IJSRM
INTERNATIONAL JOURNAL OF SCIENCE AND RESEARCH METHODOLOGY
An Official Publication of Human Journals

Human Journals
Research Article
October 2019 Vol.:13, Issue:4

Western Blot Comparison of Wet Transfer and Semi-Dry Transfer Methods

IJSRM
INTERNATIONAL JOURNAL OF SCIENCE AND RESEARCH METHODOLOGY
An Official Publication of Human Journals

Samuel Eguasi Inkabi*, Kristianna Marie Fredenburg, Mayrangela Rodriguez

Department of Pathology, Immunology and Laboratory Medicine, University of Florida, Gainesville, Florida, USA

Submission: 23 September 2019
Accepted: 28 September 2019
Published: 30 October 2019

HUMAN JOURNALS
www.ijsrm.humanjournals.com

Keywords: Western blot, protein, electrophoresis, molecular weight, transfer

ABSTRACT

Western blot is a research technique that employs the use of gel electrophoresis to separate the mixture of proteins based on molecular weight. The loss of detection of protein bands after developing the film has been a great concern to the researcher. After evaluating several factors that account for this problem in western blot, this article focuses on regulating the transfer of proteins from the gel to the membrane, comparing the wet transfer method and the semi-dry transfer method in troubleshooting the loss of protein band detection during western blot.

INTRODUCTION

Western blot is a research technique that is used to separate and identify specific protein based on the protein's molecular weight in a mixture of complex protein extracted from cells. It is a method that detect proteins and their posttranslational modifications (PTM) in biological samples (Butler et al., 2019). This technique employs the use of gel electrophoresis to separate the mixture of proteins based on molecular weight (Mahmood and Yang, 2012). Following protocol for a western blot, the separated protein based on size/molecular weight on the gel is transferred to a membrane. After transferring the proteins to a membrane, the membrane is then incubated with blocking buffer for an hour, and the membrane is incubated with label primary and secondary antibodies specific to the protein of interest. The bound antibodies to the protein of interest are then detected by developing the film which is visible by a band. The amount of protein present corresponds to the thickness of the band.

A situation of concern to researchers using western blot is the loss of detection of protein bands after developing the film. This has been a case in our lab for the past four months. We troubleshot the loss of detection by varying the amount of protein loaded for the electrophoresis, using freshly prepared primary and secondary antibodies, and having the western blot with our samples run in a collaborator's lab—only to yield the same results.

In this paper, we will describe the protocol for western blot with the appropriate modifications adapted in our lab while evaluating our own troubleshoot that led to the detection of proteins after developing the film. This article focuses on regulating the transfer of proteins from the gel to the membrane, comparing the wet transfer method and the semi-dry transfer method in troubleshooting for the problem of protein detection loss.

MATERIALS AND METHODS

Materials

Tris-Glycine SDS Running Buffer (10x) (catalog number LC2675-5), Tris-Glycine Transfer Buffer (25x) (catalog number LC3675), Novex wedgewell 4-20% Tris-Glycine Gel (XP04200BOX) were purchased from Thermofisher Scientific (USA). Trans-Blot Turbo system (Serial No. 690BR014971) and Trans-Blot Turbo Transfer Pack Mini format 0.2µm PVDF (Cat. No. 1704156) offered by Bio-Rad systems. Cell signaling ECL reagent (catalog number NC1275804) was purchased from Fisher Scientific. Primary antibody β-actin

(catalog number 60008-1-Ig) was purchased from Proteintech, and secondary antibody Goat antimouse IgG (H+L) human-HRP (catalog number 1031-05) was obtained from Southern Biotech.

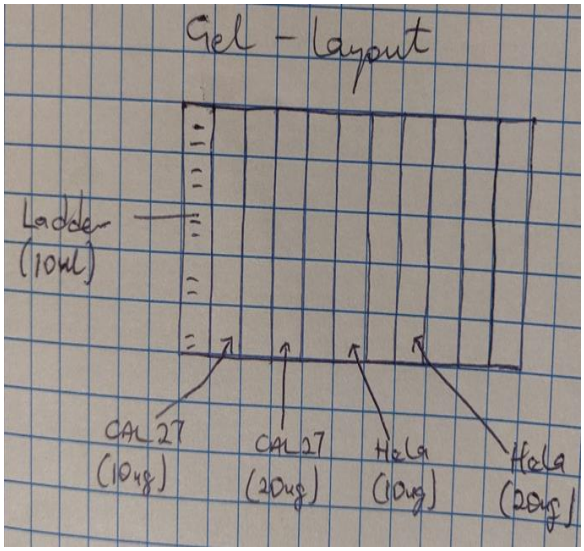
Protein lysis and concentration determination

Protein was extracted from pelleted CAL 27 and Hela cells that had confluency of 80%. The cells were lysed using RIPA buffer with Protease. The protein concentration of the samples was determined by Bicinchoninic acid assay (BCA assay) using Thermo Scientific Pierce™ BCA Protein Assay Kit (REF: 23227) and Epoch microplate spectrophotometer at A562nm set to measure total protein concentration compared to a protein standard.

Sample preparation and electrophoresis

The volume of the protein extracted from samples CAL 27 and Hela to ensure 10µg/mL and 20µg/mL in each well was respectively determined. The sample buffer of the same specified volume of the protein extracts was added to the samples in new correspondingly labeled Eppendorf tubes and mixed well. The samples were heated with dry bath (model BSH1001; Serial No. AS-BSH1-1814) for 5 minutes at 95ºC.

After heating, the samples were spun down using MyFUGE (Benchmark Scientific Inc. model: C1012) and allowed to sit on ice. Plan for sample loading in the gel was done [Figure 1]. The samples (entire volume) followed by Load marker (10µL PageRuler Plus Product No. 26619) were loaded into the wells of Invitrogen by Thermo Fisher Scientific Novex™ WedgeWell™ 4-20% Tris-Glycine Gel (REF: XP04200BOX) according to the planned loading layout. The gel was fixed in a running tank filled with 1x Running buffer up to the mark on the tank and set running at voltage 200 V for 30 minutes [Figure 2].



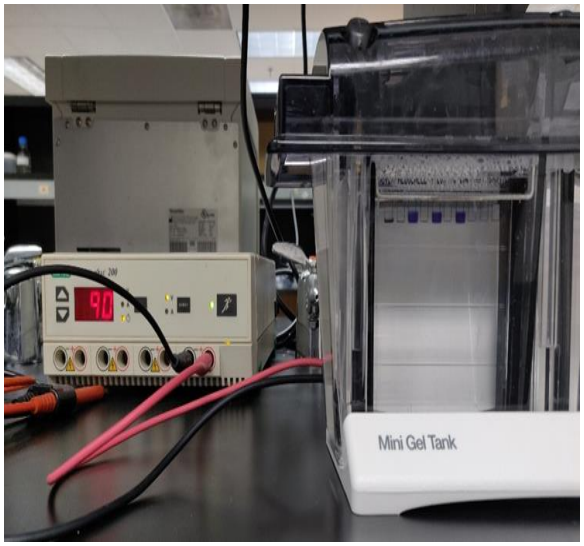


Figure No. 1: The gel loading plan **Figure No. 2: Electrophoresis**

Transfer of Protein from Gel to Membrane

After the electrophoresis, the gel was taken out of the running tank, isolated the gel from its case using electrophoresis gel cutter. The gel was carefully placed in a fresh prepared 1x Transfer buffer and allowed to soak for 10 minutes. The two methods utilized for transferring the protein from the gel to membrane were semi-dry transfer and wet transfer.

Semi-dry transfer method

The semidry transfer method was carried out using Bio-Rad Trans-Blot Turbo Transfer System (Serial No. 690BR014971) and Trans-Blot Turbo Transfer Pack Mini format 0.2µm PVDF (Cat. No. 1704156). Following the set-up protocol for the Trans-Blot Turbo Transfer System Transfer Pack Quick Start Guide, a transfer sandwich was assembled as follows:

1. The membrane and bottom stack were placed on the cassette base of the Trans-Blot Transfer system.

2. The gel was placed on top of the membrane.

3. The second, top wetted transfer stack was placed on top of the gel, serving as the top ion stack. The sandwich was rolled with a blotting roller to expel any air bubbles that might be trapped.

4. The cassette lid was closed, then locked and inserted into the Trans-Blot Transfer system. Detailed instructions were followed in the instruction manual to begin the transfer. The system was programmed at 1.3A constant; 25V; timed 7 minutes for transfer [Figure 3a].

Wet transfer method

The nitrocellulose membrane was activated by soaking it 1x Transfer buffer for 5 minutes. To avoid scratching the surface, a tweezer was used to handle the membrane. Four reasonable size-cut filter papers and two sponges were soaked in the 1x Transfer buffer for 10 minutes prior to assembly of the transfer sandwich. The transfer sandwich assembled was adopted from the Novus Biological Protein transfer from the gel to a membrane in western blot protocol, arranging on the cassette denoted-anode part:

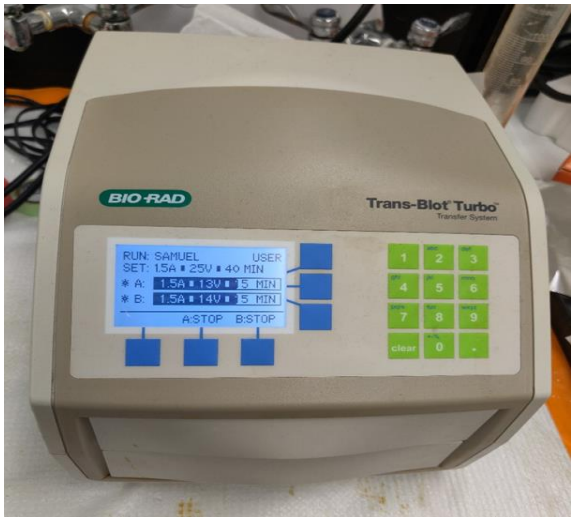
1. 2 Filter Papers

2. Nitrocellulose

3. Gel

4. 2 Filter Papers

The assembly was done in sufficient 1x Transfer buffer. The sandwich was rolled with a blotting roller to expel any air bubbles that might be trapped between the gel and the nitrocellulose membrane, also squeezing out any excess liquid. The sandwich was placed in the transfer tank with 1x Transfer buffer covering it (with which had a 'nodic gel ice pack' placed in it to maintain 4ºC. The electrodes were placed on top of the sandwich, ensuring that the gel side of the cassette holder faced the cathode (-) while the membrane side faced the anode (+). The power system (PowerPac 200 Serial No. 284BR05910) was set to run for 1 hour at 100V. [Figure 3b].



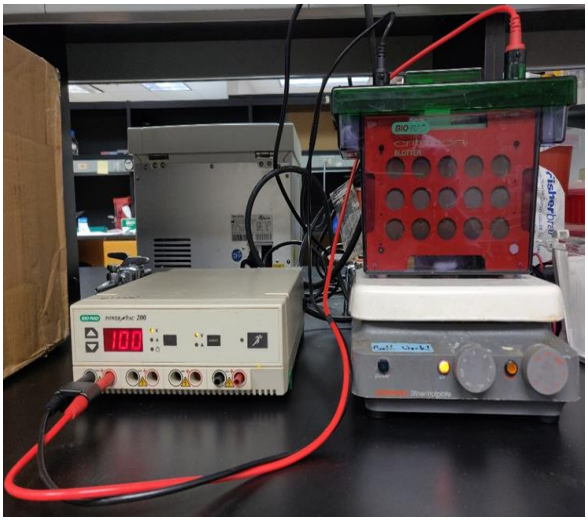


Figure No. 3a: Semi-dry transfer with Trans- Blot Turbo Transfer System **Figure No. 3b: Wet transfer**

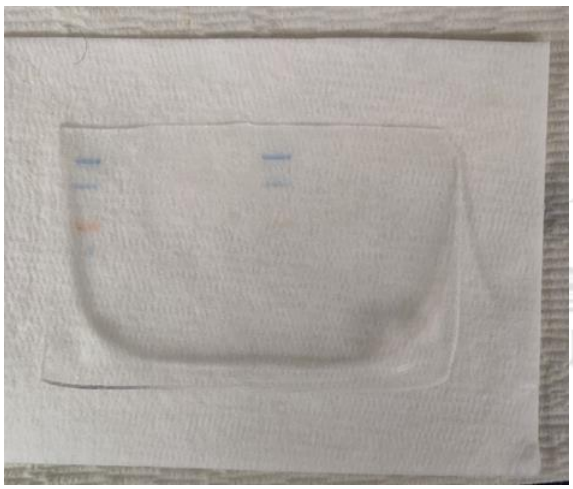


Figure No. 4a: Gel after semi-dry transfer

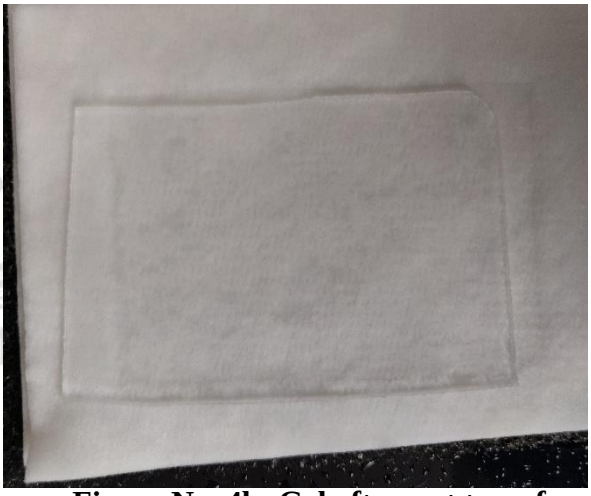


Figure No. 4b: Gel after wet transfer

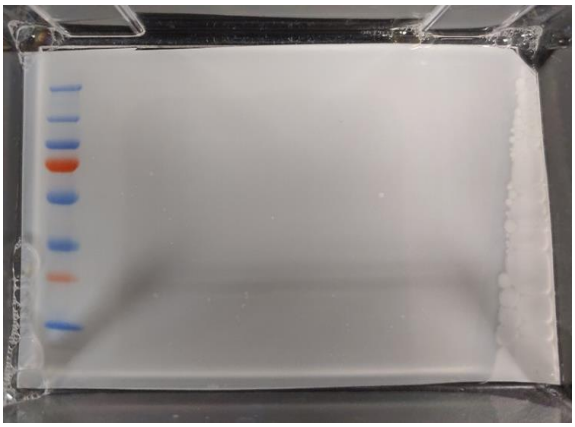


Figure No. 5a: Membrane blot after semi-dry transfer

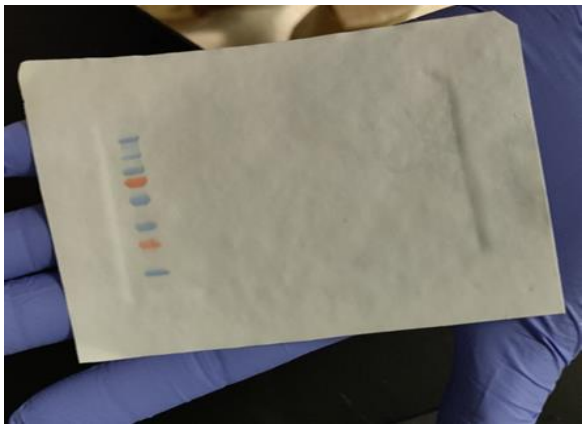


Figure No. 5b: Blot after wet transfer method

Blocking and gel staining

After running the transfer in each experiment [Figure 4a, 4b, 5a, 5b], the membrane was incubated with Blocking buffer (5% non-fat dry milk in 1xTBS) for 1 hour. The gels were stained with 20mL of EZBlue Gel Staining Reagent (Cat No. G1041) and incubated on a shaker for 1 hour. The gels were rinsed with running distilled water and allowed to soak in distilled water after incubation [Figure 6].

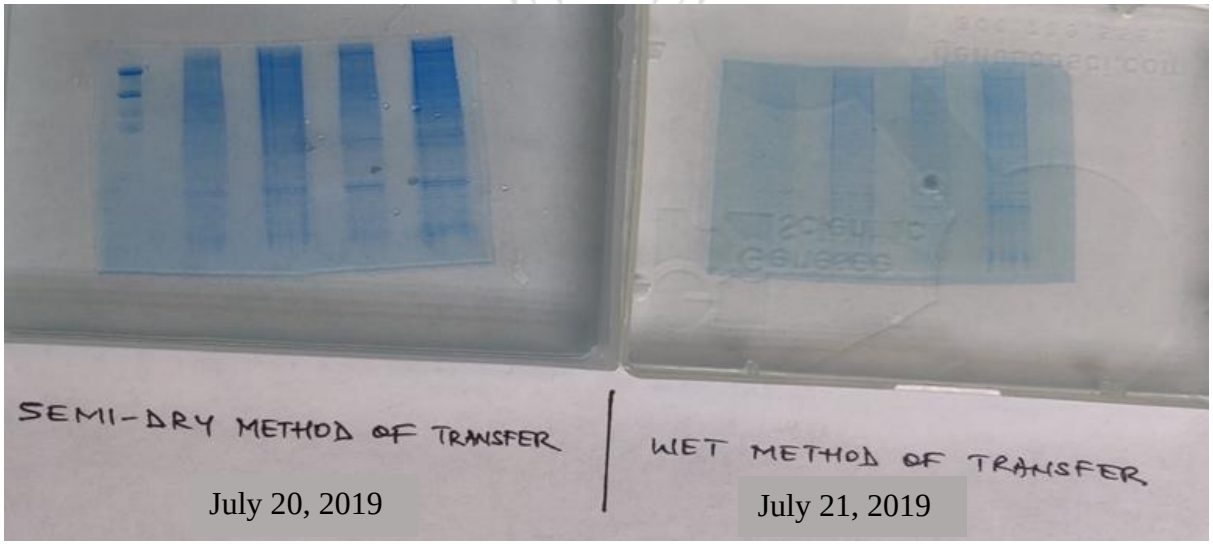


Figure No. 6: Stained gels showing the difference in lane intensity between semi-dry transfer and wet transfer

Antibody incubation

Primary antibody, 1:2000 Beta-actin (2µL Beta-actin Mouse McAb Cat No. 60008-I-Ig in 4µL 1x TBST 5% non-fat dry milk) was prepared and incubated on the membrane overnight in 4ºC, rocking gently on a shaker for each experiment conducted.

The membrane was washed with 1x TBST 2 times for 3 minutes on a shaker at room temperature to ensure even agitation. A 4µL Goat Anti-Mouse secondary antibody (Southern Biotech Cat No. 1031-05) in 8µL 1x TBST 5% non-fat dry milk was prepared and incubated on the membrane for 2 hours, rocking gently on a shaker for each experiment conducted.

The membrane was washed with 1x TBST 2 times for 3 minutes on a shaker at room temperature to ensure even agitation.

Enhanced chemiluminescence substrate and imaging

Enhanced chemiluminescence (ECL) mix (Cell Signaling Technology Cat No. 6883P3) was prepared following the proportion of 1:1 reagent A and B provided by the manufacturer. The ECL mix was incubated on the membrane for 1 minute.

Films were exposed to the membrane for each experiment at 5 minutes and 1 second to detect the protein bands and visualize the result in the dark room, marking out the molecular weight by lining up the developed film over the lot to visualize and indicate the ladder [Figure 7a and 7b].

RESULTS AND DISCUSSION

The image developed from the ran western blot showed a better detection of protein from the wet transfer method blot than the semi-dry transfer method [Figure 7a and 7b].

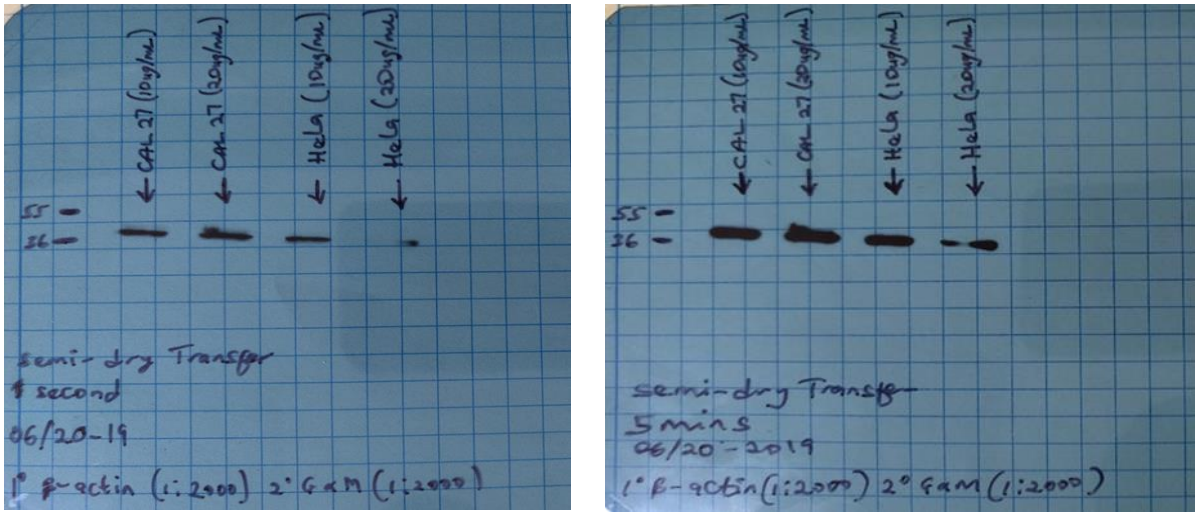


Figure No. 7a: Films showing the protein band intensity after exposure to the membrane blot from semi-dry transfer

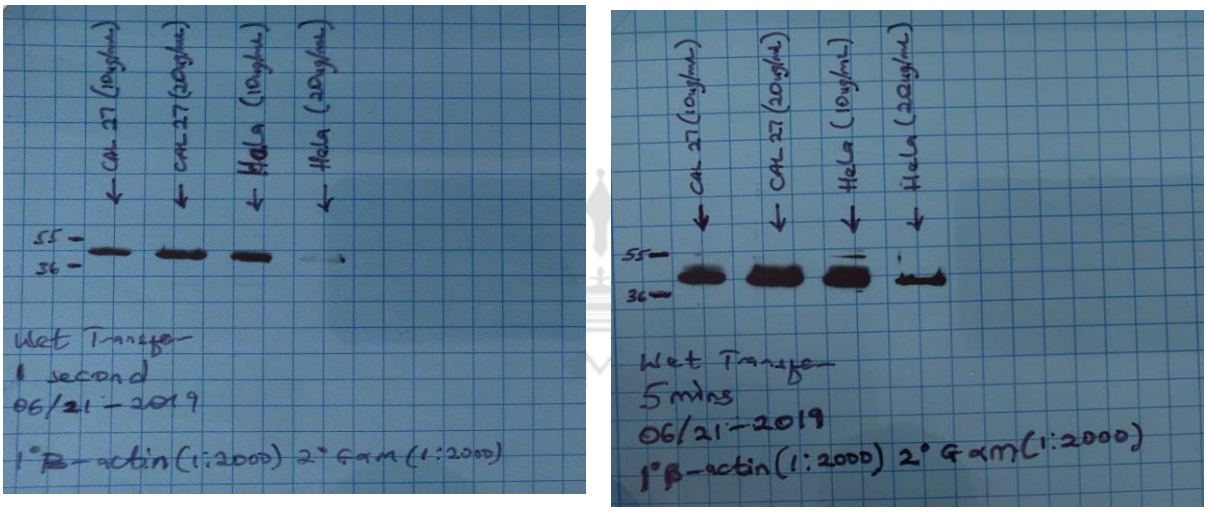


Figure No. 7b: Films showing the protein band intensity after exposure to the membrane blot from wet transfer

Troubleshooting

As earlier stated, the loss of protein detection was a huge concern to us as to researchers using the western blot technique. There are several factors that have been observed to account for this problem. These factors include using inappropriate secondary antibody, wrong concentration of antibody or low affinity to the target protein, antibody losing its activity due to long term or improper storage, the specific antigen not expressed in the source sample, not enough antigen loaded on the gel with regards to concentration, transfer not working

properly, washes being too stringent, blocking agent interfering with signal, and ECL detection reagents been contaminated (www.bio-rad-antibodies.com).

Each of these factors except for transfer technique, which we had been running semi-dry transfer, was critically assessed and addressed, varying these factors and observing the same result-either weak signal or no band detection. For example, we used a fresh sample which had been kept on ice for each Western blot. Also, freshly prepared antibodies were used for each incubation as suggested by Mahmood and Yang, 2012. Although according to Mahmood and Yang, 2012, increasing the washing time helps decrease the background, we reduced the washing time to prevent excessive loss of the blotted protein on the membrane. Other recommendations suggested according to www.bio-rad-antibodies.com were followed to maximize protein band detection but proved futile.

In fixing this problem, we decided to compare the effectiveness of semi-dry transfer method to wet transfer method. It is interesting that after comparing the stained gels from both semi-dry and wet transfer methods, the gel from the wet transfer method showed more of the proteins blotted onto the membrane than the gel from the semi-dry transfer method [Figure 8a and 8b].

Another Western blot was run using protein extracted from Hela (used as control), laryngeal cancer tissues labeled UM-SCC-98 and UM-SCC-12 at 10µg/mL and 20µg/mL for each sample respectively. Using the wet transfer method, and the consecutive steps stated above, protein bands were detected compared to previous experiments using the same samples where weak and subsequently loss of protein band was observed. Stained gel and developed film corresponding to Hela, UM-SCC-98, and UM-SCC-12 at 5µg/mL and 10µg/mL for each sample is shown in Figure 8.

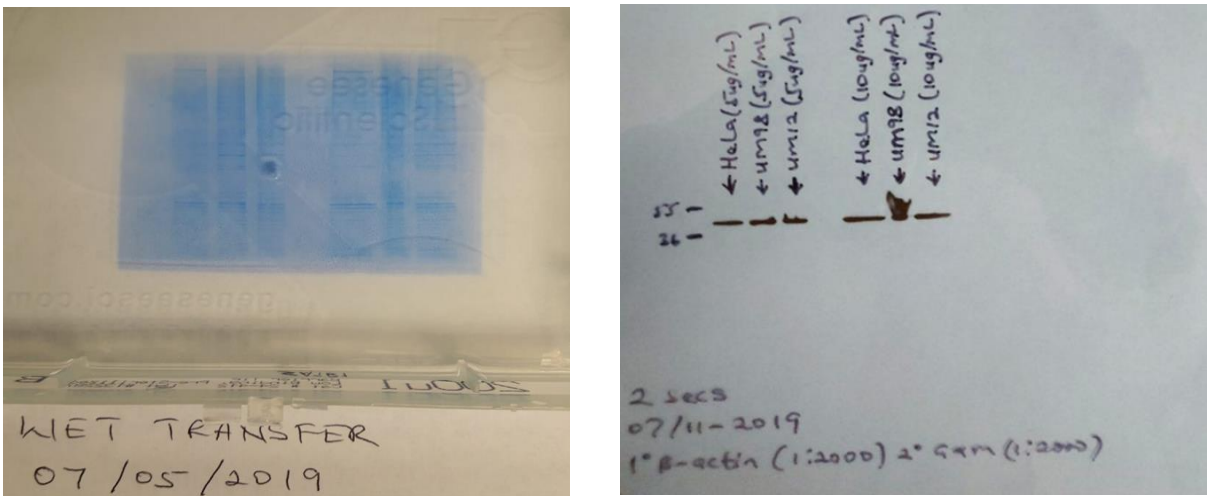


Figure No. 8: Stained gel from wet transfer and developed film showing Hela, UM-SCC-98 and UM-SCC-12 confirming successful western blotting after troubleshooting

CONCLUSION

Western blot is undoubtedly a useful technique for protein detection, allowing for qualitative and quantitative expression. In this article, modified western blot protocol used in our lab was covered while outlining some problems encountered by researchers using Western blot and troubleshooting the transfer blotting by comparing the semi-dry transfer method to the wet transfer method. We suggested the more preferred transfer method, wet transfer, due to the observed optimization of protein blotting onto the membrane and subsequent strong protein band detection.

Conflicts of Interest

The authors declare no conflicts of interest.

REFERENCES

1. Mahmood T., Yang P. Western blot: Technique, theory, and trouble shooting. North Am J Med Sci 2012; 4: 429-34.
2. Butler T.A.J., Paul J.W., Chan E.-C., Smith R., and Tolosa J.M. Misleading Westerns: Common Quantification Mistakes in Western Blot Densitometry and Proposed Corrective Measures. BioMed Research International 2019; 2019: 1-15.
3. https://www.bio-rad-antibodies.com/western-blot-no-bands-western-blotting.html, 07/15/2019 12:40 PM.