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Research Article

Novel chemiluminescent Western blot blocking and antibody incubation solution for enhanced antibody-antigen interaction and increased specificity

Western blotting is a ubiquitous tool used in protein and molecular biology research, providing information about the presence, size, relative abundance, and state of a protein in a mixture. First, the proteins in a sample are separated by size using SDS-PAGE then transferred onto a membrane for detection with a set of primary and secondary antibodies. High-quality Western data requires high signal-to-noise ratios, which depend upon reduction of nonspecific antibody interactions. Blocking is a critical step in the Western blot method as it prevents the antibodies from binding nonspecifically to the membrane and irrelevant proteins. A solution of nonfat dry milk (NFDM) in physiological buffer is commonly used for this purpose, but does not perform well with every type of antibody and is not optimal for low-abundance proteins. We present a novel blocking solution for chemiluminescent Western blots, AdvanBlock™-chemi, which outperforms NFDM in experiments with 20 unique antibodies by increasing signal-to-noise ratios and minimizing nonspecific binding. This solution enhances protein detection by Western blot and provides consistent results for detection of low abundant and modified proteins.

Keywords:
Blocking solution / Immunoblotting / Nonfat dry milk / Protein research / Western blot
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1 Introduction

Western blotting, or immunoblotting, is a widespread biochemical technique used to evaluate individual proteins. Mixtures of proteins or whole cell lysates are first separated by a SDS-PAGE. The proteins are then transferred from the gel to a protein-adsorbing membrane of nitrocellulose or PVDF [1]. Separation of proteins by SDS-PAGE is largely dependent on molecular weight, which is preserved after the proteins have been transferred to a membrane. Once the proteins are immobilized onto a membrane, the proteins are then detected with primary followed by secondary antibody conjugates. Visualization is typically attained through a secondary antibody conjugated to an enzyme or a fluorescent molecule. Chemiluminescence detection is most commonly used, in which a horseradish peroxidase enzyme that is covalently linked to the antibody causes oxidation of luminol in the presence of hydrogen peroxide to produce light that can be imaged on *x*-ray film or digitally with a CCD camera [2]. Data generated by traditional Western blots come in the form of "bands" that can be used to determine the relative abundance of a protein, assess the effect of a chemical treatment on cells, reveal protein cleavage patterns, and evaluate PTMs. Advances in proteomics have led researchers to further characterize proteins using Immunoproteomic, 2D Immunoblotting, and Far Western approaches [3–5]. Clear, measurable bands depend on a number of factors: the primary antibody must interact in a stable manor with its target protein; nonspecific interactions must be minimized between antibodies and the membrane as well as the irrelevant proteins in the sample. Initial optimization efforts are typically focused on determining the appropriate amount of sample to load on the gel and concentration of primary and secondary antibodies to use; then, there is optimization of salt and detergent concentration, evaluation of interfering substances and other factors that can impact protein–protein interactions, e.g. temperature, incubation time, etc. Importantly, optimal conditions can differ for each antibody.

To reduce background signal and nonspecific interactions, the blot is blocked before probing with antibodies. Since membranes are designed to bind protein, antibodies can easily adhere to the exposed membrane. A blocking solution contains molecules that coat the exposed membrane as

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Abbreviations: **BCA**, bicinchoninic acid assay; **CCD Camera**, charge coupled device camera; **NFDM**, nonfat dry milk; **PVP**, polyvinylpyrrolidone; **RIPA**, radioimmunoprecipitation assay buffer

Colour Online: See the article online to view Fig. 5 in colour.

well as reduce binding to the "sticky" areas of proteins where antibodies may adhere nonspecifically. Various proteins are utilized to prevent nonspecific interactions, but the most ubiquitous and cost-effective reagent is nonfat dry milk (NFDM). Dehydrated NFDM is composed of a diverse mixture of proteins and sugars that is reconstituted in a physiological buffer prior to use, providing a convenient, and economical blocking solution [6]. Other common blocking reagents include BSA, normal serum, purified casein, fish gelatin, and protein-free solutions consisting of water-soluble polymers. Each of these has advantages and disadvantages that depend on the experiment. In the case of NFDM, the powder must be fully dissolved to prevent artifacts, it is subject to spoiling quickly, and it can lead to elevated background with phospho-specific antibodies due to the presence of phosphorylated proteins. Similar cross-reactivity issues can arise with the alternative blocking reagents, as well as practical and economic considerations [7].

AdvanBlock™-chemi is a novel blocking solution that can be used effectively with a variety of samples and antibody types. AdvanBlock™-chemi successfully prevents general background, and most notably, it yields enhanced specific signal and increased signal-to-noise ratios relative to blocking with NFDM. The superior performance of AdvanBlock™-chemi was demonstrated with a panel of 20 unique antibodies, two solid support membranes, and three target protein sources (see Table 1).

2 Materials and methods

2.1 Cell lysate preparation

HeLa cells were purchased from ATCC and cultured in Eagle's Minimum Essential Medium supplemented with FBS, penicillin and streptomycin. Once the cells reached 80% confluency they were washed with 1X PBS then lysed with ice cold 1X RIPA buffer containing protease and phosphatase inhibitors. The cells were scraped from the flask and placed in a chilled centrifuge tube on ice for 30 min. The cells were centrifuged at 4600 rpm for 20 min at 4°C. The supernatant was carefully removed and the concentration was measured using the bicinchoninic acid assay (BCA). The supernatant was diluted to 2 mg/mL with 1X RIPA buffer, then an equal volume of 2X Laemmli buffer was added before they were aliquoted into fresh tubes for storage. Jurkat cell lysate at 1 mg/mL was purchased from EMD Millipore (Catalog #: 12–303).

2.2 Protein extraction from rat and mouse tissues

To one gram of each tissue, 3 mL of ice cold 1X RIPA buffer containing protease and phosphatase inhibitors was added. The samples were sonicated for 30 s on ice then allowed to cool on ice for 30 s. The sonication/cooling cycle was repeated three times. The samples were centrifuged at 4600 rpm for 20 min at 4°C. The concentration of the tissue lysates was determined by BCA and the samples were each diluted to 1 mg/mL with Laemmli sample buffer.

2.3 Protein extraction from urine

To extract proteins from urine samples, the Afyon™ SDS-PAGE sample preparation kit (Advansta, Inc) was used. Proteins were extracted from 1 mL of urine with 20 μL of Afyon™ resin. After addition of the Afyon™ resin to the urine, the samples were vortexed for 30 s then centrifuged to pellet the resin. The supernatant was removed and discarded. Protein was eluted from the resin by adding 15 μL of Laemmli reducing sample buffer and vortexing for one minute. Each sample was then transferred to a 0.45 μm spin filter and centrifuged.

2.4 SDS-PAGE

Samples were diluted with Laemmli sample buffer and heated at 95°C for 5 min then cooled to ambient temperature prior to being loaded onto Bio-Rad Mini-PROTEAN® TGX™ gels. Electrophoresis was performed with the Bio-Rad Mini-PROTEAN® Tetra Cell at 200 V using 1X Tris-Glycine-SDS running buffer until the dye front reached the bottom of the gel.

2.5 Electroblotting

Proteins were transferred from the gel to membrane using the Bio-Rad TransBlot®-Turbo™ Transfer System and mini-PVDF or Nitrocellulose transfer packs for 7 min at 1.3 A constant and up to 25 V.

2.6 Advanstain Ponceau membrane staining

After the transfer step was complete the blots were rinsed with water for 10 min. Blots were then submerged in 10 mL of Advanstain Ponceau for 5 min on an orbital shaker. Excess stain was drained from the blots before they were placed in water to remove residual stain and reduce background.

2.7 Western blotting

Postelectroblotting, the membranes were blocked for one hour in the respective blocking buffers before incubation with primary antibody diluted in blocking solution. Incubation with the specific primary antibody was performed at 0.2 μg/mL diluted in the respective blocking buffer for one hour. The secondary antibodies were all diluted at 1:3000 in the respective blocking buffer and incubated for one hour. Membranes were washed after each antibody incubation

three times with 1X AdvanWash™ (Advansta, Inc.) for 5 min per wash. After the final washing step, membranes were incubated for 5 min with a working solution of WesternBright™ ECL substrate (Advansta, Inc) or Clarity™ Western ECL Substrate (Bio-Rad), then imaged with a FluorChem HD2 system (ProteinSimple).

2.8 Analysis of signal intensities

Signal intensities of the protein bands were measured using AlphaView software (ProteinSimple) by drawing a box around the band of interest and using the same sized box for each measured band. The average signal intensity of three randomly placed boxes was used to measure background signal, then the background was subtracted from the quantitation for the protein band of interest. To get the fold increases as displayed in Table 1, the signal from the highest loaded protein concentration (5 μg) was used, and the ratio was calculated for the signal from the AdvanBlock™-chemi Western blot over the signal using NFDM. For the urine samples, the displayed number is based on Donor 1.

3 Results and discussion

To compare the effectiveness of AdvanBlock™-chemi as a blocking solution relative to NFDM, Western blotting was performed with an array of antibodies diluted with either AdvanBlock™-chemi or NFDM and incubated on blots blocked with the respective buffer. A total of 20 unique antibodies were evaluated, of different specificities and clonalities from various vendors (see Table 1). Notably, in all cases Western blotting with AdvanBlock™-chemi led to an improvement in signal, ranging from very mild (1.2-fold higher) to drastically improved (20 to 100-fold higher) (Table 1, Fig. 1A). For the 27 conditions in Table 1, the median improvement in signal for AdvanBlock™-chemi was 4.1-fold greater than the blots processed with NFDM.

Depending on the experiment, Western blotting results can greatly differ between the two most commonly used membranes, PVDF, and nitrocellulose. Both materials provide a high-affinity binding surface for proteins, but depending on the experimental design the advantages and disadvantages of each should be taken into consideration. For this reason, the effectiveness of AdvanBlock™-chemi on both

Table 1. Summary for the 20 unique antibodies that were evaluated for fold increase in chemiluminescent signal using AdvanBlock™-Chemi versus NFDM

	Antibody	Manufacturer	Catalog number	Membrane material	Sample type	Host/Clonality	Fold increase*
1	STAT-1	Santa Cruz Bio	SC-346	Nitrocellulose	HeLa	Goat/pAb	1.5
2	STAT-1	Millipore	06-501	PVDF	HeLa	Rabbit/pAb	4.1
	STAT-1			Nitrocellulose	Jurkat		3.6
3	pSTAT-1	CST	7649	PVDF	HeLa	Rabbit/mAb	3.8
	pSTAT-1			Nitrocellulose	HeLa		4.9
4	STAT-3	CST	12640	PVDF	HeLa	Rabbit/mAb	3.5
	STAT-3			Nitrocellulose	HeLa		21.0
5	pSTAT-3	CST	9145	PVDF	HeLa	Rabbit/mAb	3.4
	pSTAT-3			Nitrocellulose	HeLa		10.6
6	ERK2	Genscript	A01194	PVDF	HeLa	Rabbit/pAb	17.6
	ERK2			Nitrocellulose	HeLa		4.6
7	Ikkα	Novus	NB100-56704	PVDF	HeLa	Mouse/mAb	5.9
8	TRF-2	Novus	NB100-56506	PVDF	HeLa	Mouse/mAb	20.7
9	GAPDH	Millipore	MAB374	PVDF	HeLa	Mouse/mAb	1.2
	GAPDH			Nitrocellulose	Jurkat		1.1
10	Actin	Millipore	MAB1501R	PVDF	HeLa	Mouse/mAb	2.1
	Actin			Nitrocellulose	Jurkat		104.1
11	Tubulin	Millipore	MAB5564	PVDF	HeLa	Mouse/mAb	3.5
12	Tubulin	Millipore	MAB1864	Nitrocellulose	Jurkat	Rabbit/mAb	12.4
13	Fibronectin	SAB	38073	Nitrocellulose	Urine	Mouse/mAb	4.2
14	Albumin	R&D	MAB1455	Nitrocellulose	Urine	Mouse/mAb	1.9
15	Cytokeratin18	Novus	NB500-306	Nitrocellulose	Urine	Mouse/mAb	6.9
16	Myoglobin	SAB	42039	Nitrocellulose	Urine	Mouse/mAb	2.7
17	Haptoglobin	SAB	23017	Nitrocellulose	Urine	Rabbit/pAb	8.6
18	Fibrinogen	SAB	22542	Nitrocellulose	Urine	Rabbit/pAb	1.2
19	Collagen I	Abcam	ab34710	Nitrocellulose	Urine	Rabbit/pAb	3.6
20	Collagen III	Abcam	ab7778	Nitrocellulose	Urine	Rabbit/pAb	4.5
						Median	**4.1**

* In the fold increase column, background corrected mean signal for each band was measured and then normalized to the background corrected mean signal of NFDM.

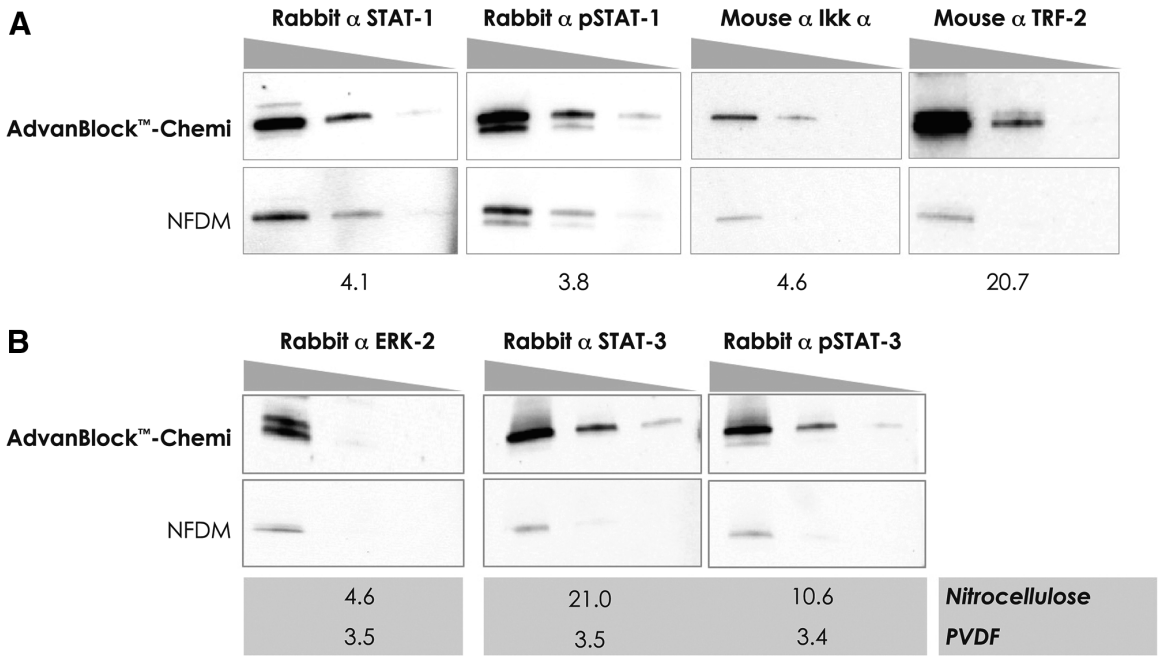

Figure 1. Western blotting with AdvanBlock™-chemi blocking buffer leads to enhanced signal compared to NFDM. (A) IFNα-treated HeLa whole cell lysate (5, 1, and 0.2 μg) was resolved, transferred to a PVDF membrane, and then Western blotted with either AdvanBlock™-chemi or NFDM blocking solutions using the indicated antibodies. Blots were treated identically with the exception of the blocking solution. Fold change is based on the highest concentration of lysate (5 μg). (B) AdvanBlock™-chemi enhances Western blot signal compared to NFDM on both PVDF and nitrocellulose membranes. IFNα-treated HeLa whole cell lysate (5, 1, and 0.2 μg) was resolved, transferred to a PVDF or nitrocellulose membrane, and then Western blotted with either AdvanBlock™-chemi or NFDM blocking solutions using the indicated antibodies. Blots were treated identically with the exception of the blocking solution. Fold change is based on the highest concentration of lysate (5 μg). Under the blots, the fold increase of signal using AdvanBlock™-chemi compared to NFDM is listed (gray box) for the blotted membranes of nitrocellulose (pictured) and PVDF (not pictured).

PVDF and nitrocellulose membranes was evaluated with the same set of antibodies (Fig. 1B). For both types of membranes AdvanBlock™-chemi demonstrated enhanced signal compared to NFDM.

Western blotting can be used to assay proteins from a variety of sources, including whole cell lysates, fractions from protein purification procedures, extracts from biological fluids, etc. Each sample has different excipients and the potential for nonspecific interactions with the antibodies used for probing. The solutions used for the Western blotting procedure must be chosen carefully based on the sample type and probing antibodies. The efficacy of AdvanBlock™-chemi for reducing background and improving signal more efficiently than NFDM with different sample types was evaluated by comparing Western blots of whole cell lysates from HeLa and Jurkat cell lines, protein extracted from urine (Fig. 2) and tissue lysates prepared from various mouse and rat organs (Fig. 3). For all antibodies tested, blocking with AdvanBlock™-chemi led to improved Western blots when compared to blocking with NFDM.

In addition to improving signal-to-noise ratios relative to traditional blocking solutions, AdvanBlock™-chemi also reduces signal from nonspecific binding of antibodies to other proteins in the sample. For example, a phospho-Stat3 (Tyr705) rabbit mAb known to detect endogenous levels of Stat3 only when phosphorylated at tyrosine 705 was used for comparison. This antibody does not cross-react with phospho-EGFR or the corresponding phospho-tyrosines of other Stat proteins. Use of this anti-phospho-STAT3 antibody led to nonspecific bands on the Western blot when traditional blocking buffers were used. Nonspecific bands were greatly reduced with AdvanBlock™-chemi blocking solution (Fig. 4A). After the transfer step was complete, the blots were stained with Advanstain Ponceau to demonstrate that equivalent levels of protein were blotted on each membrane (Fig. 4B).

The bioscience community is currently suffering from a reproducibility crisis when it comes to Western blot data consistency and lab-to-lab reproducibility. The use of low cost reagents with inherent variability such as NFDM and antibodies with high nonspecific binding has contributed to this problem. AdvanBlock™-Chemi was developed as a tool to address this issue as it provides a consistent means of blocking nonspecific binding while enhancing specific antibody-antigen interactions. AdvanBlock™-Chemi is comprised of a proprietary blend of an artificially processed carbohydrate, a nonionic detergent, a modified PEG, an anti-microbial agent and a processed and denatured mammalian protein component. In order to demonstrate technical reproducibility, five sets of serially diluted IFNα treated HeLa lysate samples were analyzed (Fig. 5). The variability was low as demonstrated by

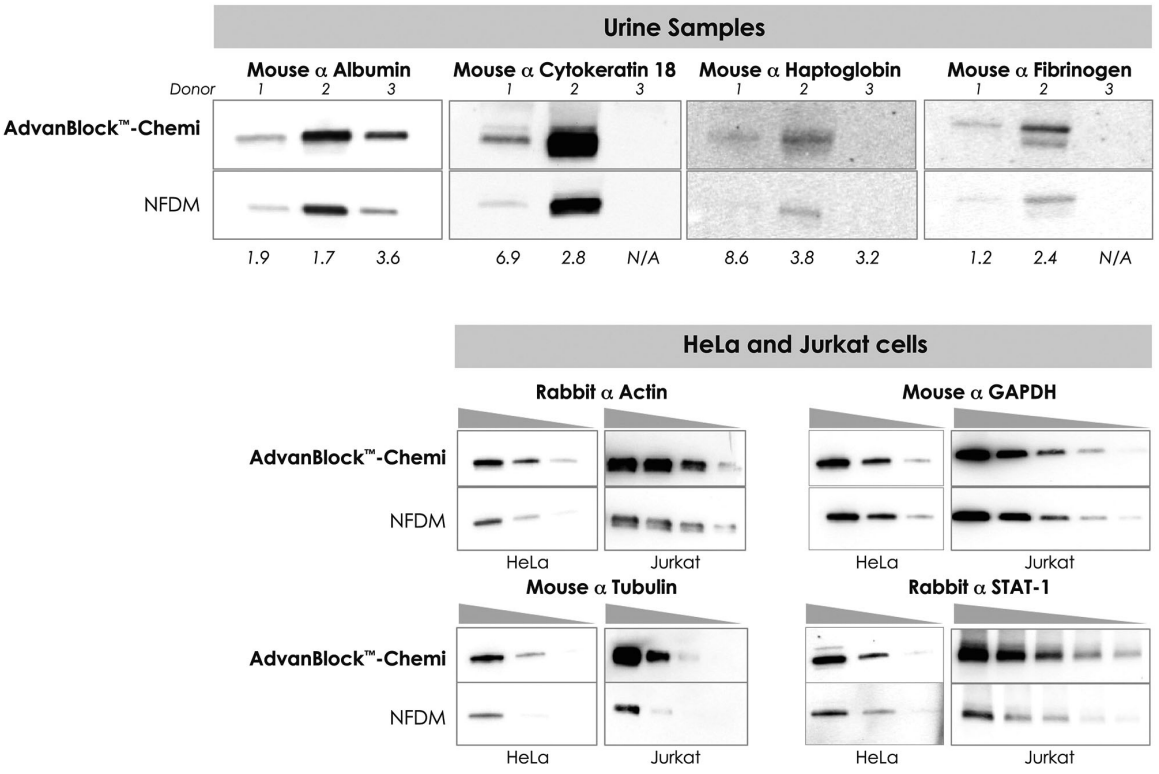

Figure 2. AdvanBlock™-Chemi is compatible with samples collected from diverse sources. Western blot data is improved with AdvanBlock™-chemi compared to NFDM for various samples including protein extracts from three unique urine samples purified with Afyon™ SDS-PAGE Sample Preparation Kit and whole cell lysates from Jurkat (0.5, 0.25, 0.125, 0.06, and 0.03 μg) and HeLa cells (5, 1, and 0.2 μg). The proteins from urine as well as Jurkat whole cell lysate were transferred onto nitrocellulose membranes, while the HeLa whole cell lysate was transferred onto PVDF. The Westerns were performed as described above with either AdvanBlock™-chemi or NFDM blocking solutions using the indicated antibodies.

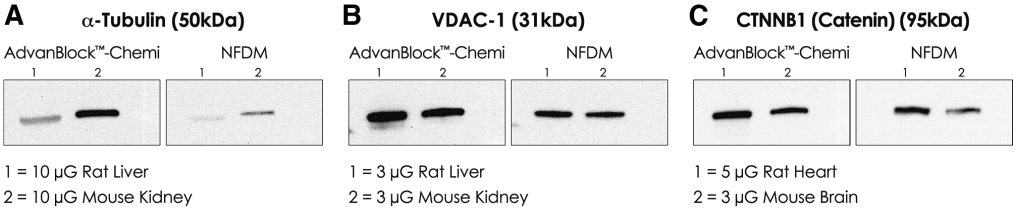

Figure 3. AdvanBlock™-Chemi is compatible with proteins extracted from various mouse and rat tissue lysates. (A) A 10 μg rat liver and 10 μg mouse kidney lysate was loaded on SDS-PAGE and probed with an anti-α Tubulin primary antibody (Boster #MA1107)). (B) A 3 μg rat liver and 3 μg mouse kidney lysate was loaded on SDS-PAGE and probed with an anti-VDAC-1 primary antibody (Boster #PA1780). (C) A 5 μg rat heart and 3 μg mouse brain lysate was loaded on SDS-PAGE and probed with an anti-Catenin primary antibody (Boster #PA1212).

% CVs less than 15% for each band detected, even at the lower limit of detection (Table 2).

Compared to NFDM, AdvanBlock™-chemi demonstrated superior signal-to-noise ratio in 27 unique antibody-antigen, sample source, and membrane combinations, including HeLa and Jurkat whole cell lysates, protein extracted from urine, PVDF, and nitrocellulose membranes, antibodies that recognize housekeeping proteins, low-abundance proteins, and phosphorylated proteins (Table 1). This improvement, across such variation in conditions, can be confidently extrapolated to the majority of Western blotting conditions for the average researcher. Not only is nonspecific background signal decreased, the specific signal is generally enhanced through stabilization of the antibody-antigen complex.

Aside from selecting a high-quality primary antibody and optimizing the sample load and antibody concentrations, the blocking agent is one of the most important variables to consider in devising a Western blot protocol. NFDM in physiological buffer is commonly used because it is convenient and economical; however, the perishable and inconsistent nature of NFDM solution means it often falls short when it comes to detecting low-abundance or posttranslationally modified antigens.

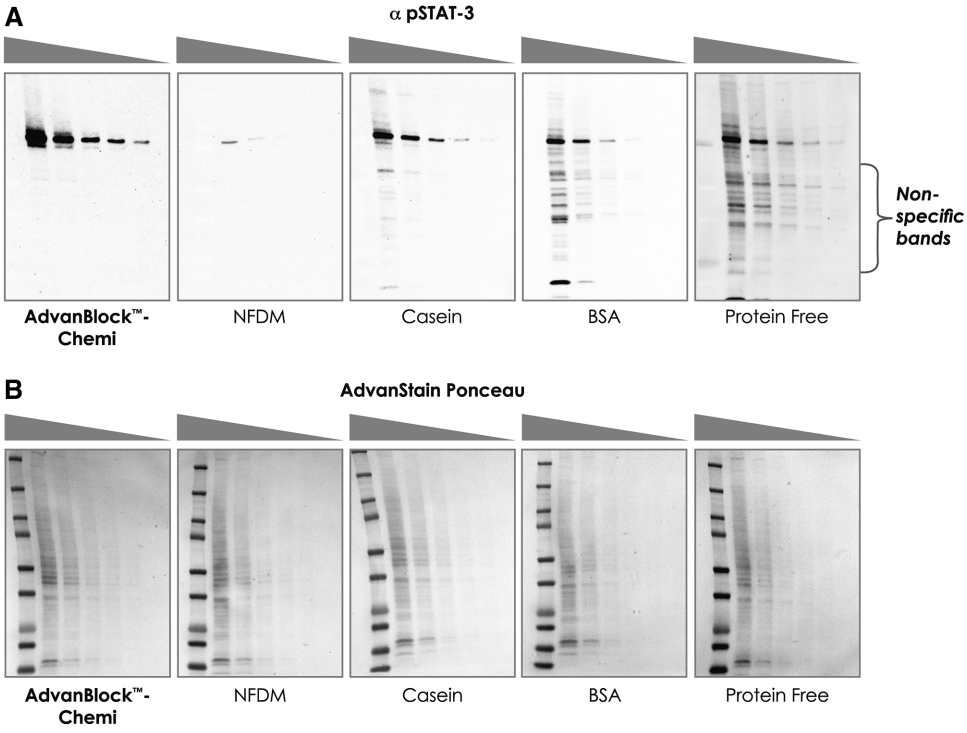

Figure 4. AdvanBlock™-Chemi outperforms common blocking solutions. (A) The appearance of nonspecific bands on the Western blot is reduced with AdvanBlock™-chemi, while the specific signal is enhanced compared to traditional buffers. IFNα-treated HeLa whole cell lysate (4, 2, 1, 0.5, and 0.25 μg) was subjected to SDS-PAGE followed by Western blotting with an anti-phospho-STAT3 polyclonal antibody. All conditions were identical with the exception of the blocking buffer (AdvanBlock™-Chemi, 5% NFDM-PBST, 1% Casein-PBST, 5% BSA-PBST, and Protein Free-PBST), which is indicated below the blot. Signal was detected with Clarity™ Western ECL substrate. (B) Blots stained with Advanstain Ponceau posttransfer.

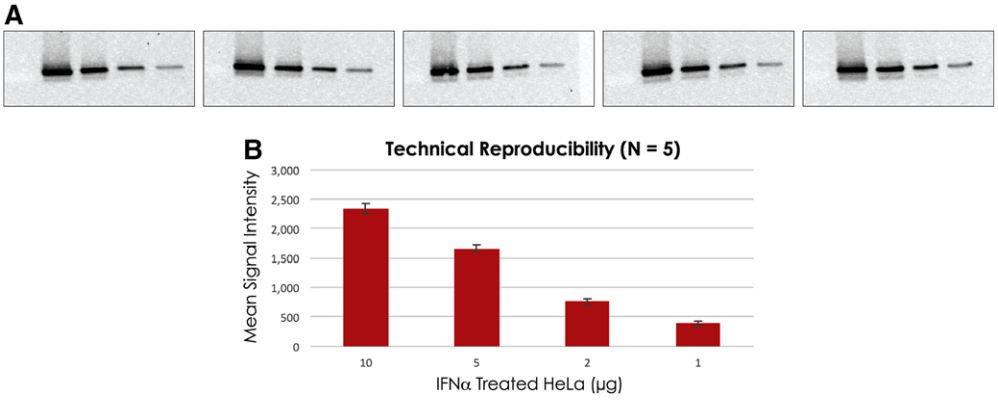

Figure 5. AdvanBlock™-Chemi demonstrates high technical reproducibility. (A) IFNα-treated HeLa whole cell lysate (10, 5, 2, and 1 μg) was subjected to SDS-PAGE followed by Western blotting with an anti-phospho-STAT3 polyclonal antibody. (B) Graphical representation of five technical replicates.

Table 2. Mean band signal intensity for five technical replicates of IFN alpha treated HeLa cell lysate (10, 5, 2 and 1ug) probed with an anti-phospho STAT3 polyclonal antibody

IFNα-HeLa (μg)	Blot 1	Blot 2	Blot 3	Blot 4	Blot 5	Mean	SD	%CV
10	2285	2242	2377	2471	2322	2340	89	4%
5	1618	1597	1725	1716	1665	1664	57	3%
2	680	794	756	732	815	755	53	7%
1	364	439	338	353	417	382	43	11%

4 Concluding remarks

For the most sensitive and reproducible results, AdvanBlock™-chemi blocking solution is the superior choice, as it provides consistently enhanced signal and increased signal-to-noise ratios. Because of the enhanced signal, the AdvanBlock™-chemi blocking solution allows more information to be ascertained from a single Western blot while conserving precious sample and expensive primary antibodies.

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The authors declare that Advanblock™-Chemi is a commercial product sold and distributed by Advansta Inc. Both authors are employed by Advansta Inc.

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