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Western Blotting (WB) Technique Guideline for Separation and Isolation of Protein

Rachmat Hidayat[1*], Patricia Wulandari[2]

[1] Department of Biology, Faculty of Medicine, Universitas Sriwijaya, Palembang, Indonesia
[2] Cattleya Mental Health Center, Palembang, Indonesia

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***Corresponding author:**
Rachmat Hidayat

E-mail address:
dr.rachmat.hidayat@gmail.com

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ABSTRACT

Western blotting is an important technique used in cell and molecular biology. Using the western blot, researchers can identify specific proteins from the complex mixture of proteins extracted from cells. This technique uses three elements to accomplish this task: (1) separation by size, (2) transfer to a solid support, and (3) marking target proteins using appropriate primary and secondary antibodies to visualize. This paper will attempt to explain the techniques and theory behind western blot, and offer several ways to solve the problem

1. Introduction

Western blots are often used in research to separate and identify proteins. In this technique the protein mixture is separated by molecular weight, and thus by type, via gel electrophoresis. These results are then transferred to the membrane producing a band for each protein. The membrane is then incubated with specially labeled antibodies for the desired protein.

Sample preparation

Cell lysates are the most common form of sample used for western westerns. Protein uptake tries to collect all of the protein in the cell cytosol. This should be done in cold temperatures with a protease inhibitor to prevent protein denaturation. Since tissue samples display a higher level of structure, mechanical discoveries, such as homogenization, or sonication are required to extract proteins.

After extracting protein, it is very important to have a good idea of the extract concentration. This ultimately allowed the researcher to ensure that the samples were compared on an equal basis. Protein concentrations are often measured using a spectrophotometer. Using these concentrations

it is possible to measure the mass of the protein being loaded into each well by the relationship between concentration, mass and volume.

After determining the appropriate sample volume, it is diluted into the loading buffer, which contains glycerol so that the sample sinks easily into the gel well. The tracer dye (bromophenol blue) was also present in the buffer which allowed researchers to see how far the separation had progressed. The sample is heated after being diluted into the loading buffer, to change the higher frame structure, while maintaining the sulfide bridge. The high denaturing of the structure ensures that the negative charge of the amino acids is not neutralized, allowing the protein to move in an electric field (applied during electrotransfer).

It is also very important to have positive and negative controls for the sample. For positive control, a known source of the target protein, such as purified protein or control lysate is used. This helps to confirm protein identity, and antibody activity. Negative controls are zero cell lines, such as β-actin, used also to confirm that the staining is nonspecific.

Gel electrophoresis

Western blots use two different types of agarose gel: stacking and separating the gel. Increasingly, the gel stack was slightly acidic (pH 6.8) and had a lower concentration of acrylamide to create a porous gel, which separates proteins poorly but allows them to form thin, sharp bands. The lower gel, called the separating or regulatory gel, is basic (pH 8.8), and has a higher polyacrylamide content, making the pores of the gel narrower. Proteins are thus separated by their deeper size in this gel, because the smaller protein travels easier, and therefore faster, than the larger protein.

Proteins when loaded on the gel have a negative charge, because they are denatured by heating, and will travel towards the positive electrode when a voltage is applied. Gel is usually made by pouring it between two plastic cups or plates, using the solution described in the protocol section. Samples and markers are loaded into the well, and empty wells are loaded with sample buffers. The gel is then connected to a power supply and allowed to run. Voltage is very important, as high voltages can overheat and distort the ribbon.

Blotting

After separating the protein mixture, it is transferred to the membrane. The transfer is carried out using an electric field that is oriented perpendicular to the surface of the gel, causing the protein to exit the gel and onto the membrane. The membrane is placed between the gel surface and the positive electrode in the sandwich. The sandwich contains a fiber pad (sponge) at each end, and filter paper to protect the gel and blotting membrane [Figure 12]. Here two things are very important: (1) close contact of the gel and membrane to ensure a clear image and (2) placement of the membrane between the gel and the positive electrode. The membrane must be placed in such a way that negatively charged proteins can migrate from the gel to the membrane. This type of transfer is called electrophoretic transfer, and can be carried out in semi-dry or wet conditions. Wet conditions are usually more reliable because they tend to dry out the gel, and are preferable for larger proteins.

The membrane, the strong support, is an

important part of this process. There are two types of membranes: nitrocellulose and PVDF. Nitrocellulose is used for high affinity for protein and retention ability. However, it is brittle, and does not allow the membrane to be used for reprobing. In this case, the PVDF membrane provides better mechanical support and allows the stain to be repurposed and stored. However, the background is higher in PVDF membranes and therefore, careful washing is essential.

Washing, blocking and incubation of antibodies

Blocking is a very important step of western blotting, as it prevents antibodies from binding to the nonspecific membrane. Blocking is often done with 5% BSA or dry nonfat milk diluted in TBST to reduce the background

Lean powdered milk is often preferred because it is cheap and widely available. However, milk protein is not compatible with all detection labels, so care must be taken to choose the right blocking solution. For example, BSA blocking solutions are preferably labeled with biotin and AP antibodies, and antiphosphoprotein antibodies, because milk contains casein, which is also a phosphoprotein and biotin, thereby interfering with the test results. It is often a good strategy to incubate primary antibodies with BSA because they are usually required in higher amounts than secondary antibodies. Placing them in the BSA solution allows the antibodies to be reused, if the stain does not produce good results.

Antibody concentration depends on the manufacturer's instructions. Antibodies can be diluted in a wash buffer, such as PBS or TBST. Washing is very important because it minimizes background and removes unbound antibodies. However, the membrane should not be washed for a very long time, as this can also reduce the signal.

The membrane is then detected using labeled antibodies, usually with an enzyme such as horseradish peroxidase (HRP), which is detected by the signal it generates according to the position of the target protein. This signal is captured on film that is usually developed in a dark room.

Count

It's important to recognize that data generated with western blots are usually considered semi-quantitative. This is because it provides a comparison of relative protein content, but not a measure of absolute quantity. There are two reasons for this; first, there are variations in loading and transfer rates between samples on separate separate lines on separate blots. These differences need to be standardized before more precise comparisons can be made. Second, the signal generated by detection is not linear across the sample concentration range. Thus, since the resulting signal is not linear, it should not be used to model concentration.

Problem solving

Although the procedure for the western blot is simple, many problems can arise, which lead to unexpected results. The problems can be grouped into five categories: (1) unusual or unexpected bands, (2) no bands, (3) faint bands or weak signals, (4) background bands that are high on smudges, and (5) a) evenly or uneven spots on the stain.

Unusual or unexpected bands can be caused by protease degradation, which results in the bands at unpredictable positions. In this case, it

is advisable to use fresh samples that have been stored on ice or altered antibodies. If the protein appears to be in a too high position, then reheating the sample can help break down the quaternary protein structure. Likewise, the opaque tape is often caused by the high tension or air bubbles present during the transfer. In this case, it must be ensured that the gel is run at a lower voltage, and that the transfer sandwich is properly prepared. In addition, changing the running buffer can also help with the problem. The nonflat bands can be the result of too fast traveling through the gel, due to their low resistance. To overcome this the gel must be optimized to fit the sample. Finally, the white (negative) band on the film is caused by too much protein or antibody.

Another problem: no band can also arise for many reasons related to the antibody, antigen or buffer used. If inappropriate antibodies are used, either primary or secondary, the bands will not appear. In addition, the antibody concentration must also be appropriate; if the concentration is too low, the signal may not be visible. It is important to remember that some antibodies are not used for western blots. Another reason for not seeing the bands is the lowest concentration or absence of the antigen. In this case, antigens from other sources can be used to confirm whether the problem lies with the sample or with other elements, such as antibodies. In addition, prolonged washing can also degrade the signal. Buffers can also contribute to the problem. It must be ensured that buffers such as transfer buffer, TBST, running buffer and ECL are all new and uncontaminated. If the buffer is contaminated with sodium azide, it can deactivate HRP.

Likewise, a weak signal can be caused by low concentrations of antibodies or antigens. Increasing the exposure time can also help make the bands clearer. Another reason is that nonfat dry milk masks the antigen. In this case, use BSA or reduce the amount of milk used.

The high background is often caused by too high a concentration of antibodies, which can bind to the PVDF membrane. Another problem is the buffer, which may be too old. Increasing the wash time can also help reduce the background. Apart from that, exposure that is too high can also cause this problem. Therefore, it is advisable to check different exposure times to achieve the optimal time.

Patchy and patchy spots on blemishes are usually the result of improper transfers. If there are air bubbles trapped between the gel and the membrane, they will appear darker on the film. It is also important to use a shaker for all incubations, so that there is no uneven agitation during incubation. Again, washing is most important as well as washing the background. This problem can also be caused by antibodies binding to an inhibiting agent; in this case another blocking agent should be tried. Filtering blocking agents can also help remove some contaminants. Finally, this problem can also be caused by secondary antibody aggregation; in this case, the secondary antibody must be centrifuged and filtered to remove aggregates.

The unbound antibody is washed leaving only the antibody bound to the desired protein. The bound antibody is then detected by expanding the film. Since the antibody binds to only the desired protein, only one band should be visible. Band thickness according to the amount of protein present; thus doing the standard can indicate the

amount of protein present. This paper will describe the protocol for western blot, accompanied by pictures to help readers and theorists to rationalize the protocol. This will be followed by a theoretical explanation of the procedure, and in the next section, troubleshooting tips for common problems. Cell lysis technique for extracting protein Proteins can be extracted from various types of samples, such as tissues or cells.

Below is a protocol for extracting proteins from adherent cells.

1. Wash cells in a flask or tissue culture dish by adding cold phosphate buffered saline (PBS) and shake gently. Discard PBS. (Tip: Keep the tissue culture dish on top of the ice).
2. Add PBS and use a cell scraper to remove cells. Pipette the mixture into the microcentrifuge tube.
3. Centrifuge at 1500 RPM for 5 minutes and discard the supernatant.
4. Add 180 µL of cold cell lysis buffer with 20 µL of fresh protease inhibitor cocktail. (Tip: If the protein concentration is not high enough at the end, it is advisable to repeat the procedure with a higher proportion of the protease inhibitor cocktail)
5. Incubate for 30 minutes on ice, and then clarify the lysate by turning for 10 minutes at 12.000 RPM, at 4 °C.
6. Transfer supernatant (or protein mixture) to a fresh tube and store on ice or freeze at -20 °C or -80 °C
7. Measure the protein concentration using a spectrophotometer.

Sample Preparation

$$\textbf{Use, Concentrate } = \frac{massa}{volume}$$

1. Determine the volume of protein extract to ensure 50 µg in each hole.
2. Add 5 µL of sample buffer to the sample, and make the volume in each row equalized using double distilled H2O (dd H_2O). Mix it well. (Tip: A total volume of 15 µL per row is recommended).
3. Heat the sample with a dry plate for 5 minutes at 100 ° C.

Gel Preparation

1. After preparing a 10% batching gel solution, install a rack for compacting the gel [Figure 1]. (Tips: 10% AP and TEMED solidifies the solution; therefore, both gels can be prepared at the same time, if the aforementioned reagents are not added to the end).
2. Add the stacking gel solution carefully until it is level with the green stick holding the glass plate [Figure 2]. Add H_2O to the top. Wait 15-30 minutes for the gel to rotate to solidify. (Tip: Using a suction pipette can make the process of adding gel to a glass plate easier)
3. Overlay the buildup gel with the separating gel, after removing the water. (Tip: It is better to tilt the utensils and use paper towels to remove water).
4. Insert the comb, making sure there are no air bubbles.
5. Wait for the gel to solidify. (Tip: Solidification can be easily checked by leaving some of the gel solution in the tube).

Electrophoresis

1. Pour the run buffer into the electrophorator [Figure 3].
2. Place the gel in the electrophorator and plug it into the power supply. (Tip: When connecting

to a power source always connect red to red, and black to black).

3. Make sure the buffer completely covers the gel, and carefully lift the comb.
4. Marker load (6 µL) followed by samples (15 µL) to each well [Figure 4].
5. Run the gel at low voltage (60 V) to separate the gel; use a higher voltage (140 V) to accumulate the gel [Figures 5a and 5b].
6. Run the gel for approximately one hour, or until the dye front has drained from the base of the gel [Figure 6].

Electrotransfer

1. Cut 6 sheets of filters to fit the gel size, and one polyvinylidene fluoride (PDVF) membrane of the same dimensions.
2. Moisten the sponge and filter paper in transfer buffer, and moisten the PDVF membrane in methanol.
3. Separate glass plate and take the gel.
4. Make a transfer sandwich as follows
   - Sponge
   - Filter Paper
   - PVDF Gel
   - Filter Paper

   (Tip: Make sure there are no air bubbles between the gel and the PVDF membrane, and squeeze out the extra liquid)
5. Relocate the sandwich to the transfer device, which must be placed on ice to maintain 4 °C. Add transfer buffer to the equipment, and ensure that the sandwich is covered with buffer. Place the electrodes on the sandwich, ensuring that the PVDF membrane is between the gel and the positive electrode [Figure 7]
6. Transfer for 90 minutes [Figure 8]. (Tip: The running time should be proportional to the thickness of the gel, so this can be reduced to 45 minutes for 0.75 mm of gel)

Antibody blocking and incubation

1. Block membrane with 5% skimmed milk in TBST * for 1 hour
2. Add primary antibody in 5% bovine serum albumin (BSA) and incubate overnight in 4 °C on shaker [Figure 9].
3. Wash the membrane with TBST for 5 minutes. Do this 3 times. (Tip: All wash and antibody incubation steps should be carried out on a shaker at room temperature to ensure even agitation).
4. Add secondary antibody in 5% skim milk at TBST, and incubate for 1 hour.
5. Wash the membrane with TBST for 5 minutes. Do this 3 times
6. Prepare the ECL mixture (following the proportion of solutions A and B provided by the manufacturer). Membrane incubation for 1-2 minutes [Figure 10]. (Tip: Use a 1000 µL pipette to ensure that the ECL covers the top and bottom of the membrane)
7. Imagine the result in a dark room [Figure 11]. (Tip: If the background is too strong, reduce the exposure time).

Recipe

1. Dissolve the following in 800 ml distilled H2O
   - 8.8 g NaCl
   - 0.2 g KCl
   - 3g Tris base
2. Add 500ul Tween-20
3. Adjust the pH to 7.4
4. Add distilled H_2O to 1L
5. Sterilize by filtering or autoclaving

2. Conclusion

The western blot is a very useful technique for protein detection as it allows the user to measure protein expression as well. This paper discusses protocols, the theory behind them, and some troubleshooting techniques. The western blot can be seen as a delicate balance, as researchers seek to derive a signal that is not specific, but strong.

Table Gel preparation

10% Stacking gel	dd H_2O	3 mL
	1 M Tris-HC1	2.1 mL (pH 8.9)
	30% Acr Bis	2.8 mL
	10% SDS	80 µL
	10% APS*	56 µL
	TEMED*	6 µL
6% Separating gel	dd H_2O	2 mL
	1M Tris-HC1	400 µL (pH 6.7)
	30% Acr Bis	600 µL
	10% SDS*	36 µL
	10% APS*	24 µL
	TEMED	4 µL

*, APS: Ammonium Persulfate ; TEMED: Tetramethylethylenediamine; SDS: Sodium dodecyl sulfate

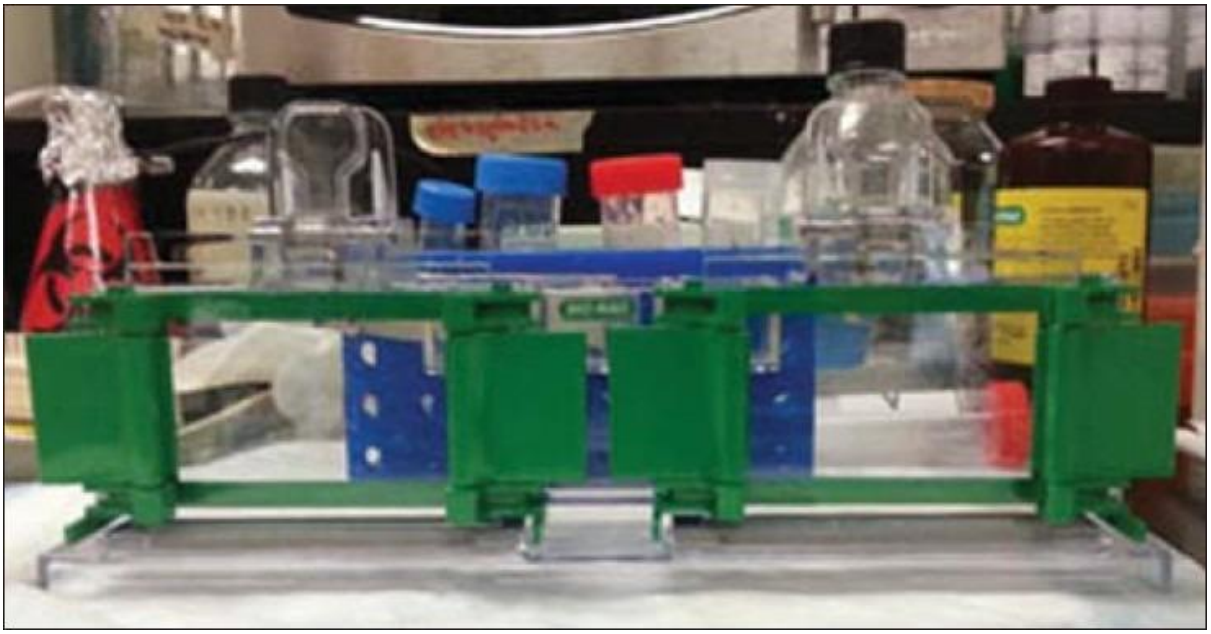

Figure 1

Figure 2

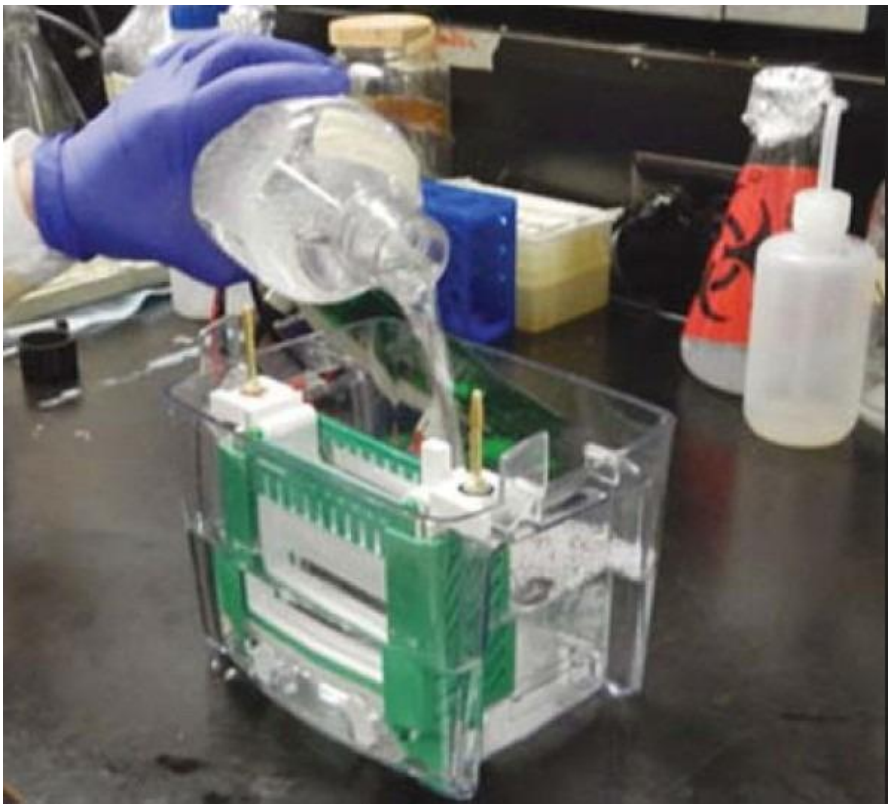

Figure 3

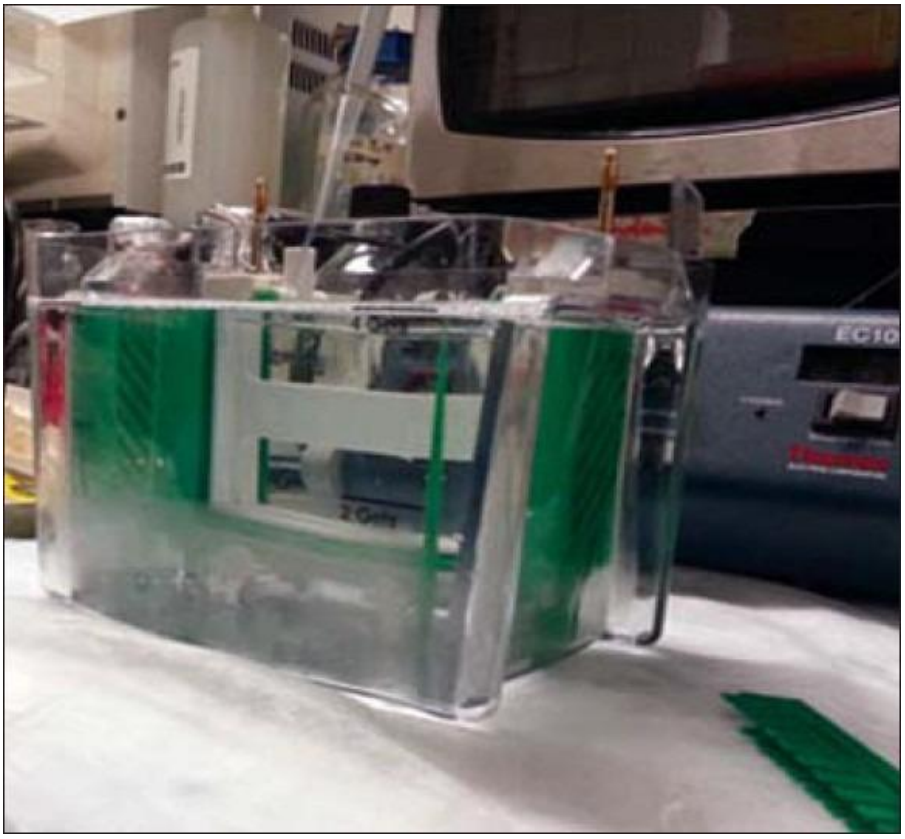

Figure 4

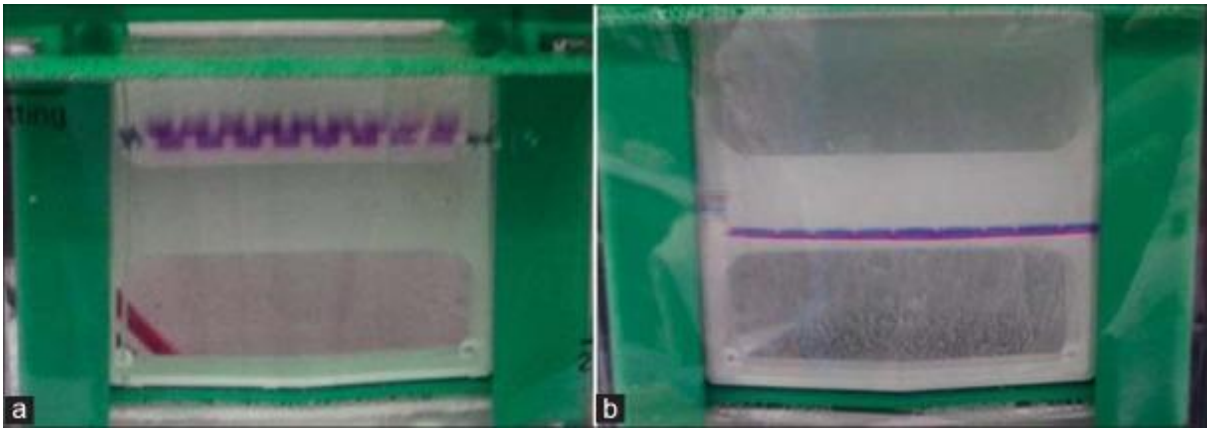

Figure 5 a and b

Figure 6

Figure 7

Figure 8

Figure 9

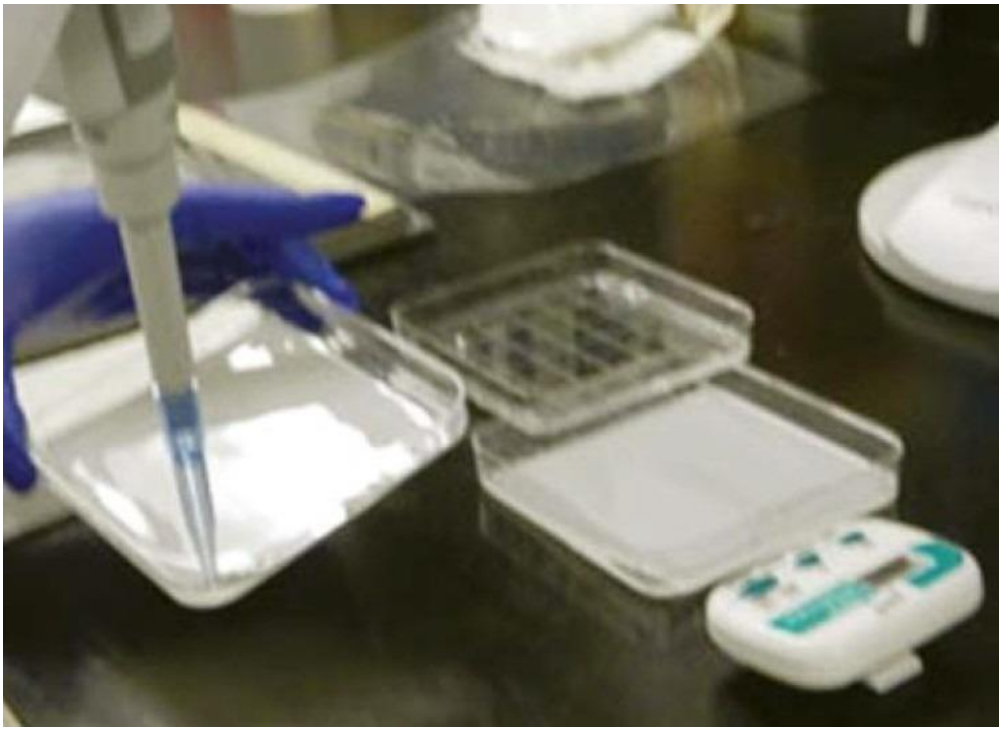

Figure 10

Figure 11

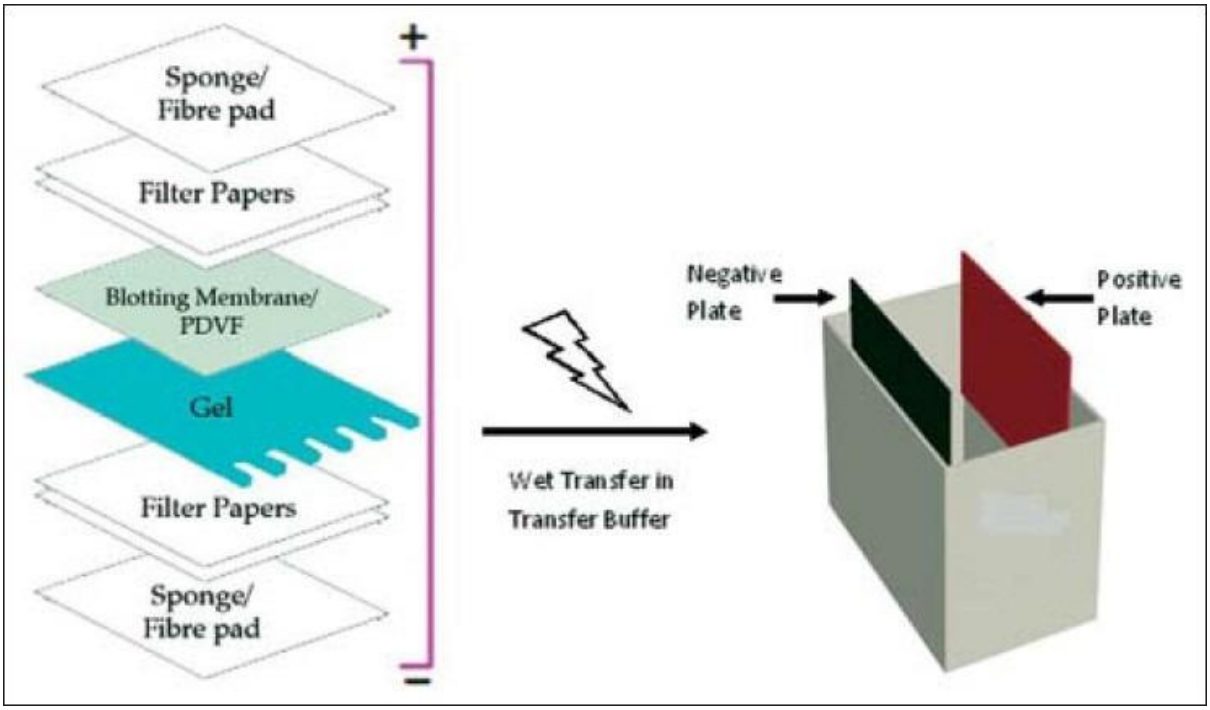

Figure 12

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