Chapter 5

Lysis Buffer Choices Are Key Considerations to Ensure Effective Sample Solubilization for Protein Electrophoresis

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Abstract

The efficient extraction of proteins of interest from cells and tissues can be challenging. Here we demonstrate the differences in extraction of the focal adhesion protein Kindlin-2 and the transcriptional repressor Snail from choriocarcinoma cells using NP-40 and RIPA lysis buffer. We also show the use of a more denaturing urea/thiourea lysis buffer for solubilization, by comparing its effectiveness with the often utilized RIPA lysis buffer for solubilization of heat shock proteins (HSP) B1 and B5 and the cytoplasmic adapter protein integrin-linked kinase (ILK) from smooth muscle. Overall, the results demonstrate the importance of optimizing lysis buffers for specific protein solubilization prior to finalizing the experimental workflow.

Key words NP-40 lysis buffer, RIPA lysis buffer, Urea/thiourea lysis buffer, Protein extraction, Protein solubilization, HSPB1, HSPB5, Kindlin-2, Integrin-linked kinase, Snail

1 Introduction

The appropriate extraction of proteins from cells and tissues can be fraught with challenges. For example, intracellular proteins may represent only a tiny fraction of total cellular protein, and therefore it would be difficult to extract these proteins in reasonable quantities without proper lysis and extraction protocols [1]. The hydrophobicity of membrane proteins can also be a significant stumbling block, particularly to 2D gel electrophoresis [2]. The advent of proteomics accelerated the development and modification of strategies for cell lysis as well as protein extraction at both small- and large-scale levels. The result is that in addition to more traditionally known cell and tissue lysis buffers [3, 4], there are now quite a number of commercially available reagents for use in such procedures [1]. Nonetheless, one must consider if a particular lysis buffer is optimally designed to extract the protein(s) of interest,

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particularly the use of the appropriate denaturants and detergent(s) for protein extraction and solubilization.

Several basic criteria are considered for the production of an effective lysis buffer for protein extraction: (1) pH, (2) ionic strength, (3) detergents and denaturants, and (4) constituents to improve protein stability or prevent proteolysis. Specific details on these parameters are described in detail elsewhere (*see* **Note 1**) [1, 5–8]. Although Gromov et al. [9] have reported the development of a lysis buffer that could be used for several different proteomic strategies, it has so far proven difficult to design a lysis buffer effective for isolation of a broad array of proteins from many subcellular compartments. This has necessitated the tailoring of buffers and protocols for individual use.

The small heat shock protein B (HSPB) family is comprised of 11 small molecular weight proteins (HSPB1–HSPB11; 15–40 kDa) that are key for cellular homeostasis and are induced by physiological stressors such as temperature, oxidative stress, and biophysical forces [10, 11]. These proteins have the ability to form low and high molecular mass (>300 kDa) complexes with other heat shock proteins and possess a dynamic quaternary structure [12–14]. In periods of stress, HSPB family members can bind denatured proteins and prevent their irreversible aggregation [15], thus aiding in the assembly, disassembly, stabilization, and internal transport of intracellular proteins [16]. Some HSPB members, such as HSPB1, can be found in high quantities (e.g., 2 mg per g of tissue protein) within specific tissues such as different types of muscle [15]. HSPB members like HSPB1 and HSPB5 also appear to interact with integrins and the actin cytoskeleton at focal adhesions and to be present in endosomes [10]. These characteristics and interactions present challenges to the effective extraction and solubilization of such proteins for SDS-PAGE and immunoblot analysis. Clearly, the determination of optimal lysis buffer conditions would be especially important if relative abundance of a specific protein(s) was going to be calculated between control and experimental conditions with downstream immunoblot analysis. Efficient immunoprecipitation of proteins could also be affected by suboptimal lysis conditions.

The use of more standard tissue lysis buffers such as NP-40 and RIPA lysis buffers is quite common for extraction of proteins from tissues, such as muscle, or established mammalian cell lines [17–22]. Using the focal adhesion protein Kindlin-2 and the transcriptional repressor Snail as examples, differences in extraction of these proteins from BeWo choriocarcinoma cells using NP-40 and RIPA lysis buffers were demonstrated. Tyson and colleagues [23] showed very effective extraction of small stress proteins from uterine smooth muscle with a urea/thiourea lysis buffer (*see* **Note 2**) and subsequent SDS-PAGE and immunoblot analysis. Thus, using stress proteins such as HSPB1 and HSPB5 and the

cytoplasmic adapter protein integrin-linked kinase (ILK) as examples, the ability of RIPA lysis buffer and urea/thiourea lysis buffer to solubilize such proteins from uterine smooth muscle was assessed. Overall, the data shown here demonstrate that lysis buffer choice should be evaluated prior to the establishment of an experimental workflow.

2 Materials

All aqueous solutions utilized ultrapure water (~18 MΩ/cm^2), and all reagents used were electrophoresis or analytical grade.

1. Phosphate-buffered saline: Dissolve 4 g NaCl, 0.1 g KCL, 0.72 g Na_2HPO_4, and 0.12 g KH_2PO_4 in 500 mL of ultrapure water, and adjust the pH to 7.4 with HCl. Filter sterilize and store at room temperature.
2. Modified RIPA lysis buffer: 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% (v/v) Triton X-100, 1% (w/v) sodium deoxycholate, and 0.1% (w/v) SDS. Dissolve 3.02 g Tris base, 4.38 g NaCl, 5 g deoxycholic acid, and 0.5 g SDS in 400 mL of ultrapure water. Add 5 mL Triton X-100; mix and bring up the volume to 500 mL following adjustment of pH to 7.5 with HCl. Filter sterilize and store at 4 °C (*see* **Note 3**).
3. NP-40 lysis buffer: 50 mM Tris (pH 8.0), 150 mM NaCl, 1% Nonidet P-40 [3]. Dissolve 0.88 g Tris base and 0.61 g NaCl in 80 mL of ultrapure water. Add 10 mL NP-40 detergent; mix and bring up the volume to 100 mL following adjustment of pH to 8.0 with HCl (*see* **Note 3**).
4. Urea/thiourea lysis buffer: 7 M urea, 2 M thiourea, and 4% (w/v) CHAPS, in 30 mM Tris–HCl solution (pH 8.5) [23]. Combine 4.2 g urea, 1.52 g thiourea, and 0.4 g CHAPS in 10 mL of 30 mM Tris–HCl (pH 8.5). Dissolve one tablet each of Mini EDTA-free protease and PhosSTOP phosphatase inhibitors (Roche Applied Science, Indianapolis, IN, USA), and store the buffer in 1 mL aliquots at −80 °C (*see* **Note 4**).
5. 1.5 M Tris–HCl resolving gel buffer: Dissolve 18.2 g of Tris base in ultrapure water to a final volume of 100 mL following adjustment of pH to 8.8 with HCl. Store at 4 °C.
6. 0.5 M Tris–HCl stacking gel buffer: Dissolve 6.1 g Tris base in ultrapure water to a final volume of 100 mL following adjustment of pH to 6.8 using HCl. Store at 4 °C.
7. SDS-PAGE 12% resolving gel composition: Combine 6 mL 30% acrylamide mix (29:1), 3.8 mL 1.5 M Tris-Cl (pH 8.8), 0.15 mL 10% SDS, 0.15 mL freshly made 10% ammonium

persulfate (w/v), and 0.006 mL TEMED in ultrapure water to a final volume of 15 mL.

8. SDS-PAGE 4% stacking gel composition: Combine 1 mL 30% acrylamide mix (29:1), 2 mL 0.5 M Tris–HCl (pH 6.8), 0.08 mL 10% SDS, 0.04 mL freshly prepared 10% ammonium persulfate, and 0.008 mL TEMED in ultrapure water to a final volume of 8 mL.
9. SDS-PAGE running buffer (5×): Dissolve 15.1 g Tris base and 94 g glycine in ultrapure water. Add 50 mL 10% SDS and adjust volume to 1 L in ultrapure water. Refrigerate until use. Dilute to 1× with ultrapure water when required.
10. SDS-PAGE loading dye (2×): Mix 2 mL 0.5 M Tris–HCl (pH 6.8), 4 mL 10% SDS, 2 mL glycerol, 1 mL 2-mercaptoethanol, and 0.02 g bromophenol blue. Adjust volume to 10 mL with ultrapure water. Aliquot and store at −20 °C.
11. Transfer membranes: 0.22 μm nitrocellulose membranes.
12. Gel transfer buffer: Dissolve 2.9 g glycine, 5.8 g Tris base, and 0.37 g SDS in 800 mL of ultrapure water, and then add 200 mL of methanol for a final volume of 1 L.
13. TBST: Dissolve 2.42 g Tris base and 8.0 g NaCl in ultrapure water, and adjust the pH to 7.6 with HCl. Add 1.0 mL of Tween-20 and bring the volume to 1 L. Buffer can be stored in the refrigerator.
14. Immunoblot blocking buffer: Dissolve 5 g fat-free skim milk powder in TBST buffer, and mix by vigorous shaking. For antisera requiring bovine serum albumin (BSA) for blocking, dissolve 5 g BSA (Fraction V) in 100 mL TBST buffer and mix vigorously.
15. Colloidal blue protein stain: Colloidal Blue Staining Kit.
16. Electrophoresis and transfer system: Mini-PROTEAN 3 system.
17. Whatman paper: 3 mm chromatography paper.
18. Molecular weight protein markers: Precision Plus All Blue Protein Standards.
19. Pierce Reversible Protein Stain Kit for nitrocellulose membranes.
20. Antisera: Rabbit polyclonal anti-Kindlin-2 (Sigma-Aldrich Chemical Co., St. Louis, MO, USA), anti-GAPDH (Abcam, Inc., Cambridge, MA, USA), anti-ILK (Cell Signaling Technology, Inc., Danvers, MA, USA), anti-HSPB1 (EMD-Millipore, Etobicoke, ON, Canada), anti-HSPB5 (Enzo Life Sciences, Farmingdale, NY, USA), or mouse monoclonal anti-Snail (Cell Signaling Technology).

21. Tissue: Rat uterine smooth muscle tissue from d23 of pregnancy.
22. Cells: BeWo choriocarcinoma cells (American Type Culture Collection, Manassas, VA, USA).

3 Methods

3.1 Tissue Collection

Tissue samples should be isolated quickly and placed in ice-cooled PBS for rapid washing. Subsequently, place tissues in polypropylene vials, and freeze in liquid nitrogen (*see* **Note 5**).

3.2 Protein Solubilization

1. For BeWo cells, wash cells with PBS, then add 0.2 mL of lysis buffer to the cells, and collect with a plastic cell scraper. Place the mixture in a pre-chilled 0.5 mL lysis tube (Precellys CK14 lysis kit) containing ceramic beads and homogenize cells with a Minilys Bead Mill using a 10 s burst. Centrifuge the sample at full speed for 15 min in a microcentrifuge, and collect supernatants for protein analysis.
2. For uterine smooth muscle samples, chip off pieces of frozen tissues, weigh the fragments (~100–250 mg) in pre-cooled weigh boats, and place in a pre-cooled mortar on dry ice. Grind the samples into a fine powder with a pestle under liquid nitrogen. The use of a fume hood is suggested to avoid inhalation of vapors from the liquid nitrogen. Transfer the powdered samples to 1 mL urea/thiourea lysis buffer (*see* **Notes 2** and **4**) or 1 mL RIPA lysis buffer in 15 mL polypropylene tubes and homogenize up to 1 min on ice with a Polytron PT10–35 homogenizer (*see* **Note 6**).
3. For samples homogenized in urea/thiourea lysis buffer, allow the lysates to settle at room temperature for 30 min while RIPA tissue lysates are kept on ice. Subsequently, transfer all sample lysates to appropriately labeled microcentrifuge tubes, centrifuge at full speed for 15 min in a microcentrifuge, and collect supernatants for protein analysis.
4. Determine sample protein concentrations using the Bradford assay [24].

3.3 SDS–PAGE and Electroblotting

1. Prepare a polyacrylamide gel casting module according to the instructions provided by the appropriate manufacturer (*see* **Note** 7).
2. Immediately after the addition of TEMED to the resolving gel mixture, add the mixture to the prepared gel cassette with a Pasteur pipet (*see* **Notes 8** and **9**). Add isopropanol over the top of the resolving gel to ensure that gel polymerization is not inhibited. After 45 min, remove the overlaid isopropanol by

tipping the gel molds to pour off the solvent, and soak up residual isopropanol with Kimwipes. Pour the stacking gel in the same manner as the resolving gel. Insert the appropriate gel comb into the stacking gels ensuring that no air bubbles are trapped under the teeth of the comb.

3. Once gel polymerization is complete and the gel is assembled in the electrophoresis tank, incubate protein samples (e.g., volumes equivalent to 20 μg protein) with equal volumes of 2× SDS-PAGE loading dye at 95 °C for 5 min prior to gel loading (*see* **Note 10**). Run the gel at 50 V until samples and the prestained molecular mass standards enter the resolving gel; then separate proteins at 100 V until the dye front reaches the bottom of the gel (*see* **Note 11**).
4. Following electrophoresis, gently pry open the gel plates with a plastic wedge to recover the gel.
5. To help assess the effective solubilization of sample proteins with the different lysis buffers, stain the polyacrylamide gel with a Colloidal Blue Staining Kit according to the manufacturer's instructions. Photograph the gel with a gel documentation system.
6. For immunoblot analysis skip **step 5** and place the gel in transfer buffer. Cut a nitrocellulose membrane to the same size as the gel, and also place it in transfer buffer. Assemble the gel for electroblotting as has been described in detail elsewhere [25, 26] (*see* **Note 12**), and conduct electroblotting for 1 h at 300 mA in transfer buffer with constant buffer stirring at 4 °C.
7. To help assess effective solubilization and transfer of sample proteins in an alternative way to **step 5** above, reversibly stain the immunoblot using a Pierce Reversible Protein Stain Kit according to the manufacturer's instructions, and photograph the blot with a digital immunoblot imaging system. Following erasure of the protein staining, proceed to Subheading 3.4, **step 1**.

3.4 Immunoblot Analysis

Unless otherwise stated, all incubations should be conducted at room temperature and with constant agitation.

1. Rinse the membrane with TBST for 5 min.
2. Block the blot in 5% skim milk powder/TBST or 5% BSA/TBST (depending on antisera) for 1 h (*see* **Note 13**).
3. Incubate the membrane in appropriate antisera, diluted in blocking solution, for 1 h.
4. Rinse the blot 1 × 15 min in TBST, followed by 2 × 5 min in TBST.

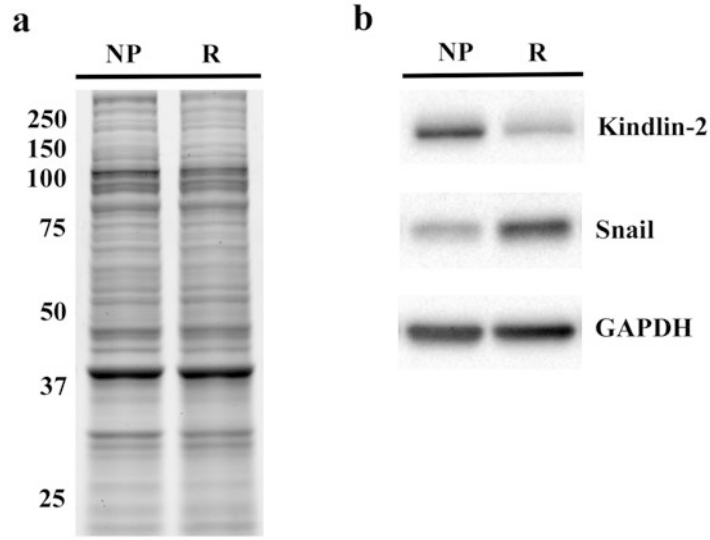

Fig. 1 SDS-PAGE and immunoblot analysis of Kindlin-2 and Snail expression using NP-40 and RIPA lysis buffers. Proteins were extracted from BeWo choriocarcinoma cells with NP-40 (NP) or RIPA (R) lysis buffer followed by SDS-PAGE and immunoblot analysis. (**a**) A Mini-PROTEAN TGX stain-free precast acrylamide gel (Bio-Rad) was utilized for SDS-PAGE. Thus, total protein was activated and photographed with a ChemiDoc MP imaging system. (**b**) Immunoblot analysis demonstrated that Kindin-2 was solubilized to a greater degree in NP-40 lysis buffer, while Snail was solubilized to a greater degree in RIPA lysis buffer even though the overall extent of total protein extraction was comparable. These results indicate the care that must be taken to predetermine the optimal lysis buffer prior to an experimental workflow as proteins of interest could remain in the insoluble fraction. Representative immunoblots are shown ($n = 4$)

5. Incubate membranes for 1 h in HRP-conjugated goat anti-rabbit IgG (H + L) or HRP-conjugated goat anti-mouse IgG (H + L) antisera (1:10,000 dilution) diluted in blocking solution.
6. Wash the blot 1 × 15 min in TBST and then 4 × 5 min in TBST.
7. Detect proteins on the immunoblot using the Pierce Super-Signal West Pico Chemiluminescent Substrate detection system (Figs. 1 and 2). Generate multiple images by capturing a timecourse of chemiluminescence signal with a digital imaging documentation system.

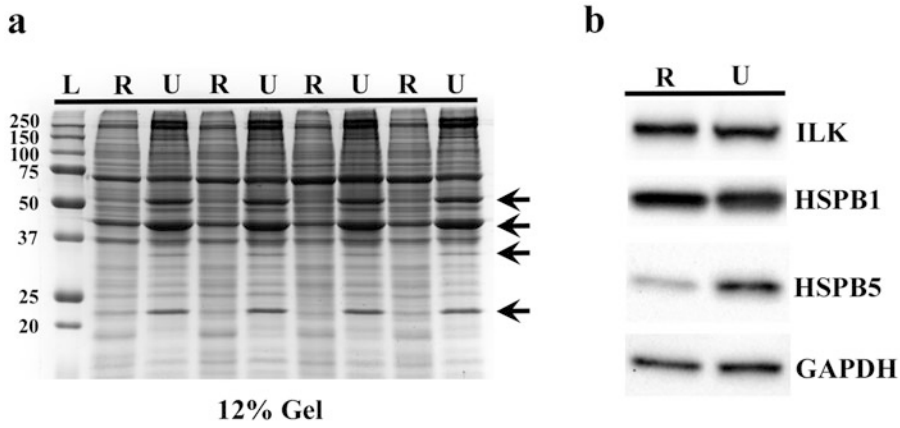

Fig. 2 SDS-PAGE and immunoblot analysis using RIPA and urea/thiourea lysis buffer. (**a**) Proteins were extracted from pregnant rat uterine smooth muscle with RIPA (R) or urea/thiourea (U) lysis buffer and loaded on a 12% polyacrylamide gel for SDS-PAGE. The gel was stained with Colloidal Blue and destained. Arrows indicate examples of protein species that appeared to solubilize to different extents in the two lysis buffers. L, protein molecular mass ladder listed in kDa. Paired lanes represent tissue lysates obtained from different animals ($n = 4$). (**b**) Representative immunoblot analyses of ILK, HSPB1, HSPB5, and GAPDH. Proteins extracted were separated by SDS-PAGE and electroblotted. Protein-specific primary antisera were then used for immunoblot analysis of the proteins obtained from the different lysis conditions. Both ILK and HSPB1 were comparably detected from smooth muscle tissue lysates prepared in both lysis buffers. In contrast, HSPB5 was more readily detected from lysates prepared in the urea/thiourea lysis buffer indicating it may be a more optimal lysis buffer for solubilization of this protein of interest

4 Notes

1. Some researchers like to modify or develop their own lysis buffers, and Harlow and Lane [3] recommended considering the following range of variables to optimize the lysis buffer for downstream western blot analyses: salt concentrations 0–1 M, nonionic detergents 0.1–2%, ionic detergents 0.01–0.5%, divalent cation concentrations 0–10 mM, EDTA concentrations 0–5 mM, and pH 6–9.
2. The urea/thiourea lysis buffer appears to have been originally used for extraction of skeletal muscle-specific proteins in 1983 by Yates and Greaser [27, 28]. Their utilization of thiourea was based on the report of Pace and Marshall [29] indicating thiourea was a potent protein denaturant. The use of this type of buffer, particularly in 2D gel electrophoresis, has been described in detail elsewhere [30, 31]. The combined use of urea and thiourea increases protein solubility since urea is effective at disrupting hydrogen bonds aiding protein unfolding and denaturation, while thiourea is much better at reducing hydrophobic interactions between proteins [32]. The volumes

of lysis buffers utilized are also very important for lysis efficiency. Gorg and colleagues [31] have previously reported the use of 1 mL of this lysis buffer with ~50–100 mg of mammalian tissue. In our hands, it has proven reasonable to use up to a maximum of 250 mg of tissue with a 1 mL volume. It is recommended that the appropriate volume be determined by the investigator on a case-by-case basis.

3. When required for lysis, take 10 mL aliquots, and completely dissolve one tablet each of Mini EDTA-free protease and PhosSTOP phosphatase inhibitors prior to use. Any unused buffer can be frozen at −80 °C for future use. As with just about every lysis buffer, care should be taken to ensure that all components are in solution prior to use as cold storage can lead to precipitation of some constituents (e.g., SDS).
4. It is recommended that the urea/thiourea lysis buffer be prepared fresh whenever possible, but it can also be aliquoted (1 mL) and stored at −80 °C for up to several months. It has also been reported that once the buffer is thawed, it should not be re-frozen [30, 31].
5. Cells and tissues should be frozen rapidly with liquid nitrogen to avoid protease degradation of proteins in the sample or collected and lysed quickly, preferably while chilled. Since proteases as well as phosphatases can be released during lysis and act on your target protein(s), protease and phosphatase inhibitors should be included in the lysis buffers. Many of these are produced as cocktails in tablet form for easy purchase, and their use is as simple as dissolving the tablet in the lysis buffer prior to utilization.
6. Methods utilized for tissue disruption clearly depend on the tissue origin. There are a large number of other means to lyse cells/tissues, and readers are directed to Simpson [33] for specific details and discussion of these protocols. When using mechanical homogenization, it is important to avoid the production of excessive amounts of foam as this could decrease your recovery volume (i.e., becomes difficult to recover from the homogenizer). Short bursts of mechanical homogenization, while the sample(s) is cooled with ice, are usually best.
7. Place two to three folded Kimwipes under the thermoplastic rubber gaskets of the casting module and a strip of Parafilm on top of each gasket. This prevents leaks by increasing the thickness of the rubber gaskets upon which the glass plates are held against with a spring-loaded lever. Leakage can be a problem as the gaskets age and lose their flexibility and overall thickness from constant use.

8. It is critical to use high-quality SDS from a single source and polyacrylamide that is free of contaminating metal ions. Sambrook and Russell [25] have reported that the migration pattern of polypeptides can change significantly when SDS from different manufacturers are interchanged. Purchase of pre-made acrylamide from reputable companies is becoming the normal procedure. Acrylamide solutions with a 1:29 bisacrylamide/acrylamide ratio are usually employed as they are capable of resolving polypeptides differing in size as little as 3% [25], but solutions can be purchased with different ratios if necessary to vary the pore size of the gel [26]. In addition, Tris base should always be used for the preparation of gel buffers to avoid production of diffuse protein bands and even improper polypeptide migration [26].
9. Leave approximately 1 cm of space below the eventual bottom of the combs for the later addition of the stacking gel mixture.
10. It has been noted that heating of samples containing urea for 2D gel electrophoresis can result in some decomposition of urea and release of isocyanate leading to protein carbamylation and charge heterogeneities of the samples [34]. However, in this instance there is no need to worry about protein carbamylation during heating of the samples in 2× SDS loading dye at 95 C as the samples are not being used for isoelectric focusing. This heating step is necessary to produce SDS-polypeptide complexes for subsequent SDS-polyacrylamide electrophoresis.
11. The system used here is a discontinuous buffer system. As a result, the SDS-polypeptide complexes in the 4% stacking gel become deposited and concentrated on the surface of the resolving gel. The SDS-polypeptide complexes are then separated in the resolving gel according to size by molecular sieving in a zone of uniform voltage and pH. Greater details on the mechanism of polyacrylamide gel electrophoresis are found elsewhere [25].
12. There are now many options for transfer of polypeptides to membranes, and the reader is directed to a review of these procedures [26]. It is also imperative that no air bubbles be trapped between the nitrocellulose membrane and the polyacrylamide gel as this will result in the lack of polypeptide transfer to the membrane in these regions. Use a blot roller to remove any bubbles between the gel and membrane.
13. The researcher should consider the blocking buffer that is most appropriate for the specific antiserum (e.g., skim milk powder vs BSA). Blocking a blot serves two important purposes. The first is well known in that it can help mask any potential non-specific binding sites on the membrane itself. The second

purpose, being less known and even less understood, is that blocking a membrane can promote renaturation of antigenic sites [35]. However, it has been reported that prolonged blocking times (>24 h) can actually remove antigens [36].

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