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Innovations

Optimizing Western blotting immunodetection: Streamlining antibody cocktails for reduced protocol time and enhanced multiplexing applications

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Abstract

Adaptive, rather than innate, immunity relies mainly on antigen–antibody recognition. This recognition is driven by the binding of specific antibody paratopes to distinct epitopes found on antigens. This interaction is pivotal for immune responses that have been re-purposed for diagnostic and therapeutic purposes. This article focuses on Western blotting, an *in vitro* technique performed for protein immunodetection. Traditionally, this technique requires separate incubations of both primary and secondary antibodies, for which these antibodies recognize different antigen epitopes (conventional method). We propose a modified protocol combining both antibodies, involving a single incubation step that reduces time and conserves reagents (non-conventional/improved method). This improved protocol will enhance efficiency without compromising detection accuracy. It will support multiplexing, enabling the simultaneous detection of multiple proteins. Despite the positive results found by applying available antibodies, further optimization is required for a more thorough evaluation, to ensure that all antibodies consistently yield successful results in every detection attempt for broader use. Our findings indicate that the tested antibody cocktails remained stable over time, which suggests potential for commercialization of this modified Western blot protocol with a wide scope towards multiplex diagnostic application.

Keywords: Western blotting; immunoblotting; multiplexing; antibody cocktails; shortened protocol

Introduction

The structural basis of antigen–antibody recognition lies in the functionality of antibody paratopes and their ability to specifically bind to antigen via antigenic determinants known as epitopes [1]. Within the variable region of antibodies, there are six hypervariable loops known as complementarity-determining regions that are responsible for the specificity towards antigen binding and recognition [2]. The association of antibodies with antigens happens through various non-covalent interactions that play a major role in achieving the high specificity and affinity that is required for reversible binding [3]. This interaction not only plays a key role in adaptive immunity but also may be applied as an experimental tool in diagnostics, biomedical research, and may also be exploited for therapeutic approaches [4].

Antigen–antibody interactions may be divided into two types: *in vivo* and *in vitro*, with the former occurring under natural conditions, while the latter happens under artificial conditions. Among the well-known *in vitro* methods applied is the Western blot technique required for immunodetection of proteins [5, 6]. It is a simple yet powerful technique that is used to investigate protein–protein interactions by identifying presence, relative mass, and abundance [7]. Moreover, it may also be useful when attempting to examine the presence of post-translational modifications [8]. Towbin *et al.* and Burnette were the first to describe and apply this technique. The inception of this protocol relied on specific interactions of antibodies towards target antigens, by initially separating the sample mixture via electrophoresis based on differences in the molecular weight of each protein [6]. Once these proteins are separated on the gel, they are then transferred to a membrane where they will encounter an incubation period with blocking reagent to minimize non-specific binding before the addition of primary antibodies with the intention of probing the target protein [7]. Further incubation and washing steps will be performed that will be followed by the addition of secondary antibodies conjugated with labeled chemiluminescent molecules or similar labelling to provide signal detection via densitometry or similar analysis of the specific probed protein bands [7]. Therefore, primary and secondary antibodies are added, at different steps, each binding specifically to their intended counterparts. Additionally, primary antibodies will specifically bind to proteins on the membrane since they have the ability to directly bind to antigens of the target protein [7]. The secondary antibody is unable to bind to the target protein, but interacts with the heavy chains of the primary antibody that has been previously bound to the target protein [7]. Moreover, it is known that secondary antibodies are most often used for immunolabeling rather than initial antigen–antibody binding [8]. This secondary antibody may need to be pre-adsorbed to reduce non-specific binding and increase binding specificity [8].

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The complete Western blot protocol involves multiple interdependent steps that may be considered complex and tedious, sometimes dependent on expertise and subjective choices when it comes to protocol adaptation [9]. Accordingly, such subjectivity may potentially impact the reproducibility of obtained results and may substantially become the source of error due to the variations made while performing the methodology [9]. Nevertheless, a major concern with Western blotting is the complex and somewhat tedious protocol involved [9]. A solution to this might be to co-incubate primary and secondary antibodies with the protein-target-containing membrane, instead of them being added at different times. The reasoning behind this concept is to reduce the number of steps and incubation time within the protocol. Many have warned against this method, as it was believed that it may cause false-negative results due to the prozone effect [10]. This is an interference that is caused by high antibody titers, inhibiting antigen–antibody lattice formation and therefore will disrupt the interaction between the primary antibody and antigens on the blot [10]. Assumptions have been made, but solid data are greatly lacking in this narrative. Understanding the dynamic between primary and secondary antibodies relative to protein interaction by performing Western blot experiments is of high importance [7]. This article aims to define and test this hypothesis, in which combining and co-incubating primary and secondary antibodies together might show no effect on protein band density and will not be materially different from having them added and incubated at separate steps within the protocol.

Materials and methods

Cell culture

Ovarian cancer cell lines (PEO4, PEO6, OVCAR4, and SKOV3) and HCT-116 colon cancer cells were kindly supplied by Dr Khaldoon Alsamman, from Imam Abdulrahman bin Faisal University (IAU). A master mix was prepared using RPMI-1640 medium (Cat. # 22409015, Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS) (Cat. # 16000044 Gibco), 1% (w/v) penicillin streptomycin (Pen/strep) (Cat. # 15140122, Gibco), and 1% (w/v) L-glutamine (Glutamax reagent) (Cat. # 25030081, Gibco). Cells were seeded from an initial vial containing 1×10^6 cells into T25 flasks (T-25 CytoOne® Flask, TC-Treated, Vented, Cat. #CC7682-4825, Starlab) and grown to ~80% confluency for all experiments. Cell lines were cultured at 37°C in a 5% (v/v) CO_2 atmosphere incubator.

Passaging of cell lines in culture was performed once they reached ~80% confluency. Flasks were washed twice using PBS (Cat. # 10010023, Gibco) to remove excess FBS, as the presence of FBS would hinder the trypsinization process. Three milliliters of Trypsin-EDTA (1X) (Cat. #25300054, Gibco) was added. Culture flasks were incubated for 3 min at 37°C to detach cells, afterward flasks were carefully shaken to remove any remaining adherent cells. Then, 5 ml of media was added to inhibit further trypsinization that may lead to cell degradation. Further mixing was performed using a pipette; to help cells completely detach, all media was transferred to a 15 ml Falcon (Cat. # E1415-0100, Starlab) tube to be centrifuged at 1500 rpm for 5 min. After centrifugation, the supernatant was completely discarded, and 1 ml of media was added and mixed well with the pellet. An additional 5 ml of media was added to the Falcon tube and mixed well. The contents of the Falcon tube were divided equally and placed into new labeled T25 flasks containing 10 ml of RPMI-1640 media (Cat. # 22409015, Gibco). These flasks were placed in 5% (v/v) CO_2 atmosphere incubator at 37°C. Media was changed, when necessary, once cells reach 80% confluency. The passage protocol was repeated, and cells split into new T25 flasks to collect suitable protein amounts for further testing.

Protein extraction

Protein extraction was performed using RIPA lysing and extraction buffer (Cat. # 89901, ThermoFisher). Cell lysis has been shown to disrupt cellular components, creating a release from compartmentalization of endogenous enzymes that may affect result outcomes by increasing degradation. Protease and phosphatase inhibitors (Cat. # CH-M398 and CH-M393, respectively, Molequle-On) were added (as explained later) to help in the prevention of degradation of extracted proteins, and the preservation of phosphorylated residues at the time of cell disruption.

The culture medium was removed, and cells seeded in T25 flasks were washed twice with ice-cold PBS. All reagents were placed on an ice tray with the cell culture flask (as this protocol should be performed on ice). Then, 1 ml of RIPA buffer (Cat. # 89901, ThermoFisher) was added to the flask with 10 µl of both protease and phosphatase inhibitors (Cat. # CH-M398 and CH-M393, respectively, Molequle-On) (after a careful mix of the bottles). The flasks were mixed and placed on ice for 30 min with the reagents evenly distributed in the flask. After the end of the 30 min incubation period, flasks were scraped using a cell scraper to remove all attached cells and the flask was examined under the microscope making sure all cells have detached. Afterward, the mixture was transferred into labelled Eppendorf tubes, vortexed and centrifuged in a microcentrifuge (MiniSpin®, Cat. # 5452000018, Eppendorf) for 5 min at 13 500 rpm. Supernatant containing protein was then transferred into new labelled Eppendorf tubes and stored in −20°C, while pellets were discarded.

Protein quantification

A bicinchoninic acid (BCA) assay (BCA kit, Cat. # KT1501, Molequle-On) was performed, following a protocol by ThermoFisher [11], on extracted protein lysates to determine the concentration of total protein for each sample. Quantification of protein concentrations was needed to unify protein amounts for subsequent Western blotting experiments. Protein lysates were thawed on ice and then diluted with PBS in a 1:10 ratio. Eighty microliters of each sample was prepared by diluting 8 µl of the sample with 72 µl of PBS. The BCA solution was prepared by mixing reagent A with reagent B in a 50:1 ratio. A total of 12.5 ml of reagent A (BCA kit, Cat. # KT1501, Molequle-On) was mixed with 0.25 ml of reagent B (BCA kit, Cat. # KT1501, Molequle-On). In a microtiter plate (96-well plate) placed on ice, 20 µl of the blank (PBS), 7 protein standards, and sample lysates were added to predefined wells in triplicate. Two hundred microliters of prepared BCA solution was added to all the wells. The plate was then placed on a rotator and thoroughly mixed for 30 s. The microtiter plate was covered in foil as the reagents were light sensitive, and incubated at 37°C for 30 min. The microtiter plate was then removed from the incubator and placed onto the absorbance plate reader (BioTek, ref. ELX808) to measure the absorbance at 562 nm. The average of the triplicate samples was calculated by the automated plate reader and then analyzed against the standard curve obtained using Microsoft Excel software.

Protein analysis

Sample preparation

Protein lysate samples were prepared based on protein concentrations calculated from the BCA assay. Aliquots of protein corresponding to known amounts of total protein for each cell line were prepared and diluted with calculated amounts of RNase-free water in Eppendorf tubes. All sample concentrations were adjusted to 50 µl of sample (7 and 5 µg for ovarian cancer and HCT-116 cells, respectively) mixed with water. The sample buffer was prepared by mixing 475 µl of 2X Laemmli buffer (Cat. # 1610737 BIO-RAD) with 25 µl of β- mercaptoethanol (Cat. # 17-1317-01, GE Healthcare), and 50 µl of this sample buffer was added to each sample. Eppendorf tubes containing the sample mixture were sealed with parafilm, vortexed, and placed on a heat block for 5 min at 95°C.

Gel electrophoresis

An electrophoresis chamber was filled with SDS-PAGE running buffer, which contains 25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS, pH 8.3. The buffer was prepared by adding 100 ml 10X Tris-Glycine-SDS-PAGE (TGS) running buffer (Cat. #: 1610732, BIO-RAD) to 900 ml of deionized water. Two Mini-protean TGX precast gels (4%–15%, 10-well comb, 50 µl/well) were placed inside the chamber (after removing the comb and tape), and SDS-PAGE buffer was poured to fill the inner chamber until it reached the top of the gels. Fifty microliters of samples was then loaded on the gel starting from the second well leaving the first well for 10 µl protein standard ladder (Cat. # 1610363, BIO-RAD). Electrodes were then connected for the gel to run on 100 V for approximately 60 min, separating protein–SDS complexes based on their electrophoretic mobility.

Western blot technique (conventional vs. non-conventional/improved protocol)

Conventional method

Once the electrophoretic run was completed, cassettes were opened for gel retrieval. Gels were rinsed with the transfer buffer. The transfer buffer was prepared by dissolving 5.82 g tris (hydroxymethyl) methylamine, 2.93 g glycine, and 0.0375 g SDS in 200 ml of methanol. The solution was then made up to 1 L using distilled water. The final concentrations of Tris base, glycine, and SDS are 0.048, 0.04, and 0.013 M, respectively. Transfer-Blot Turbo Transfer System (serial no. 690BR013881, BIO-RAD) was used to transfer bands from the gel onto Polyvinylidene fluoride (PVDF) membrane (Trans-blot turbo transfer pack, Cat. # 1704156, BIO-RAD). Mini PVDF transfer packs were used and assembled via the sandwich method in the transfer cassette for each gel. The transfer was at standard settings for 25 min.

The blocking buffer was prepared by adding 1× TBST to dissolve 5% (w/v) of dried milk powder (Regilait skimmed milk). The TBST was prepared by dissolving 1.21 g tris (hydroxymethyl) methylamine and 8.76 g sodium chloride in 800 ml distilled water. The pH was then adjusted to 8 using 1M HCl and the solution was made up to 1 L containing 0.1% (v/v) Tween. The final concentration of tris (hydroxymethyl) methylamine and sodium chloride was 0.01 and 0.15 M, respectively. The PVDF membranes were blocked for 30 min to 1 h in the fridge, ensuring full coverage of the membrane.

Primary antibodies were diluted with blocking buffer at optimized concentrations, as described later in Supplementary Tables S1 and S2. Membranes were incubated with primary antibodies overnight at 4°C. The membranes were then washed three times in 1X TBST for 5 min each on a rotator. Then, they were incubated with HRP-linked secondary goat anti-mouse/rabbit diluted in blocking buffer for 1 h in the fridge, ensuring full coverage of the membranes. The membrane was washed three times in 1× TBST for 5 min each on a rotator. A 1:1 ratio of ECL Western blot detection reagents (Cat. # 1705061, BIO-RAD) was mixed and used on the membrane and incubated for 1 min. A BIO-RAD ChemiDoc MP imaging system (serial no. 731BR02984) was used to develop the membrane using chemiluminescent blot standard settings. Band analysis was performed using Image J software as indicated later. Detection of the housekeeping protein was carried out using β-actin (a mouse anti-β-actin monoclonal antibody). Experiments were carried out in triplicate on three separate occasions for each ovarian cancer cell line, or as indicated otherwise.

Non-conventional/improved method

Once the electrophoretic run was completed, and cassettes were opened for gel retrieval, gels were rinsed with the transfer buffer, which was prepared as described earlier. Transfer-Blot Turbo Transfer System (serial no. 690BR013881, BIO-RAD) was used to transfer bands from the gel onto polyvinylidene fluoride (PVDF) membrane (Bio-Rad, Trans-blot turbo transfer pack, Cat. # 1704156, BIO-RAD). Mini PVDF transfer packs were used and assembled via the sandwich method in the transfer cassette for each gel. The transfer was at standard settings for 25 min.

Alternatively, probed membranes could be stripped and re-probed with the proposed antibody cocktails, indicating that these cocktails are working in different experimental settings (blots of stripped membranes will be highlighted in the Results section).

The blocking buffer was prepared by adding 1× TBST to dissolve 5% (w/v) of dried milk powder (Regilait skimmed milk). PVDF membranes were blocked for 30 min to 1 h in the fridge, ensuring full coverage of the membranes.

Primary and secondary antibodies were diluted together with the blocking buffer, which was described earlier, at optimized concentrations (antibodies used are summarized in Supplementary Tables S1 and S2) and membranes were covered with antibody cocktails and incubated in the fridge for 1 h. The membrane was then washed three times in 1× TBST (composition was described above) for 5 min each on a rotator. A 1:1 ratio of ECL Western blot detection reagents (Cat. # 1705061, BIO-RAD) were mixed and used on the membrane and incubated for 1 min. A BIO-RAD ChemiDocMP imaging system (serial no. 731BR02984, BIO-RAD) was used to develop the membrane on chemiluminescent blot standard settings. Experiments were carried out on ovarian and colon cancer cell lines as illustrated below.

Stability checks

Moreover, it is an important consideration to test the stability of the proposed antibodies cocktails; therefore, different cocktails (Supplementary Table S2) were re-used after being stored in -20°C for approximately 1 year. This was carried out after an initial experiment, in which a pre-made mixture of "stock" primary and secondary antibodies was stored at 4°C for 72 h before being diluted and used to probe β-actin (bottom of Supplementary Table S1). This opens up the possibility of manufacturing these cocktails for future research.

Results

Western blot analysis is used to evaluate levels of protein expression in cell lines to explain abundance, modifications, and or interactions of certain proteins. This evaluation may assist in diagnosis of many diseases, which would be helpful in developing appropriate individualized treatment regimens for patients. The protein band densities were analyzed by performing two different Western blot protocols: conventional and non-conventional/improved methodologies. The latter was proposed as a modified version of the former to provide an improvement on the conventional more-tedious procedure. We started by detecting a single protein using a simultaneous incubation with primary and secondary antibody cocktails using both protocols. The results showed approximately similar band densities (statistical analysis was performed for β-actin (Supplementary Fig. S1)), for which no statistical differences were obtained between the two methods (the conventional vs. non-conventional/improved) as shown in Fig. 1 depicting detection of β-actin, GAPDH, and TAP-1 proteins in a series of ovarian cancer cell lines as described in Fig. 2.

Additionally, we investigated the potential multiplexing of the improved protocol for concomitant detection of multiple proteins. The results showed that β-actin and GAPDH were co-detected on the same blot in the same ovarian cancer cells (Fig. 2) using the antibody cocktails listed in Supplementary Table S1. Moreover, and in a similar context, β-actin and Lamin

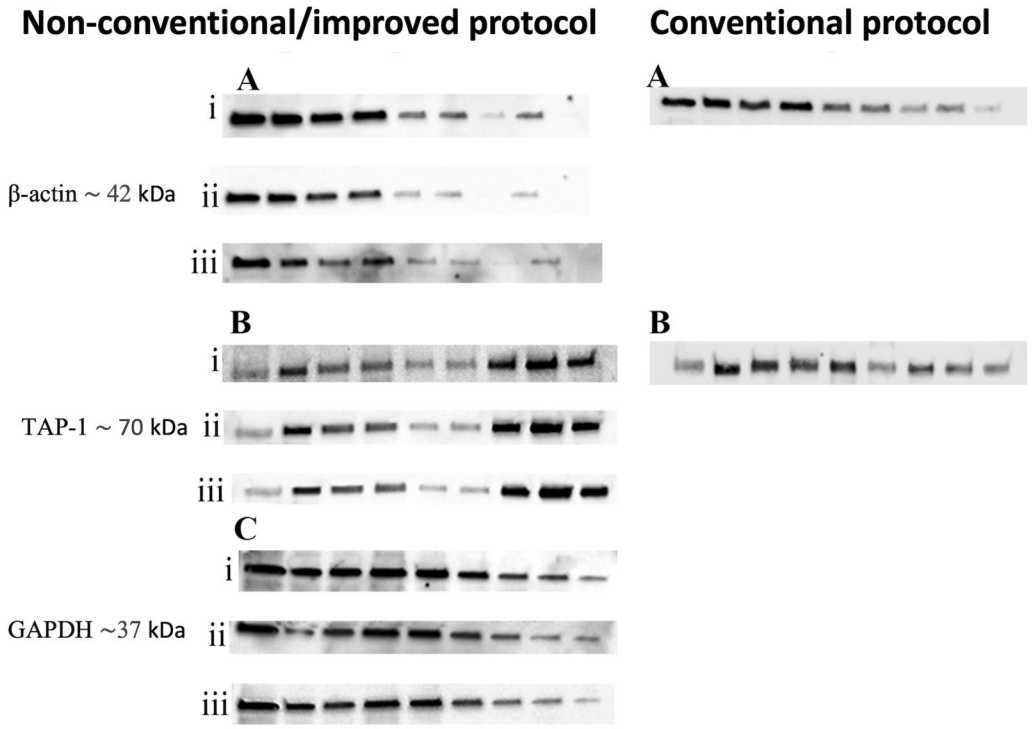

Figure 1 Western blotting using the conventional and non-conventional/improved protocols. The band densities of β-actin (**A**) and TAP1 (**B**) obtained by the non-conventional/improved protocols are comparable to that generated by the conventional protocol. GAPDH (**C**) was detected by employing the nonconventional/improved protocol with clear band intensities, as observed for the other two proteins. Original, uncropped blots are provided in Supplementary Fig. S2

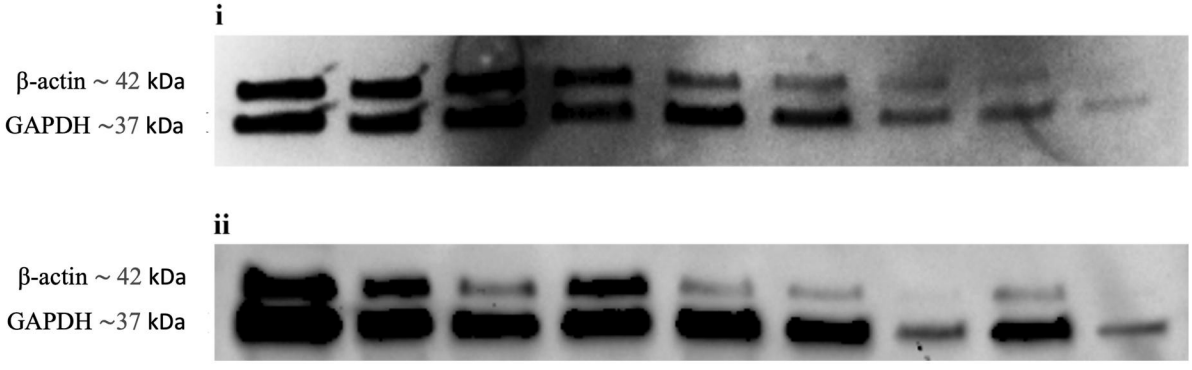

Figure 2 Co-detection of β-actin and GAPDH proteins in different ovarian cancer cell types. β-actin and GAPDH were co-detected by the non-conventional/improved protocol in lysates of different passages of ovarian cancer cell lines (from left to right: PEO4, PEO6_1, PEO6_2, PEO6_3, OVCAR4_1, OVCAR4_2, SKOV3_1, SKOV3_2, and SKOV3_3). _1, _2 and _3 indicate different passages. Original, uncropped blots are provided in Supplementary Fig. S3

B1 were co-detected using the non-conventional/improved protocol (Fig. 3).

Examining the stability of the proposed antibody cocktails

Figure 4 illustrates the detection of β-actin using a pre-made cocktail of stock primary and secondary antibodies that was incubated in the fridge for 72 h before being diluted as indicated before and applied on the membrane to probe β-actin. The results were compared with the previously-explained method (non-conventional/improved protocol) involving simultaneous addition of primary and secondary antibodies together for detection without preparing a pre-made mixture. Both signals were comparable, which provides evidence for the stability and usability of the proposed cocktails.

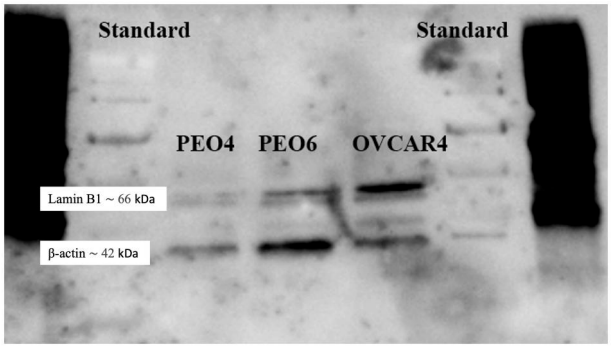

Figure 3 Co-detection of β-actin and Lamin B1 by the non-conventional/improved protocol in different ovarian cancer cell lines. The membrane was co-incubated with primary antibodies against β-actin and Lamin B1 + secondary antibodies simultaneously for 1 h. Membrane was then washed, and the signal was generated according to the routine protocol (original blot is provided)

Examining the stability of long-stored cocktails at −20°C

Different antibody cocktails were used then stored at −20°C for approximately 1 year before being utilized again for probing of their targets. The results demonstrated that these cocktails were stable and are effective for reuse after this storage period to probe targets either on freshly blotted membranes or stripped ones. For example, Fig. 5 illustrates the co-detection of β-actin and GAPDH in OVCAR4 ovarian cancer cells on a stripped membrane using an antibody cocktail that was stored as described above and listed in Supplementary Table S2 (cocktail 1). Likewise, Fig. 6 shows co-detection of p62 and GAPDH or p62, β-actin and GAPDH in HCT-116 colon cancer cells using antibody cocktails that were stored as described above. Moreover, the stability was further confirmed by co-detection of AMPK, CHOP, and JNK proteins in OVCAR4 cells on a stripped membrane as shown in Fig. 7. Additionally, another stored cocktail of primary and secondary antibodies was used to detect TAP-1 protein in PEO6 ovarian cancer cells (Fig. 8).

These results together support our hypothesis that primary and secondary antibody cocktails can be prepared, stored for long periods, and reused in Western blotting technique either for singular or multiplex detection purposes. The results also support the possibility of commercializing these cocktails based on the stability checks as for the investigated antibodies and suggest the possibility of making these cocktails from other antibodies that enable co-detection of a complete pathway targeting multiple components in a single run.

Discussion

Western blotting has classically been used in research laboratories for immunodetection of proteins using antibodies recognizing specific epitopes in the target proteins [6]. Despite the long-lasting and successful use of the method, diagnostic applications are very limited, perhaps due to the lengthy procedure, the need

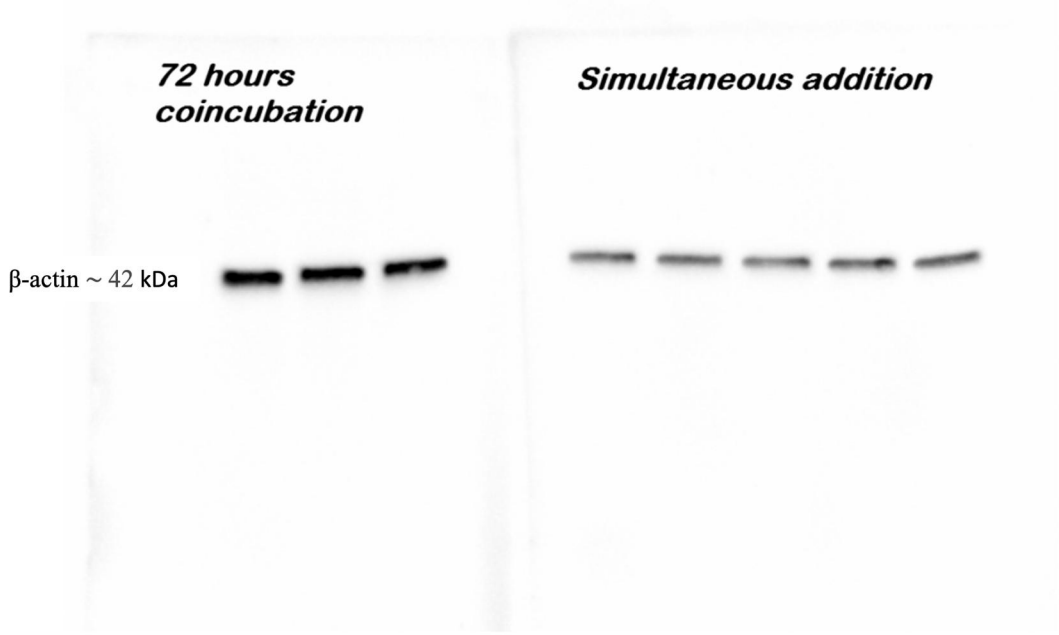

Figure 4 Detection of β-actin with a pre-made antibody cocktail and simultaneous addition of primary and secondary antibodies. Pre-made antibody cocktail, which was mixed as a single reagent and stored for 72 h in the fridge (left), was then diluted and produced a slightly better signal than simultaneous addition of antibodies (right). The blot is for detection of β-actin protein in PEO6 cells (original blots are provided)

for multiple incubations and washes, and the traditional lack of multiplexing. In addition, the Western blotting technique requires appropriate optimization and troubleshooting of each step. To this end, with little modifications of the technique, the expansion of the diagnostic use of Western blotting will remain limited, particularly because of the limited multiplexing applications that have been reported to date. Therefore, we introduced a modified protocol in this article, which should reduce the time of the experiment, save reagents, and expand multiplexing for the potential expansion of Western blotting's diagnostic applications. With this in mind, careful adjustment of the optimum secondary antibody concentration during the manufacturing of the proposed antibody cocktails should be considered. This will depend on the number of protein targets and the number of secondary antibodies that is dependent on the host species in which primary antibodies were raised. However, it is recommended that the cocktail be designed so that a single secondary antibody is included with primary antibodies, whenever possible.

The improved method involves mixing multiple primary and secondary antibodies in a single cocktail for a one-step immunodetection of target proteins. The results presented here are from experiments performed using the available antibodies in our laboratory and showed the possibility of preparing efficient, effective, and stable cocktails of primary and secondary antibodies

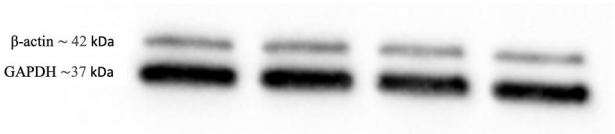

Figure 5 Co-detection of β-actin and GAPDH with a stored antibody cocktail on a stripped membrane in OVCAR 4 ovarian cancer cells using the non-conventional/improved method. The antibody cocktail included mouse monoclonal antibody to β-actin, goat anti-mouse-HRP conjugated secondary antibody, rabbit polyclonal antibody to GAPDH, and goat anti-rabbit-HRP conjugated secondary antibody. The results demonstrate the stability of the antibody cocktail for 1 year at −20°C. The original blot is provided in Supplementary Fig. S4.

(Supplementary Tables S1 and S2) for the detection of single or multiple proteins with one incubation step.

In this context, a method for detecting multiple proteins on the same blot was described by Upadhaya *et al.* [12]; however, this protocol is fundamentally different from our method as the authors detected multiple proteins in a sequential order involving multiple steps. In contrast, our improved protocol involves the addition of multiple primary and secondary antibodies in a single cocktail enabling the co-detection of multiple target proteins in a single step, which may be considered a promising protocol that reduces the time required for a somewhat tedious protocol and may potentially broaden the diagnostic applications of this immunodetection technique.

Another modified immunodetection method for botulinum neurotoxin type D has also been reported [13]. In this method, a secondary antibody was immobilized within the membrane, then the detection of the target was carried out using a specific primary antibody, which is a multi-step protocol requiring immobilization of the secondary antibody followed by a detection step with the primary antibody [13].

A one-step immunoblotting was recently described by incubating the detection strip with a secondary antibody and a good-grade primary antibody that is recognized by a multi-step protocol [14]. While this method might have shortened the time of the experiment as we did in our improved protocol, there are fundamental improvements in our method that were not described in the reported one-step method [14]. In our method, we have used pre-made cocktails of several primary and secondary antibodies, tested their stability, and proved multiplexing using this improved protocol showing the possibility of commercializing these antibody cocktails and simultaneous detection of multiple proteins facilitating the potential diagnostic use of Western blotting. In addition, our method showed the possible co-detection of target and house-keeping proteins, which allows accurate comparison of results between different samples.

Likewise, a double-labeled kit for the immunohistochemical detection of S100 and cytokeratin was described in a published patent application [15]. Similarly, another invention described

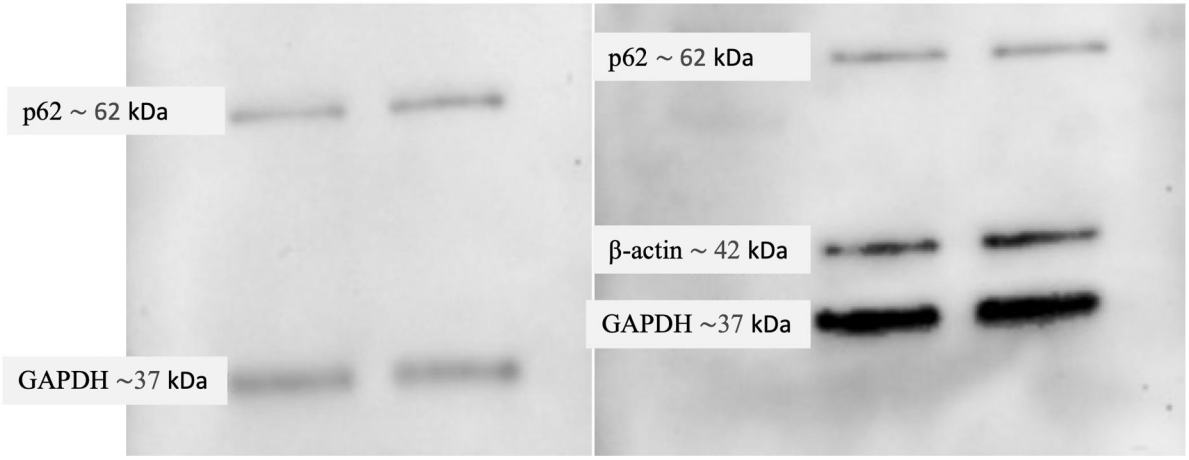

Figure 6 Co-detection of GAPDH and p62 (SQSTM1) (left) and GAPDH and p62 (right) in HCT colon cancer cells β-actin. For detection of GAPDH and p62 (left), the antibody cocktail consists of rabbit polyclonal antibodies to GAPDH and p62 and goat anti-rabbit-HRP conjugated antibodies. For co-detection of β-actin, GAPDH and p62, the antibody mixture contains mouse monoclonal antibody to β-actin, rabbit polyclonal antibodies to GAPDH and p62, goat anti-mouse-HRP conjugated and goat anti-rabbit-HRP conjugated secondary antibodies. The antibody cocktails were re-used after being stored for 1 year at −20°C. The original blot was provided in Supplementary Fig. S5

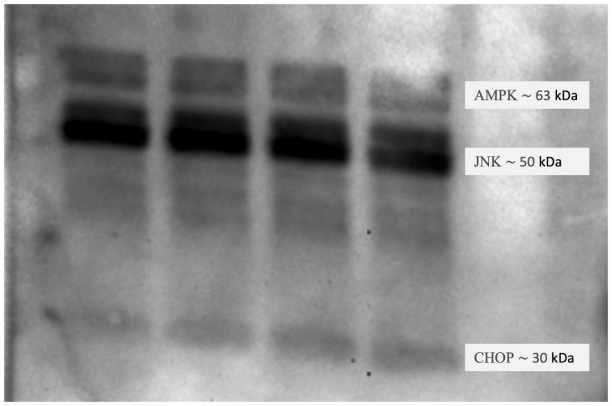

Figure 7 Co-detection of AMPK, CHOP and JNK in OVCAR 4 cancer cells (stripped membrane). For co-detection of AMPK, CHOP, and JNK, the antibody mixture contains mouse monoclonal antibodies to JNK and CHOP, rabbit polyclonal antibodies to AMPK, goat anti-mouse-HRP conjugated and goat anti-rabbit-HRP conjugated secondary antibodies. The antibody cocktail is 1 year old and was stored at −20°C

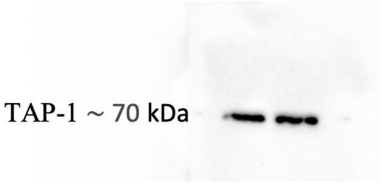

Figure 8 Detection of TAP-1 in PEO6 ovarian cancer cells. A cocktail of mouse monoclonal antibody to TAP-1 and goat anti-mouse-HRP conjugated secondary antibody was stored at −20°C for 1 year and re-used to detect TAP-1 in PEO6 cells. The original, uncropped blot is provided in Supplementary Fig. S6

the co-detection of CD8 and CD66b by immunohistochemistry using a mixture of primary antibodies [16]. In these methods, primary antibodies were incubated simultaneously, followed by a second incubation step with secondary antibodies and several washes [15]. These methods are also different from our improved protocol, where co-detection of two proteins was conducted by immunohistochemistry not immunoblotting and did not shorten the time of the protocol. Additionally, we described the preparation of a variety of antibody cocktails for multiplexing that have been proven stable upon proper storage.

Conclusion

This work presented a modified, shortened protocol of Western blotting that demonstrates the possibility of making cocktails of antibodies for multiplexing of immunoblotting/immunodetection of proteins. This might enable the co-detection of components to a single pathway or partner proteins in a co-expression network. Moreover, the successful use of long-stored cocktails facilitates the possible commercialization of these antibody mixtures. Additionally, this modified protocol reduced the duration of Western blotting, conserving reagents by decreasing the number of washing steps, and saved on gels with various other consumables as it incorporated multiplexing.

However, the work presented herein was limited to the available antibodies in our laboratory at the time of experiments and we must, therefore, flag up the need for optimization of experimental settings when using other antibodies.

Author contributions

Lamya Z. Yamani (Conceptualization [supporting], Methodology [equal], Writing—review & editing [equal]), Khaldoon Alsamman (Methodology [supporting], Resources [supporting], Writing—review & editing [equal]), and Omar S. El-Masry (Conceptualization [lead], Data curation [equal], Methodology [lead], Writing—original draft [lead], Writing—review & editing [equal]).

Supplementary data

Supplementary data is available at *Biology Methods and Protocols* online.

Conflict of interest statement. All authors have no competing interest to disclose except the filing of the data as a patent application in the United States Patent and Trademark Office (reference: 549094US)/application number 18/812,963.

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None declared.

Data availability

All data generated in the study are presented in the manuscript and the supplementary file. The data were also filed as a patent application in the United States Patent and Trademark Office (reference: 549094US)/application number 18/812,963.

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