Aptamer Validation by Western Blot–an overview

Lamees Jamal Talib *, Basma Talib Al-Sudani **, Mustafa Ghazi Al-Abbassi***
Central Teaching Hospital of Pediatric, Baghdad, Iraq
****Department of Clinical Laboratory Sciences, College of Pharmacy, Mustansiriyah University, Baghdad, Iraq***
*****College of Pharmacy, Alkafeel University, Iraq.***
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Corresponding Author email:
basma_alsudani@uomustansiriyah.edu.iq
orcid: https://orcid.org/0000-0001-6253-3599

Abstract:

Western blot is the main and basic technique in cellular and molecular biology. The principle of the western blot is the isolation and detection of the target molecule usually from a cellular extract. The whole process of western blot consists of three stages and can be described briefly as separation of protein. followed by transportation to a solid membrane and finally detection of the target by an antibody. Western blot technique is usually used for the detection of proteins but also can be used to detect other molecules such as aptamers. Aptamers can be defined as a short-stranded DNA or RNA that bind with the target with high specificity and affinity. Aptamers highly resemble antibodies with many advantages. In this review, there is a focus on the aptamers that had validated by western blot technique other than other methods. This method has the advantage of less time required, no antibodies needed, and introducing the possibility of multiplexing detection.

Key words: Western blot, Aptamer, DNA.

التحقق من الأبتمار بواسطة اللطخة الغربية- مراجعة عامة

لميس جمال طالب * , بسمة طالب السوداني ** , مصطفى غازي العباسي***
**** مستشفى الطفل المركزي التعليمي, بغداد, العراق***
***** قسم العلوم المختبرية السريرية , كلية الصيدلة , الجامعة المستنصرية, بغداد, العراق***
****** جامعة الكفيل , كلية الصيدلة, العراق***

الخلاصة:

لطخة ويسترن (بالإنجليزية: Western Blot) (تعرف أيضا باللطخة المناعية Immunoblot)) هي التقنية الرئيسية والأساسية في علم الأحياء الخلوي والجزيئي. مبدأ اللطخة الغربية هو عزل وكشف الجزيء المستهدف عادة من مستخلص خلوي. تتكون عملية اللطخة الغربية بأكملها من ثلاث مراحل ويمكن وصفها بأنها فصل البروتين متبوعًا بالنقل إلى غشاء صلب واكتشاف الهدف بواسطة جسم مضاد في النهاية. عادة ما تستخدم تقنية لطخة غربية للكشف عن البروتينات ولكن يمكن استخدامها أيضًا للكشف عن جزيئات أخرى مثل الأبتامرات. يمكن تعريف الأبتامرات على أنها جزيء قصير من الحمض النووي والحمض النووي الريبوزي يرتبط بالهدف مع خصوصية عالية وألفة عالية. تشبه الأبتاميرات الأجسام المضادة بشكل كبير مع العديد من المزايا. في هذه المراجعة ، هناك تركيز على الأبتامرات التي تم التحقق منها بواسطة تقنية لطخة غربية بخلاف الطرق الأخرى.

الكلمات المفتاحية: اللطخة الغربية , ابتامر , الحمض النووي الريبوزي

Introduction

Immunoblotting and protein blotting is an alternative name to the western blot technique, one of the most important analytical techniques in biology (1). Western blot technique was used for a very long period but the first complete description of the process was introduced in 1979 by Towbin (2). Since 1979 western blot is used in almost all laboratories all over the world for the quantitative and qualitative detection of proteins in highly complex homogenates obtained from tissue or cells (3). The importance of western blot has been increased by the increased flexibility, sensitivity, and efficiency over time (4). In addition to that, this technique has the advantage of a broad range of accurate detection of different proteins even if the protein has a low expression this is attributed to the development of ultrasensitive imaging systems (5).

The major factor that determines the accuracy of the qualitative detection by western blot is the antibody validation (6). The importance of the antibody is represented by the assurance of specific interaction between the selected antibody and the antigen, also in the determination of the dilution factor which is essential for sample loading (2). In addition to the critical role of the antibody, the determination of the normalization method in a suitable manner is another important factor in the western blot technique (7). Usually, there are two methods of normalization based on reference signals and they include housekeeping proteins and total protein intensity, the normalization step is essential to recognize and rectify the error (6,7).

According to Tahrin, et al, western blot protocol includes cell lysis, sample preparation, gel preparation, electrophoresis, electrotransfer, blocking, and antibody incubation (8). In cell lysis step, the cells were washed with cold phosphate buffer saline along with gentle rocking then the washing buffer cast away. After that, phosphate buffer saline added again to the cells and centrifuge for five minutes at 1500 rpm and the supernatant discarded. Following that, 180 µl of ice-cold cell lysis buffer and 20 µl of protease enzyme inhibitor in order to inhibit protein denaturation. The mixture incubated on ice for 30 minutes followed by centrifugation at 12000 rpm for 10 minutes at 4 °C and the supernatant transferred to a fresh tube and store on ice. Using a spectrophotometer, the protein concentration was measured. It is important to use positive and negative control where the positive control necessary to approve the antibody activity and the identity of the detected protein. On the other hand, the negative control is necessary to ensure specific staining.

In sample preparation, after the determination of the protein extract volume, 5 µl of sample buffer added and the final volume in each well complete to 15 µl with double distilled water. Finally, the sample was heated at 100 °C for 5 minutes.

The third step includes the preparation of 10% of stacking gel and added to the rack to the specified level and left for solidification for 15-30 minutes. The separating gel added over the stacking gel and after clarifying that there is no air bubble, the comb placed and left for a while for gel solidification. Usually, the concentration of acrylamide in stacking gel is low and this is responsible for sharp and thin defined bands of protein. While the separating gel contains a higher concentration of acrylamide resulting in separation of proteins according to their size.

In the electrophoresis step, the running buffer is added into the electroporator and then the gel is placed inside the electroporator and connects it to the power supply. The samples and the marker loaded in the well and run the separating gel with 60 V and stacking gel for 140 V for about one hour.

After electrophoresis, electrotransfer step followed by the formation of a transfer

sandwich that consists of three layers of filter papers that were wet with transfer buffer, then the gel layer, polyvinylidene fluoride membrane soaked with methanol, finally other three filter papers. The transfer sandwich locates in the transfer apparatus and the temperature should keep at 4 °C by using ice. The transfer buffer added and the running continue for about 90 minutes, the time of running can be changed according to the thickness of the gel (8).

Finally, the membrane blocked with 5% milk in tris-buffered saline tween 20 for about one hour followed by the addition of the primary antibody in 5% of bovine serum albumin and incubated on a shaker in 4 °C for overnight. Then, the membrane washed three times for about 5 minutes with tris-buffered saline tween 20 and the secondary antibody added after that. The secondary antibody added in 5% milk in tris-buffered saline tween 20 and incubated for about one hour. Again, the membrane was washed three times with tris-buffered saline tween 20 for five minutes each time. At last, the membrane incubated with Enhanced Chemiluminescence mixture for one or two minutes, and the results were visualized in a dark room (8).

By these steps there will be an accurate detection of the target protein whether it exists or not in addition to the quantity, also there will be a determination of size and any modification or decay in the detected protein (9).

Due to the multiple advantages of the western blot technique, it uses not limited to the identification of proteins but also it has been used for validation of aptamer. Aptamer also has known as antibody analog and can be defined as artificial, short, single-strand DNA, or RNA (10). Aptamers can be designed in three dimensions structure that provides the high specificity and selectivity of binding between the aptamer and the target which may include protein, drug, or any molecule (11). Despite that, the aptamers are analog to the antibodies but there are a revolutionary widespread and applications of aptamers in different aspects due to the multiple advantages over antibodies (12).

Aptamers have the advantages of high stability, selectivity, specificity (13). Furthermore, aptamers' properties can be changed according to the needs, and also the condition of the synthesis process of the aptamer can be changed to obtain the desired properties of the aptamer (14). Also, when the aptamer denaturated by heat, it can be restored and be functional (13,14). The aptamers synthesized for the first time in 1990 by a process known as SELEX, Systemic Evolution of Ligands by Exponential Enrichment (15).

Western blot technique had been used for the validation of multiple aptamers, in this short review, we will outline these aptamers.

Aptamer validation by western blot

Aptamers detection and validation has been started with the SELEX process since 1990 and with the increasing applications, using and advantages of aptamers there were many alternative methods used for aptamers validation (16). Aptamers have been validated by different means including western blot technique due to multiple advantages including the short time required when compared with other methods, the absence of the antibody's disadvantages and also the ability of multiple targets other than one target in a single experiment (17)

Aptamer in clinical diagnosis

Both DNA and RNA aptamers have been studied to be used as a tool for diagnosis of different diseases, therapeutic means for viral infection and cancer and other diseases, development of novel drugs and delivery systems of drugs (18). In the last two decades, there are many aptamers have been investigated to be inhibitors of different targets such as aptamers that inhibit thrombin and vascular endothelial growth factor (VEGF) (19). Aptamers have a large importance in the development of

viral diseases especially HIV (Human immunodeficiency virus) since the aptamers show that they can interfere with different stages of the viral infection, also in 2004 there was the first approved for the clinical use of aptamer against age-related macular degeneration which is known as Macugen aptamer (20). One of the most important applications of the aptamers is the treatment of cancers, AS1411 aptamer is one of the aptamers under clinical trials for the treatment of acute myelocytic leukemia (AML) (21). After all, aptamers are considered to be one of the most promising tools with unique properties and advantages to be a revolutionary advance in the diagnosis and treatment of different diseases.

Aptamer validation by western blot for *E. coli* outer membrane proteins

Western blot used for the validation of the proteins or aptamer for target protein of different bacteria, one of these bacteria is general and pathogenic *E. coli* in which validation of reactive aptamer against *E. coli* was done and also aptamer validation against Shiga-like toxin 2 (verotoxin 2) (22). There are numerous serotypes of pathogenic *E. coli* that can reach up to 70 types (23). In this study, the pathogenic types of O157: H7, Big 6 non- O 157 Shiga toxin-producing *E. coli* were selected along with other types of gram-positive bacteria for comparison (22). According to Bruno and Sivils, the aptamers used in their study were designated against the whole structure of *E. coli* and they are known as EcO 3R and EcO 4F, these two aptamers subjected to manipulation by elongation about 100 bases by T spacer and became to be known as T-EcO 3R and T-EcO 4F (24). After bacteria culture on blood agar overnight, the bacteria converted to pellet by centrifugation then, 75 µl of bacterial pellet added to 25 µl of 4X SDS-PAGE loading buffer containing beta-mercaptoethanol followed by mixing for about 1 minute and the boiling at 100 °C for five minutes. After that, the sample subjected for centrifugation for five minutes at 14000 rpm, from the supernatant fluid 30 µl was taken and loaded into the wells of 12% Bis-Tris plus polyacrylamide gels. Before electrophoresis, the gels loaded with either ColorBurst or EZ-Run marker associated with the orange reference band at 72 kD, the gels run for one hour at 125 V. Aptamers gels were coated with pre-wetted nitrocellulose membrane in Tris transfer buffer which contains 20% methanol. The bands of aptamers were transferred in cold temperature overnight at 4 mA, then, using 10 ml of Super Block the membranes were blocked for one hour associated with mixing. The blocking step followed by the addition of DNA aptamer containing 5` biotin labels in Super Block and the volume added about 5 µl of 100 µM DNA associated with further mixing for about one hour. Using 10 ml of Tris-buffered saline containing 0.1% tween 20, the membranes were washed for five minuted five times followed by the addition of Streptavidin-Alkaline phosphate included in 10 ml of Tris-buffered saline with 0.1% Tween 20 and the dilution was 1:100000. The membranes were incubated for one hour along with mixing at room temperature. Additional washing of the membranes done for five times with Tris-buffered saline contained 0.1% Tween 20 for five minutes each time and then washing with Tris-buffered saline alone for three times, five times for each time. To 10 ml of Alkaline Phosphatase substrate, the membranes were transferred and incubated at room temperature for five minutes. Finally, the membranes were placed in the X-ray film cassette for examination.

The result showed that the aptamer provides specific binding with Shiga-like toxin 2 and virulence factor of the *E. coli* surface, in other words, the aptamers can be used in western blot technique for the highly specific binding with the pathogenic *E. coli* other than the related gram-negative or unrelated gram-positive bacteria and this specificity can be obtained even in the

highly complex cellular lysates. This is the most important advantage of aptamer against other techniques such as ELISA, dot blot, and lateral flow test in which there is no possibility to determine the molecular weight of the detected targets (18). Another important advantage of aptamer use in western blot validation is that the binding of aptamer with a protein has a molecular weight that differs from the target may indicate that this protein has an epitope found in the target protein (22).

Validation of DNA aptamers with selective targeting of *Leishmania infantum*

Leishmaniasis is a parasitic disease that is endemic in about 88 countries, leishmaniasis affects about 12 million people in the world and is associated with a death rate of 80000 human beings (25). The human infection with Leishmania can cause a mild, moderate, and severe sign and symptoms and this depend on the type of species that infect the human where the simple form of infection usually associated with *L. major* and the severe form occurs due to *L. donovani* (26). The diagnostic tools usually used in Leishmaniasis are either serological test, microscopic examination, PCR, and LAMP (Loop-mediated isothermal amplification) (27). Both PCR and LAMP tests are still expensive and not available in all countries also, in a chronic phase of Leishmaniasis the blood contains a low number of the parasite so it is difficult to be diagnosed by PCR and LAMP tests (28). According to that, there are many attempts to develop aptamers with selectivity for Leishmania to be used as a diagnostic and detection tool such as KMP-1 aptamer that targeting *L. infantum* (29).

According to Martin, et al, their study attended to investigate multiple aptamers to select the most selective one for the target which was histone H2A of *Leishmania infantum* to be a new tool for treatment and diagnosis since H2A protein considered to be the best candidate (28, 29,30). The study included multiple steps starting by cell culture and preparation of extraction, followed by expression and purification of H2A protein of *Leishmania infantum*, then aptamer sequencing and cloning step where three aptamers have been selected including KMP-1, LiH2A, and LiH3 (31, 32,33). After that, the kinetic and affinity and selectivity of the aptamers were studied by enzyme-linked oligonucleotide assay followed by validation of the aptamers by western blot (28).

In immunoblotting step, about 30 µg of the total, nuclear, and cytosolic extract of *Leishmania infantum* used along with one µg of recombinant LiH2A and all of them undergo separation on 15% SDS-PAGE gels. The gels were then transferred to the nitrocellulose membranes followed by incubation at room temperature for one hour with 40 nM of digoxigenin-labeled AptLiH2A number one and two mixed with selection buffer along with gentle rocking. The membranes were washed three times with selection buffer and examined at room temperature for one hour with an anti-digoxigenin-POD antibody after its dilution to 1:1000. The final step included washing the membranes with selection buffer for three times (28).

The result of this study shows that two of the selected aptamers detect the target protein of *Leishmania infantum* according to the three-dimension structure of the aptamer, in addition, the charge of the nucleic acids. As a result of the previous reason, the aptamers provide a highly selective detection with much high signal comparing to the control aptamer associated with very low cross-reactivity with other parasites (28).

Screening for DNA aptamer against mouse prion protein

Prion disease is a neurodegenerative disease in which the normally occurring prion protein undergoes structural changes resulting in abnormal accumulation in the brain (34). The marker of prion disease is

the scrapie associated prion protein (scrapie is the β sheet isoform of prion protein which is resistant to protease digestion) and thus the diagnosis of the disease usually occurs by anti-prion protein antibody kit (35). There are many evidence indicate that anti-prion antibody can inhibit the formation of scrapie associated prion protein and therefore the therapy of the disease can be based on the action of the antibody (36). There were many attempts to develop both DNA and RNA aptamers against prion protein and there are already RNA aptamers that have a high affinity to prion protein (37, 38). Because of the positively charged nature of prion protein molecules, prion protein has the ability to interact and bind with the nucleic acids, and therefore it is difficult to develop DNA aptamers with high selectivity to prion protein (39,40).

According to the previous reason, it is difficult to develop DNA aptamers from the SELEX process, and therefore in this study, they tried to isolate DNA aptamers in other method known as competitive selection followed by validation with western blot (41). According to Ogasawara, et al, the first step included the prion protein preparation from mouse followed by the competitive selection of DNA aptamer in which α prion protein was used to select certain sequences of DNA (41). In competitive selection, the DNA incubated with the target protein on a membrane, and then the membrane undergoes incubation with a horseradish peroxidase-labeled anti-F1TC antibody after that the membrane washed for one hour with mixing with phosphate buffer saline in tween. After that, the membrane incubated with ECL Plus and followed by chemiluminescence. Western blot proceeded with 15% SDS-PAGE gel, the DNA and chemiluminescence detection was subsequently incubated by aptamer blotting. A successful control experiment was carried out using the anti-mouse PrP antibody 7D9 as the primary antibody and the anti-mouse IgG antibody conjugated HRP and the secondary antibody STAR105P. β-Prion protein was treated with or untreated with PK at different concentrations for 1 hour, finally the reaction ceased by the addition of a stop solution which consistS of 9 M urea, 50 mM Hepes-NaOH included with 1mg/ml Pefabloc (41). The immunoblotting step followed by binding assay of the isolated DNA clones and then detection of prion protein by fluorescence depolarization. The result showed that one DNA aptamer can specifically detect both α and β prion protein with aptamer blotting and western blotting and this aptamer can be applicable as a diagnosis tool for prion disease.

Preliminary development of DNA aptamer for sensitive detection of *Rickettsia* cells

Rickettsia is an obligatory intracellular bacterium that consist of about species in with nearly 17 of these species are pathogenic in human and animals (42). Pathogenic species are classified into the insect-borne typhus community (TG) and the spotted fever community (SFG), which is transmitted primarily by mites and rough ticks (43). The determination of effective treatment is usually dependent on accurate and fast diagnosis since the early signs and symptoms of Rickettsia highly resemble other different diseases (44). The ordinary method of diagnosis usually occurs by detection of antibodies formed by the patient by serological tests, Nucleic acid detection using PCR or isothermal amplification has been used as with many cryptic infectious diseases, but reactions based on DNA polymerase are susceptible to inhibition in biological samples from heme, collagen or other molecules and, in the case of PCR, a usually heavy and bulky thermal cycler is required (44, 45, 46). Therefore, according to Bruno, et al, they tried to develop a fast, specific, and sensitive method for diagnosis of the most common Rickettsia species (47).

The methodology of the study consists of preparation of Rickettsia samples then

DNA aptamer development and cloning followed by the screening of candidate aptamers after that the aptamer undergoes western blotting (47). Whole-cell samples of *Rickettsia* (22.5 µl) were added to 7.5 µl or 4X SDS-PAGE loading buffer containing beta-mercaptoethanol and heated at 95 °C for five minutes. Thirty µL samples were loaded into a polyacrylamide gel with a Tris-MOPS-SDS gradient of 4-20% and tested at 90 mA for 1 hour, followed by 120 mA until the front color reached the bottom of the gel. The gel was layered with a pre-wetted PVDF membrane in Tris transfer buffer containing 20% methanol and bands were transported at 4 mA in a cold room overnight. The membrane was blocked with 10 ml of Super- Block with gentle mixing for one hour followed by addition of 5 µl of 100 µM Rt-18R DNA aptamer - 5'-biotin in Super Block with an additional mixing at room temperature for one hour. The membrane was washed five times with 10 ml of phosphate buffer saline containing 0.1% of tween 20 for five minutes for each wash. Then, a dilution of Streptavidin Alkaline Phosphatase (~ 2 mg/ml of stock) in 10 ml of phosphate buffer saline plus 0.1% tween 20 was applied, and the membrane was incubated with gentle mixing for 1 hour at room temperature. The membrane was washed five more times in 10 ml of phosphate buffer saline containing 0.1% of tween 20 for 5 min per wash and three times in 10 ml of phosphate buffer saline containing 0.1% of tween 20 for 5 min per wash. The membrane was moved to 10 ml alkaline phosphatase substratum and was incubated at room temperature for 5 minutes. The wetted membrane was put in a cassette of X-ray films and grown for 10 minutes (47). The result showed that Rt-18R aptamer detected only one band in *Rickettsia typhi* on western blot and in general this work revealed the possibility of development of rapid and ultrasensitive tools based on the structure of the nucleic acids for the detection and diagnosis of Rickettsia (47).

Aptamers VS primary antibody in western blotting

A Western blot analysis is an analytical technique routinely used to quantify specific proteins. The procedure includes complicated and elaborate steps and requires many reagents, such as two types of antibodies. There are now many reagent companies that specialize in providing antibodies against tens of thousands of different proteins (12). Hah's group published a new aptamer-based Western blot strategy that has reduced the procedure to one step and easily detects the target protein using only one aptamer. Instead of two types of antibodies, the QD-conjugated RNA aptamer specific for the His-tag was employed. This method has the advantage of requiring less time, not requiring antibodies or ^{32}P, and introducing the possibility of multiplexing detection (16).

Conclusion

Western blot plays an important role to identify the ligand of protein- aptamers, the detection for target proteins-aptamer on a single blot, which thus may be able to facilitate and simplify the routinely used protein detection procedure. Aptamers can be selected to a target in 12 weeks. Coupled with limited batch-to-batch variability during synthesis, aptamers are a powerful addition to existing Western blotting methods.

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