binding by cyclic draining and replenishing (CDR) of the antibody solution in conjunction with an immunoreaction enhancing agent. Biochemical analyses revealed that the CDR method reduced the incubation time of the antibodies, and the presence of a commercial immunoreaction enhancing agent altered the affinity of the antibody, respectively. Combination of the CDR method with the immunoreaction enhancing agent considerably enhanced the output signal and further reduced the incubation time of the antibodies. The resulting high-speed immunoblot can be completed in 20 min without any loss in sensitivity. Further, the antibodies are fully reusable. This method is effective for both chemiluminescence and fluorescence detection. Widespread adoption of this technique could dramatically boost efficiency and productivity across the life sciences.

Keywords: chemiluminescence; fluorescence; immunoblot; immunoreaction enhancing agent; western blot.

Abbreviations: AP, alkaline phosphatase; CGS-1, Can Get Signal®-1 Immunoenhancer Solution; CGS-2, Can Get Signal®-2 Immunoenhancer Solution; CDR, cyclic draining and replenishing; ELISA, Enzyme-linked Immunoabsorbant assay; MTL, mass transport limitation; ODS, Odyssey Blocking Buffer; ODS-T, Odyssey Blocking Buffer containing 0.1% Tween20; PBS-T, phosphate buffer saline containing 0.1% Tween20; PMA, phorbol 12-myristate 13-acetate; PVDF, Polyvinylidene difluoride; SPR, surface plasmon resonance; TBS-T, tris-buffered saline containing 0.1% Tween20.

On-surface bioassays, such as immunoblot, surface plasmon resonance (SPR), enzyme-linked immunoabsorbant assay (ELISA) and immunostaining, have proven indispensable to biomedical research. Although immunoblot and immunostaining are performed routinely and the premise of these procedures are simple, each iteration takes hours or even days to complete, which means these assays impose an enormous time burden relative to other techniques that have benefited from the development of new, high-speed technologies. Here, we demonstrate an enhanced immunoblotting technique that overcomes this limitation in assay kinetics without sacrificing sensitivity. Our results suggest that similar enhancements may apply to other on-surface bioassays, giving these reliable but time-intensive techniques a much-needed boost into the age of high-throughput biology.

In on-surface bioassays, detecting probes (antibodies) float in solution, while their targets (antigens) are immobilized on a fixed surface. Because of their high affinity, probes close to the surface bind to their targets quickly, leaving behind a low-concentration ‘depletion layer’. The passive diffusion of more distant probes into this depletion layer may take hours, substantially increasing incubation times (1). The circulation of probes in solution is further limited by the tendency of bound probes to detach and rapidly re-bind before they can escape (2). This effect is labelled mass transport limitation (MTL). It has been proposed that repeatedly draining and replenishing the probe-containing solution may disrupt the depletion layer and overcome MTL (3) (Supplementary Fig. S1). Here, we have designed a unique technique that implements this cyclic draining and replenishing (CDR) concept to overcome MTL in a traditional immunoblotting protocol and markedly reduce the required incubation times. To preserve the sensitivity of the assay, we also utilized an immunoreaction enhancing technology. Together, these modifications yield a high-speed immunoblotting protocol that dramatically reduces procedure time without measurable loss in sensitivity.

Materials and Methods

Antibodies

Antibodies used in this study are shown in Supplementary Table S1.

Cell culture and transfection/PMA treatment

All experiments and procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) at the National Institutes of Health. Primary cultures of cerebral cortical astrocytes were prepared from newborn C57BL/6 mice as described previously (4). HEK293 (CRL-1573^{TM}, ATCC) and HeLa S3 (CCL-2.2^{TM}, ATCC) cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% foetal bovine serum and antibiotics. After the rinse of adherent HEK293 cells and mouse primary cultured astrocytes with

PBS twice, cell lysates were prepared by RIPA buffer (Thermo Fisher Scientific) in the presence of protease inhibitor cocktail (Calbiochem, Catalog #539,137). Protein concentration was measured by the BCA protein assay kit (Thermo Fisher Scientific). When DNA transfection was performed, HEK293 cells cultured in 6-well plates were transfected with pEGFP-C1 (Clontech, 2 µg/well), pLNCX chick v-src (a gift from Joan Brugge, Addgene plasmid #14,578, 2 µg/well), or pAPTAG5 (GenHunter, 2 µg/well) pre-mixed with Avalanche®-Omni Transfection Reagent (EZ Biosystems). Forty-eight hours later, cell lysates were prepared as described above for the detection of exogenous GFP expression and tyrosine phosphorylation, and the conditioned media were harvested for the detection of secreted alkaline phosphatase. To detect Erk 1/2 phosphorylation, HeLa cells were serum starved for 20 h and treated with 500 nM phorbol 12-myristate 13-acetate (PMA, Sigma) for 30 min. Cell lysates were prepared with 2 × SDS sample buffer (5) and protein concentration was measured by the BCA protein assay kit. The amount of secreted alkaline phosphatase (AP) was measured in a microplate reader (SpectraMax190, Molecular Device) at 37°C under kinetics mode with BluePhos substrate (KPL) (6).

SDS-PAGE and electrophoretic transfer

Cell lysates and conditioned media were treated with SDS Laemmli sample buffer at 95°C for 10 min and separated by SDS-PAGE (10% or 13% acrylamide) under reducing conditions (5). All gels were run as 10- or 12-well, 1.0-mm-thick, tris-glycine minigels on an XCell SureLock® Mini-Cell system (Thermo Fisher Scientific). Electrophoretic protein transfer to Immobilon®-P Polyvinylidene difluoride (PVDF) membranes (Millipore) was done with a semi-dry blotting system (ATTO, Japan) using 25 mM Tris/192 mM glycine/20% methanol.

Slot blots

Slot blots were performed with The Convertible™ Filtration manifold System (Thermo Fisher). Serial dilutions of the conditioned media containing AP-(His)₆ derived from HEK293 cell transfection were loaded onto PVDF membranes under vacuum. The membranes were completely dried and kept at room temperature. Before the blocking step (see below), the membranes were soaked in methanol for 1 min, followed by rinsing in MilliQ water for 10 min three times.

Immunoblot: membrane blocking

After transfer, PVDF membranes were incubated with 10% skim milk in PBS containing 0.1% Tween20 (PBS-T) for 1 h at room temperature otherwise stated. For the detection of tyrosine-phosphorylation, 5% BSA in 10 mM Tris-buffered saline (pH 7.4) containing 0.1% Tween20 (TBS-T) was used for blocking. Odyssey Blocking Buffer (PBS, LI-COR) was also used for fluorescence immunoblot detection.

Immunoblot: antibody probing

Primary and secondary antibodies were diluted in either 5% skim milk in PBS-T, 10% Can Get Signal®-1 Immunoenhancer Solution (CGS-1, TOYOBO), Odyssey Blocking Buffer containing 0.1% Tween20 (ODS-T) for the primary and Can Get Signal®-2 (CGS-2) for the secondary antibodies. The composition of CGS is proprietary, but the MSDS reports that it contains Casein (0.02% or less), NaCl (2.0% or less), NaH₂PO₄ (0.3% or less), isothiazoline-based compound (0.001% or less) and non-ionic surfactant (0.01% or less). Other ingredients have not been disclosed.

Static method

Membranes were incubated with diluted antibodies on sheet of parafilm for an indicated time.

CDR method

The PVDF membranes were inserted into tubes such that they adhered to the wall to place the blot protein side towards the centre of the tube. The tube was rotated horizontally in a hybridization oven (HYBAID Micro-4) such that solution washed continuously over the membrane surface at 6 rpm for the indicated times. The volume of the antibody solution was 3 ml for small membranes (2 × 8–4 × 8 cm) in Falcon™ 14 ml Round-Bottom Polypropylene Tube (Corning #352,059) and 8 ml for large membranes (4 × 8–8 × 8 cm) in Falcon™ 50 ml Conical Centrifuge Tubes (Corning #352,070).

Rocking method

Membranes were incubated with diluted antibodies (3 ml) in a container (11.5 cm × 8 cm) on an adjustable tilt rocker (Labnet) at 24 rpm for the indicated times.

Immunoblot: membrane washing

Conventional washing

After incubation with antibodies, membranes were rinsed with PBS-T (50 ml) in a container (11.5 cm × 8 cm) with agitation for 10 min three times after primary antibody incubation and for 5 min six times after secondary antibody incubation.

Rapid washing

Membranes were briefly rinsed with PBS-T in a container to remove the majority of the antibody, followed by a quick but thorough wash in an OXO Salad Spinner with 250 ml of PBS-T twice. When phosphorylation was examined, TBS-T was used.

Immunoblot: chemiluminescence detection

Bound antibodies were visualized with myECL™ Imager (Thermo Fisher) using LumiGLO® Peroxidase Chemiluminescent Substrate Kit (KPL). Non-specific binding of the antibodies to the PVDF membrane was judged by the presence of signals on the pre-stained protein standard (New England BioLabs, P7712S). When PVDF membranes were re-probed, bound antibodies were removed by Restore™ Western Blot Stripping Buffer (Thermo Fisher Scientific) following the manufacturer's protocol.

Immunoblot: fluorescence detection

Bound antibodies were visualized with an Azure c600 Imaging System (Azure Biosystems). Raw 16-bit TIF images were analysed by Image Studio™ Lite for quantitation.

Preparation of GFP-(His)₆ protein

An AgeI/HindIII digest of pEGFP-C1 fragment was subcloned into XmaI/HindIII sites of pQE30 (Qiagen). (His)₆-tagged EGFP protein was expressed in XL1 Blue *Escherichia coli* cells (0.5 mM IPTG for 16 h) and purified by TALON® Resin (Clontech), followed by dialysis against PBS at 4°C. Protein concentration was determined by the BCA protein assay kit.

Solid-phase binding assays

Solid-phase binding assays were performed in an ELISA format as previously described (6, 7). Briefly, Immulon® 4HBX plates (Thermo Fisher Scientific) were coated with purified (His)₆-tagged EGFP at 4°C for 24 h. After blocking with 5% BSA in PBS-T at 4°C for 16 h, anti-6X His tag antibody diluted with either 5% BSA in PBS-T or CGS-1 (30 µl/well) was incubated for 60 min at 22°C. After rinsing the plate with PBS-T 5 times, anti-rabbit IgG antibody conjugated with Horseradish peroxidase (1:8,000 dilution either 5% BSA in PBS-T or CGS-2) was incubated for 30 min at 22°C. HRP activity of the bound fraction was measured in a microplate reader (SpectraMax® 190, Molecular Devices) under kinetics mode with SureBlue Reserve™ TMB 1-Component Microwell Peroxidase Substrate (KPL). All experiments were performed in triplicate and data were expressed as Δmilli OD/min ± standard deviation. Dissociation constant (*K_d*) and maximal number of binding sites (*B_{max}*) were calculated using Prism6 (GraphPad).

When time course experiments were performed, a fixed concentration (3.33 nM) of anti-6X His tag antibody diluted with either 5% BSA in PBS-T or CGS-1 (30 µl/well) was incubated with purified (His)₆-tagged EGFP (1 mg/ml) immobilized on Immulon® 4HBX removable strips for the indicated periods. When the CDR method was used, Immulon® 4HBX removable strips containing 150 µl/well of the diluted antibody were sealed with films (Excel Scientific SealPlate® Films) and kept rotating at 6 rpm on HulaMixer® (Thermo Fisher). After rinsing the strips as mentioned above, HRP-conjugated secondary antibody diluted with either 5% BSA in PBS-T or CGS-2 was incubated for 30 min at 22°C in a static condition, followed by the measurement of HRP activity as mentioned above.

Results and Discussion

Immunoreaction enhancing technologies accelerate the antigen-antibody reaction, thereby improving the

Fig. 1. (A) Effect of CGS on the binding of anti-6X His tag antibody. Purified (His)₆-tagged EGFP protein (40 $\mu\text{g}/\text{ml}$) was immobilized and the binding of different concentrations of anti-6X His tag antibody under static conditions was measured. Inset, scatchard plot. Red, CGS; blue, BSA. (B) Enhanced and accelerated binding of anti-6X His tag antibody achieved by the combination of CGS and CDR. Purified (His)₆-tagged EGFP protein (1 mg/ml) was immobilized and the binding of anti-6X His tag antibody (3.3 nM) was monitored over time in the presence or absence of CGS. Red line, CGS; blue lines, BSA; solid lines, CDR method; dashed lines, static method.

signal-to-noise ratio. Among several commercially available reagents, Can Get Signal® (CGS) Solution was chosen for the current study. Solid-phase binding assays were performed with anti-6X His tag antibody in the presence or absence of CGS to characterize its effects (Fig. 1A). Scatchard plot analysis showed that CGS increased the affinity ($K_d = 2.23 \pm 0.31$ nM with CGS and $K_d = 11.23 \pm 1.07$ nM with BSA) while there was no substantial alteration in maximal binding ($B_{\text{max}} = 321.7 \pm 12.4$ with CGS and $B_{\text{max}} = 355.8 \pm 15.44$ with BSA). The proprietary composition or mechanism of action of CGS has not been disclosed, but our result indicates that increased antigen-antibody affinity is, at least in part, responsible for the enhancing effect of CGS under these conditions.

MTL is often observed when the density of the immobilized target is high and flow rate is low in kinetic SPR experiments (1). We used solid-phase binding assays to determine whether combining the CDR method with CGS showed an effect on binding kinetics where MTL was likely to be observed (Fig. 1B). CDR was performed by rotating the shielded ELISA plates containing anti-6X His tag antibody for the indicated times. In all conditions, binding reached a plateau by 60 min. Using BSA as a diluent, CDR reached the maximal binding achieved by the static method after only 15 min. A comparable acceleration was observed with CGS as the diluent. The combination of CDR and CGS reached the maximal binding achieved by static incubation with BSA after only 5 min. This observation is intriguing. MTL depends on the total number of surface sites in SPR, and it is known that the total surface density of immobilized targets strongly influences the functional distribution

Fig. 2. Optimization and validation of immunoblot solutions with chemiluminescence detection. (A) Different amounts of cell lysates (1:2 serial dilutions from 8.8 $\mu\text{g}/\text{lane}$) were separated by SDS-PAGE, followed by immunoblot with mouse anti- β -actin antibody (1:3,000 dilution). (B) Conditioned media containing the secreted (His)₆ tagged AP (8×10^{-14} mol/lane) was separated, followed by transfer to PVDF membranes. Each membrane was subjected to immunoblot 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.

of binding sites (8). Thus, CDR may alter the binding properties between soluble antibody and immobilized antigen. Having consistently demonstrated a drastic reduction of the incubation period without any loss in sensitivity, we sought to use this method to improve the efficiency of the immunoblotting technique.

CDR was performed in a hybridization oven by rotating tubes that contained PVDF membranes and the antibody solution ([Supplementary Movie S1](#)). We first compared the amounts of antigen and antibody, respectively, necessary for chemiluminescence detection on immunoblots using CDR and CGS. In both static and CDR incubations, a 2- and 4-fold increase in sensitivity was found for the antigen: the minimum amount of cell lysates required to detect β -actin was reduced by CGS from 2.2 to 1.1 μ g under static conditions and further reduced from 1.1 to 0.275 μ g using CDR ([Fig. 2A](#)). CGS also permitted a higher dilution of the primary antibody: the minimum concentration of anti-6X His tag antibody was reduced from 100 to 50 ng/ml under static conditions and from 100 to 12.5 ng/ml under CDR condition ([Fig. 2B](#)). Further, CDR incubation for either 5 min or 10 min efficiently

lowered the detection limit compared to static incubation (60 min with primary and 30 min with secondary antibodies) with both CGS and skim milk as diluents. Finally, the combination of CDR with CGS showed greater sensitivity on immunoblots (0.275 μ g of cell lysates for β -actin detection and 12.5 ng/ml of anti-6X His tag antibody) even within this remarkably short incubation period.

To further confirm that the enhancement is due to the CDR and CGS solutions rather than simply the inclusion of the CGS solution, we performed slot blot analysis ([Supplementary Fig. S2](#)) with the same antibody concentrations (100 ng/ml of anti-6X His tag antibody and 1:16,000 dilution of anti-rabbit IgG antibody-HP conjugated) and the same incubation time (5 min) for both primary and secondary antibodies. Under static conditions, the presence of CGS solutions enhanced the signals as compared to skim milk. When CGS solutions were used to dilute the antibodies, the CDR method boosted the signals substantially within such a short incubation period compared with the static incubation method. Consistent with the solid-phase binding data ([Fig. 1B](#)), we concluded that

Fig. 3. Application of enhanced chemiluminescence immunoblot to other common antibodies. (A) Detection of induced phosphorylation of Erk1/2 by PMA treatment. Cell lysates (20 μ g/lane) from HeLa cells treated with either DMSO or PMA were subjected to immunoblot with rabbit anti-phospho Erk1/2 antibody. The membranes 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 rabbit anti-Erk1/2 antibody. (B) Detection of exogenous expression of GFP. Cell lysates (20 μ g/lane) from 293 cells transfected with pEGFP were subjected to immunoblot with chicken anti-GFP antibody. The membranes 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- β -actin antibody. Source data for the re-probed blots are available in [Supplementary Fig. S7](#).

both CDR and CGS are crucial for the superior sensitivity. Notably, as reported by Li *et al.* (3), we found that the difference between static incubation and rocking agitation of the antibody solution was negligible, while CDR with CGS markedly enhanced the signals (Supplementary Fig. S3). Therefore, we consider the static condition to be equivalent to rocking agitation. Li *et al.* (3) also demonstrated that CDR can be performed at rotation rates of up to 20 rpm without loss of function in their ELISA system and that CDR performance degrades at higher rates, likely due to insufficient time provided for gravity-driven draining of solution.

We next demonstrated that this high-speed immunoblotting technique is generalizable by testing several other antigens and antibodies. Induced phosphorylation of Erk1/2 upon protein kinase C activation (Fig. 3A), induced tyrosine phosphorylation by v-src overexpression (Fig. 4A) and GFAP (glial fibrillary acidic protein) expression in primary cultured astrocytes (Fig. 4B) were readily detected in a short incubation period. More importantly, this accelerated and enhanced method still permits antibody stripping and re-probing of the membrane. Re-probing of total Erk1/2 and β -actin, respectively, was performed with 10 min incubation for primary and 5 min incubation for secondary antibodies. Moreover, we show this method is generalizable across species by testing primary antibodies raised in chicken, mouse and rabbit (Fig. 3A and B).

In addition to saving time, this technique can reduce costs by lowering the amount of antibody used because antibodies account for a significant proportion of the operational cost of immunoblot; in fact, repetitive use of the same antibody solution produces

consistent results (Supplementary Fig. S4). When the PVDF membrane was pre-washed before the incubation with the primary antibody in order to avoid the contamination of skim milk into CGS, the same antibody could be used, at least up to eight times without substantial reduction of detection. Usage of the diluted CGS solutions also contributes to a significant cost reduction.

The immunoblotting technique has become increasingly important for quantitative applications (9). Because of a wider dynamic range, fluorescence detection is the best option for quantitative comparisons between protein samples and for multiple protein detection. Thus, we sought to use this method for fluorescence immunoblot. We first compared the blocking reagents in fluorescence immunoblot (Supplementary Fig. S5). Since Odyssey® Blocking Buffer (ODS) in PBS showed a strong signal, we decided to use this reagent for blocking. Next, we determined the amount of anti-6X His tag antibody necessary for fluorescence detection (Supplementary Fig. S6). Use of CGS under CDR condition showed higher signals compared to ODS under static condition. We chose the concentration of 200 ng/ml for further studies with anti-6X His tag antibody.

To investigate antigen amounts and incubation time required for fluorescence detection, we employed the slot blot technique (Fig. 5A and B). Consistent with the data in Supplementary Fig. S6, CGS with CDR gave higher signals than ODS under static incubation at any incubation period. As the incubation times increased, the fluorescent signals were increased more effectively under CGS/CDR than ODS/static condition. More importantly, CGS with CDR displayed a wider linear dynamic range. Even with the enhanced signals with CDR and CGS, high linearity was maintained. A similar observation was obtained with lower amounts of antigen on the membrane (Fig. 5C). One hour incubation with CGS under CDR condition demonstrated better linearity than 16 h incubation with ODS under static condition. Thus, our protocol improves quantitative fluorescence-based immunoblot.

In contrast to chemiluminescence detection, fluorescence immunoblot has the unique advantage of allowing multiple targets to be assayed on the same blot at the same time without the need to strip and re-probe. Antibody pairs raised in different species are frequently multiplexed when using two-colour fluorescence detection. We applied our method to analyse multiple proteins. Simultaneous detection of AP-His and β -actin (Fig. 6A), and phosphor-Erk1/2 and Erk1/2 (Fig. 6B) were successful, but CDR incubation with CGS greatly accelerated their detection. Taken together, these findings demonstrate that the combination of CDR and CGS reliably improves immunoblotting speed and sensitivity with straightforward optimization to diverse assay conditions (Fig. 7).

Since the development of the immunoblotting method ~40 years ago (10, 11), many modified protocols and technologies have emerged to increase its speed and sensitivity (12, 13). Our protocol achieves this with several unique advantages. First, by lowering the detection limit with CGS, we reduce incubation

Fig. 4. Other applications of enhanced and accelerated chemiluminescence immunoblot. (A) Detection of tyrosine phosphorylation induced by overexpression of chicken v-src. Cell lysates (20 μ g/lane) from 293 cells transfected with pLNCX-v-src were separated by SDS-PAGE, followed by immunoblot with anti-phosphotyrosine 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- β -actin antibody. Source data for the re-probed blot are available in Supplementary Fig. S7. (B) Detection of GFAP. Cell lysates (10 μ g/lane) from primary cultured mouse astrocytes were subjected to immunoblot with anti-GFAP antibody. The blots were imaged as a single image and dotted line indicates the border of individual membranes. M, marker; L, lysates.

Fig. 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 16×10^{-14} mol/slot in duplicate) were loaded and the membranes were blocked at 4°C for 60 min with 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-T under static condition (bottom). After rapid washing, the membranes were incubated for 15 min with goat anti-rabbit IgG-IRDye 680RD (1:5,000 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. (C) Left: different amounts of the secreted (His)₆ tagged AP (1:2 serial dilutions from 8×10^{-14} mol/lane) were subjected to immunoblot. The membranes were incubated with anti-6X His tag antibody (200 ng/ml) diluted either in 10% CGS-1 under CDR condition for 1 h (top) or ODS-T under static condition for 16 h (bottom). After rapid washing, the membranes were incubated for 15 min with goat anti-rabbit IgG-IRDye 680RD (1:5,000 dilution) either in 10% CGS-2 under CDR condition (top) or ODS-T under static condition (bottom). Source data are available in [Supplementary Fig. S7](#). Right: quantitation of raw images was implemented and the fluorescent intensity was plotted.

time without sacrificing sensitivity. Reduced amounts of antigens and antibodies are sufficient to obtain equivalent signals to those with conventional methods (Fig. 2). Second, no special and expensive equipment is required ([Supplementary Movie S1](#)). Use of a hybridization oven (or 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 characterized. Our key innovation is to show that CDR removes the depletion layer in on-surface bioassays far more effectively than other, similar techniques such as rocking or shaking (Fig. 1 and [Supplementary Fig. S3](#)). Rinsing PVDF membranes in a household commercial salad spinner with a large volume of solution (rapid wash)

also reduces the washing time drastically ([Supplementary Movie S2](#)). Third, this procedure is applicable to stripping and re-probing under chemiluminescence detection. Fourth, this method can be directly applied to fluorescence immunoblot and a linear dynamic range is maintained for quantitative immunoblot analysis. Finally and again, this protocol shaves hours of the time to finish the entire immunoblotting procedure, which is almost equivalent to a period for mini-scale DNA plasmid preparation from liquid *E. coli* culture. Longer incubation with antibodies may increase the intensity of the signals; however, non-specific binding may be increased because no saturated binding up to 60 min was obtained in this immunoblotting protocol. Unsaturated binding of the

Fig. 6. Simultaneous detection of multiple targets in fluorescence immunoblot using CDR in conjunction with CGS. (A) Different amounts of lysates (1:2 serial dilutions from 10 µg/lane) from 293 cells transfected with pAPTAG5 were separated and transferred to PVDF membrane. The blot was probed with anti-6X His tag and anti-β-actin antibodies, followed by IRDye 800CW goat anti-rabbit IgG and IRDye 680RD goat anti-mouse IgG. Left: antibodies diluted with CGS under CDR condition; right: antibodies diluted with ODS-T under static condition. (B) Cell lysates (20 µg/lane) from HeLa cells treated with either DMSO or PMA were subjected to fluorescence immunoblot with rabbit anti-phospho Erk1/2 antibody and mouse anti-Erk1/2 antibody. Source data are available in [Supplementary Fig. S7](#).

Fig. 7. Schematic illustration of an enhanced and accelerated CDR immunoblot compared to a traditional static method. An immunoblot that utilizes the static method requires at least 150 min to obtain result. Whereas, the equivalent image can be achieved with the CDR method in as early as in 20 min.

antibody to its immobilized antigen could be due to complications relating to the PVDF membrane, such as the presence of pores and its thickness.

Immunoblot has diverse applications and remains a ubiquitous tool to investigate protein abundance, kinase activity, protein-protein interactions and post-translational modifications. Implementation of this simple protocol could impact many fields, slashing waiting times not only for experiments but also for clinical diagnosis that rely on immunoblotting technology.

Supplementary Data

[Supplementary Data](#) are available at *JB* Online.

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Conflict of Interest

The authors declare no conflict of interest.

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