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Western Blotting a review: Principle, protocol and problem solving

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Abstract

Western blotting is a fundamental technique widely used in molecular biology and biochemistry to quantify protein expression levels and modifications in various biological samples. This study presents a detailed protocol for performing WB, focusing on the principles and steps involved in the process. The article discusses the isolation of protein samples from cultured cells and their quantification using the BCA protein assay. The importance of protein quantification is emphasized to ensure accurate loading of proteins in gel wells. A step-by-step guide is provided for gel electrophoresis, highlighting the preparation of resolving and stacking gels and the migration of proteins under an applied voltage. Furthermore, the blotting procedure is detailed, covering membrane selection and the electrophoretic transfer process. The use of blocking agents to prevent non-specific antibody binding is explained, along with the incubation of primary and secondary antibodies. The article emphasizes the significance of negative and positive controls to validate the experimental results. Lastly, common troubleshooting strategies are provided to address potential issues encountered during the Western blotting process, such as absent or weak bands, unusual patterns, and high background. In conclusion, this comprehensive Western blotting protocol offers researchers a reliable and optimized method to analyse protein expression and modifications accurately, contributing to the advancement of molecular biology and biological research.

Keywords: Sample preparation for western blot; Protein quantification; Gel electrophoresis; Western blot

1. Introduction

Western blotting (WB), also known as immunoblotting, is a primary laboratory technique widely employed to analyze protein expression and post-translational modifications in biological samples. This powerful method relies on the specific binding of antibodies to target proteins, allowing researchers to detect and quantify proteins of interest. WB involves several intricate steps, from protein sample isolation and gel electrophoresis to antibody incubation and signal detection. In this comprehensive guide, we will walk you through the principles, procedures, and common troubleshooting tips associated with Western blotting. [1]

1.1. Principle

The technique of WB is basically used to quantify the expression level of the proteins and their modification in homogenized tissue samples and lysed cell-lines. WB depends on the principle of availability of a specific antibody paratope against the epitope of antigen (protein of interest). During WB, the antibodies which were used directed not only against the protein of interest but also their specific chemical modifications including glycosylation and phosphorylation of the amino acid residues present in them. WB includes the denaturation of the proteins in the homogenized tissue samples and lysed cell-lines which are further subjected to acrylamide gel electrophoresis by which they are separated based on size, and further transferred on to a nylon membrane. The membrane is further incubated with the antibody which reacts with the protein of interest on the membrane by binding to its specific antigen. The

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resulting Antigen-Antibody complex is generally visualized using a chemiluminescent assay system by darkening X-ray films or by using a gel documentation system. [1]

2. Protein sample isolation from cultured cells

The samples used during WB are prepared commonly by sonication or homogenization of the cells or tissue. Protein extraction technique helps to break the cell so that the cytosolic proteins can be collected. The isolation of the protein is done at a lower temperature and the protein inhibitors are used to prevent the protein from denaturation. The procedure is as follows,

- The cell culture flask or well plates should be washed with 1X Phosphate-buffered saline (1X PBS) one time to wash out the growth media completely.
- *(Remark: The cells can be proceeded immediately for processing, or it can also be stored at -80* ℃ *after the plates are wrapped.)*
- The culture plate or flask must be kept in an ice bucket during the protein isolation process.
- To isolate cells from the plate or flask add 1X PBS (e.g., 1 ml in each well of a six well plate) and the cells are scrapped out completely using a scrapper.
- The scrapped content is transferred to a 1.5 ml micro centrifugation tube and the tube is centrifuged at 2000 RPM for 5 minutes at 5 ℃
- *(Remark: Flat bottom micro centrifuge tube is better choice for sonication step)*
- After centrifugation, the supernatant is discarded and 150 to 200 µl of radio immune precipitation assay (RIPA) buffer is added to the pellet.
- *(Remark: The volume of RIPA buffer should be considered depending on the quantity of available cells for isolation. If the quantity is less, add a little volume of RIPA buffer to the pellet to increase the concentration. Also, if the quantity of cells is more and if you guess that the protein concentration might be above 2000 µg/ml, then dilute the pellet with more RIPA buffer.)*
- Using probe sonicator, homogenise the tube till the mixture turns milky white in colour.
- After sonication, the tubes should be incubated left in ice or at 2-8 ℃ for 1 hour.
- Now, centrifuge the sample at 14000 RPM for 30 minutes at 5 ℃.
- Transfer the supernatant which contains the protein to another vial and discard the pellet. Store the protein sample at -80 ℃.

3. BCA protein quantification assay

(Kit Used-ThermoScientific Pierce™ BCA Protein Assay Kit)

This is a high-precision, detergent-compatible protein assay for determination of protein concentration in the sample. By using this concentration, the mass of protein which must be loaded in the well can be quantified by evaluating the relationship between concentration, mass, and volume.

3.1. Standard preparation

Table 1 Preparation of standards

Standard	Volume of Diluent (µl)	Volume of Stock (µl)	Final Concentration (µg/ml)
A	0	300 from stock	2000
B	125	375 from stock	1500
C	325	325 from stock	1000
D	175	175 from Std. B prep.	750
E	325	325 from Std. C prep.	500
F	325	325 from Std. E prep.	250
G	325	325 from Std. F prep.	125
H	400	100 from Std. G prep.	25

I	400	0	Blank

(Remark: The diluent used for standard preparation is RIPA buffer because the same solution is used for the protein isolation also. The blank's concentration must be zero and if not, subtract the blank value from standards and samples value also.)

3.2. Preparation of working reagent

- Working reagents constitute Solution A (50 part) and Solution B (1 part).
- Per ELISA well plate 200 µl of working reagent is added according to protocol.
- Prepare working reagent total volume according to the number of wells planned to be utilized.
  (Example: Actually, we need to put 8 standards and 1 blank in duplicates, and let's say we have 6 samples. So, it is 24 wells in total. For that we need to prepare a working reagent for a total of 25 wells. And the volume is Solution A (5 ml), and Solution B is (100 µl).

3.3. Procedure

- 200 µl of working reagent should be added to all the wells.
- 10 to 25 µl of sample/ standard should be added to the respective wells with reference to ELISA plate map.
- Incubate the ELISA plate at 37 ℃ for 30 minutes, wrapped in aluminium foil.
- Measure the optical density using ELISA reader between 540 to 590 nm.

Table 2 ELISA plate map with optical density value measured

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	Std A (1.222)	Std A (1.1142)	Blk I (0.1871)									
B	Std B (0.9298)	Std B (0.9619)	Blk I (0.1871)									
C	Std C (0.7219)	Std C (0.7199)	Smp 1 (0.7588)									
D	Std D (0.5798)	Std D (0.5984)	Smp 2 (0.761)									
E	Std E (0.463)	Std E (0.4696)	Smp 3 (0.7686)									
F	Std F (0.3511)	Std F (0.3286)	Smp 4 (0.6946)									
G	Std G (0.257)	Std G (0.2539)	Smp 5 (0.1012)									
H	Std H (0.1945)	Std H (0.22)	Smp 6 (0.7528)									

(Remark: The average of standards and the sample value must be subtracted with the blank value and the standard graph is plotted using a linear trendline using MS Excel. Once the graph is plotted, we get the formula to calculate the unknown concentration of the samples)

3.4. Calculation

Once the standard graph is plotted, the formula is generated in excel,

$$(y = mx + c)$$

$$\mathbf{y = 0.0005\ x + 0.0282}$$

Where,
y is the optical density
x is the unknown concentration

Therefore, x = (y – 0.0282)/0.0005

Table 3 Unknown concentration of samples

Sample	OD	Blank adjusted	Concentration (µg/ml)	Concentration (µg/µl)	Sample volume in µl that contains 15 µg of protein	Sample volume in µl that contains 20 µg of protein
1	0.7588	0.5763	1096.2	1.0962	13.68	18.24
2	0.761	0.5785	1100.6	1.1006	13.63	18.17
3	0.7686	0.5861	1115.8	1.1158	13.44	17.92
4	0.6946	0.5121	967.8	0.9678	15.50	20.67
5	0.1012	-0.0813	The sample contains less amount protein and hence cannot be used			
6	0.7528	0.5723	1088.2	1.0882	13.78	18.38

(Remark: If the sample concentration is above 2000 µg/ml, then the sample must be diluted with RIPA because the highest standard concentration is 2000 µg/ml. And if the sample concentration is very less, we cannot use it for gel loading.)

4. Gel electrophoresis

Two different types of polyacrylamide gels are used in WB i.e., stacking and separating gel. The stacking gel is quite acidic (pH 6.8) and has lesser concentration of acrylamide, making the gel porous so the protein separates very poorly, while the resolving gel is comparatively basic (pH 8.8), with higher polyacrylamide content with narrow pore size. Therefore, the protein of interest is separated based on its size separating the smaller proteins faster than the larger proteins. When a voltage is applied, the loaded proteins having a negative charge on the gel will travel and move toward the positive electrode. In general, the gels are prepared by using the solution containing Tris-HCl, SDS, Acrylamide/Bis-acrylamide, ammonium persulfate and Tetramethyl ethylenediamine (TEMED). Along with samples and a marker loading into the wells, the power supply is provided, and the gel is run at a voltage that cannot overheat and distort the bands.

4.1. Buffers and chemicals needed

- Phosphate-buffered saline (1X) (1 Litre)
  - Measure 800 ml of distilled water
  - Add 8 g of NaCl
  - Add 200 mg KCL
  - Add 1.44 g of Na_2HPO_4
  - Add 240 mg KH_2PO_4
  - Adjust pH to 7.4
  - Make up the buffer to 1 litre using distilled water
    (Storage: Room temperature.)
- RIPA buffer (Hi-media)
  - (Storage – 2 to 8 ℃)
- Tank buffer or running buffer (1X) (1 Litre)
  - Measure 1000 ml of distilled water
  - Add 3 g of Tris base
  - Add 14.4 g of Glycine
  - Add 1 g of sodium dodecyl sulphate
    (Storage: Room temperature. Buffer loses stability after 3-4 runs.)
- Transfer buffer (1X) (1 Litre)
  - Measure 600 ml of distilled water
  - Add 200 ml of carbinol/methanol
  - Add 3 g of Tris base
  - Add 14.4 g of glycine
  - Make up the buffer to 1 litre using distilled water
    (Storage: 2 to 8 ℃. Buffer can be stored for a longer period.)
- Tris HCL (1.5 M) (100 ml)

- Measure 70 ml of distilled water
- Add 23.64 g of Tris HCL
- Adjust pH to 8.8
- Make up the solution to 100 ml using distilled water
  (Storage: 2 to 8 ℃)

- Tris HCL (1 M) (100 ml)
  - Measure 70 ml of distilled water
  - Add 15.76 g of Tris HCL
  - Adjust pH to 6.8
  - Make up the solution to 100 ml using distilled water
    (Storage: 2 to 8 ℃)
- 30% Acrylamide (100 ml)
  - Measure 100 ml of distilled water
  - Add 29 g of Acrylamide
  - Add 1 g of Bis-acrylamide
  - Use magnetic stirrer for mixing
  - Filter using Whatman paper
    (Storage: 2 to 8 ℃. Store it in a brown bottle.)
- 10% Sodium dodecyl sulphate (50 ml)
  - Measure 50 ml of distilled water
  - Add 5 g of Sodium dodecyl sulphate
  - Use magnetic stirrer for mixing
    (Storage: Room temperature.)
- 10% Ammonium persulphate (1 ml)
  - Measure 1mL of distilled water
  - Add 100 mg of Sodium dodecyl sulphate
    (Storage: 2 to 8 ℃. Store aluminium foil wrapped. Prepare fresh for usage)
- TEMED (Commercial)
  - (Storage: Room temperature)
- Loading buffer (2X) (10 ml)
  - Add 2.5 ml of distilled water
  - Add 197 mg of Tris HCL to the above (That makes 2.5 ml of 0.5 M Tris HCL pH 6.8)
  - Add 4 ml of 10 % Sodium dodecyl sulphate
  - Add 2 ml of Glycerol
  - Add 2 mg of Bromophenol blue
  - 500 µl of 2-Mercaptoethanol/β-mercaptoethanol or 310 mg of Dithiothreitol
  - Make up the solution to 10 ml using distilled water and stir using magnets.
    (Storage: -20 ℃ in aliquots)

4.2. Gel casting procedure

- Set the glass plates with spacers using clips support and seal the bottom using cello tapes.
- Meanwhile prepare fresh 10 % ammonium persulphate according to standard protocol.
- Resolving gel preparation: Prepare 12 % resolving gel for 10 ml (refer to the annexure 1 table1) Add extra TEMED if necessary and allow (15-20 minutes) the mixture to solidify without disturbing.
- After solidification add 100 µl of carbinol or N-butanol above the resolving gel to get a straight line. Leave it for 2-3 minutes and then wash it off using water twice to remove the carbinol.
- Stacking gel preparation: Prepare stacking gel 5 ml (refer to the annexure 1 table2) and pour the mixture above the solidified resolving gel and wait for 10-15 minutes after placing the comb.
- After solidification, place the gel in a tray containing a running buffer and remove the comb and spacer. Load the running buffer to the electrophoresis unit and fix the gel to it.

4.3. Protein sample preparation and Gel loading

The proteins are diluted using the loading buffer. The glycerol in the buffer makes the samples sink easily due to the density into the gel wells. The loading buffer also constitutes a tracking dye (bromophenol blue) which enables the researcher to track the position of samples inside the gel. To denature the proteins and break their secondary, tertiary and quaternary structures without disturbing the peptide bonds, the samples are heated after dilution. This denaturation helps in sustaining the transfer of negatively charged proteins under the influence of electric field. A

positive and negative control is also needed for the sample and the purified protein sample or control lysates are used as positive controls, which help in confirming the protein identity and the activity of antibody. β-actin/GAPDH is used as a negative control to confirm the nonspecific staining in the blots. The procedure is as follows,

- Pre-warm water bath at 95 ℃ and defrost samples by leaving on ice or keep it at 2-8 ℃
- Calculate the volume of samples to get 15 or 20 µg of protein.
- Label 1 ml tubes, add required volume of sample and then add equal volume of 2X loading buffer to that of sample to make it 1X.
- Vortex the mixture and spin in a spinning thing.
- Make sure all lids are closed properly and warm them into the water bath for 5 minutes.
- Spin samples after heating to get the full volume.
- Add 5 µl protein ladder to the first well of the gel followed by a full volume of samples and run the electrophoresis unit at 100 V.
- After the dye front crosses the stacking gel switch the voltage to 150 V. Roughly the dye front takes 2 hours of time to reach the bottom of the resolving gel.

4.4. Blotting

After separating the protein on the resolving gel, it is transferred to a membrane. The transfer occurs perpendicular to the surface of the gel using an electric field, causing the proteins to move onto the membrane from the resolving gel. The membrane is sandwiched between the resolving gel surface and the positive electrode which includes a sponge pad at both ends, and filter papers to protect the blotting membrane and the gel. The membrane is kept in a way so the transfer of the negatively charged proteins occurs freely from the resolving gel on to the membrane and this type of transfer is known as electrophoretic transfer. The membrane used should give solid support and plays an essential role in the electrophoretic transfer process. Two types of membranes are generally used in WB i.e., nitrocellulose and polyvinylidene difluoride (PVDF). Nitrocellulose membrane offers high affinity and retains the proteins but is quite brittle and doesn't offer re-probing. In this respect, PVDF membranes gain an upper hand due to its better mechanical support and for its re-probing and storage ability. It has a higher background possibility, which can be overcome by washing carefully. The gel transferring procedure is as follows,

- Using a metal scale like thing, open the gel. When taking the gel out, lift gently using a forceps.
- Cut the gel out and place it in a tray containing 1X transfer buffer. Cut PVDF membrane and activate it dipping in methanol.
- Get the sandwich frame support, place two rough sponge, then two soft sponges, then three Whatman paper one by one and ensure that there are no bubbles. Then place the gel on top again ensuring that there are no bubbles.
- Then place the membrane and make sure the membrane is at the correct orientation to reflect gel, again make sure no air bubble. Followed by placing Whatman paper, soft sponges and rough sponges. Close and press firmly in the middle. Make sure you close the sandwich properly.
- Put the sandwich in the transfer tank and pour in a 1X transfer buffer all over the middle back and front. Put the ice at the back of the transfer tank.
- Make sure that the gel should be on the cathode (black) electrode and the membrane must face the anode (red) electrode. Run the unit at 100 V. Wait for half an hour.

4.5. Washing, Blocking and Antibody incubation

During western blotting, blocking is a very important step which helps in preventing the non-specific binding of antibodies to the membrane, which can be made possible with 5% Bovine Serum Albumin (BSA), or non-fat dried milk diluted in TBST that reduces the background. Although non-fat dried milk is often preferred for its wide availability and inexpensive nature. Blocking solution should be used with utmost care as the milk proteins are not suitable for all detection labels. Thus, when the anti-phosphoprotein antibodies are used, the membrane is blocked using BSA because the casein protein present in milk can interfere with the results of WB. Incubation of primary antibody with BSA seems to be a good strategy because of the abundant need for the secondary antibody beside their reuse for next experiments. The antibody is diluted as per manufacturer instruction in a wash buffer, such as PBS or TBST and they are incubated along with the blots. These incubated membranes are washed accordingly to minimize background and to remove unbound antibodies. These membranes are then treated with a secondary antibody tagged with horseradish peroxidase (HRP) with the corresponding signal against specific protein of interest with signal captured on a X-ray film or by gel documentation system.

4.6. Blocking and series of washes

- 5 % Bovine serum albumin (20 ml)
  - Measure 20 ml of distilled water
  - Add 1g of BSA
  (Storage: 2 to 8 ℃. Prepare fresh for usage)
- 0.001 % PBST
  - Measure 500 ml of 1X PBS
  - Add 500 µl of Tween 20
  (Storage: Room temperature)
- Preparing the antibody: Both primary and secondary antibody must be diluted with 1 % BSA at different dilution factors.
  - Primary antibody (1:1000) (8 ml): Actually, 5 % BSA diluted to 1 % using PBST. i.e., 1.6 ml of 5 % BSA + 6.4 ml of PBST = 8 ml of 1 % BSA. Therefore, 8 ml of 1 % BSA + 8 µl of Primary antibody gives (1:1000).
  - Secondary antibody (1:5000) (8 ml): Actually, 5 % BSA diluted to 1 % using PBST. i.e., 1.6 ml of 5 % BSA + 6.4 ml of PBST = 8 ml of 1 % BSA. Therefore, 8 ml of 1 % BSA + 1.6 µl of Secondary antibody gives (1:5000).
- Enhanced chemiluminescence (ECL) (4 ml)
  - Add ECL solution A and B in equal volume
  (Storage: 2 to 8 ℃. Store aluminium foil wrapped. Prepare fresh for usage)

- Cut the membrane according to the need and immerse it completely into 5 % BSA and rock it slowly for 2 hours at room temperature.
- After 2 hours of blocking, wash the membrane 3 times by dipping it in 0.001 % PBST.
- Stock the BSA back in the fridge.
- After washing, submerge the membrane primary antibody solution prepared and rock the membrane in antibody for 2 hours slowly or this step can also be left overnight inside a cold room or at very low room temperature.
- After the primary antibody step, wash the membrane 3 times again with PBST.
- Now, put the membrane in a secondary antibody solution for 45 minutes to 1 hour and rock it slowly.
- After that wash the membrane 2 times with PBST followed by a single PBS wash. Next, immerse the membrane in water once or twice and go for visualization.
- Add ECL to the membrane and visualize the membrane under UV.

4.7. Quantification

The quantification of the WB is usually said to be semi-quantitative as it only talks about the relative comparisons of protein levels, but the absolute quantity of the protein cannot be determined as there might be the variations in the loading and transfer rates of the samples in different lanes that are different on separate blots. Secondly, the signals generated are not linear across a range of concentrations in the samples used. Hence it should be used to model the concentration.

4.8. Troubleshooting

Although the process of western blotting is simple, many issues may arise leading to unexpected results. In general, these problems can be divided into five different sets, such as the absence of bands, pale bands, or weak signal, unusual or unexpected bands, high background, and uneven spots on the blot. The absence of bands may occur due to several reasons ranging from antibody, antigen, to buffers used. The use of an improper antibody (primary or secondary) or their low concentration may result in the absence of a band on the blot beside it may also occur due to lower protein (antigen) concentration that can be confirmed through same protein from another source which confirms the real problem with the protein or an antibody.

Pale bands occur due to multiple reasons such as high voltage or air bubbles during the transfer or multiple reuses of running buffers. These issues can be overcome by simple changes such as the use of low voltage or careful placement of membrane between the resolving gel surface and the positive electrode or by preparing a fresh running buffer. The unusual or unexpected bands mainly occur due to compromised protein integrity by the proteases, for this, fresh samples should be used on ice to overcome this issue. When the position of the bands is high enough, their respective protein samples should be reheated to break the quaternary structure of the proteins. The non-flat bands, which occur due to the fast travelling of protein through the gel due to the low resistance, can be overcome by optimizing the gel used as per our sample requirements.

Additionally, prolonged washing with buffers may also lead to decreased signal beside these buffers being highly reused and contaminated with sodium azide (inactivate HRP). By using new and non-contaminated buffers running, transfer, ECL and PBST, this problem can be overcome. The issues with the weak signals can also be overcome by enhancing the concentration of antibody or antigen or increasing their respective exposure time which may help in the visualization of clear bands on the blot. Weak signals may be caused due to the masking of antigens by the non-fat dry milk, in such a case it should be replaced with BSA, or it has to be used in decreased amounts. Higher concentration of antibodies and use of old buffers can also lead to the high background, which can be overcome by increased washing time (optimised). Sometimes, the uneven and patchy blots appear because of the improper transfer of the proteins, or the air bubbles trapped in between the gel and the membrane which can be overcome by properly placing the membrane in between the gel and the positive electrodes. [2, 3]

5. Conclusion

In summary, this comprehensive guide to Western blotting has elucidated the intricate techniques, procedures, and troubleshooting strategies essential for successful protein analysis. By understanding the principles behind Western blotting, mastering the intricacies of protein sample isolation, and ensuring accurate quantification, researchers can harness the full potential of this powerful tool in molecular biology. As we move forward, this knowledge will not only advance our understanding of cellular processes and disease mechanisms but also contribute to the development of novel diagnostics and therapeutic strategies, ultimately benefiting society through improved healthcare and scientific innovation. The path ahead involves continued refinement of Western blotting protocols and the integration of emerging technologies to enhance the precision and scope of protein analysis in the pursuit of scientific excellence and societal well-being

Compliance of ethical standard

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Disclosure of Conflict of Interest

The author declare no conflicts of interest associated with the content presented in this article. This work is driven solely by the intention to provide accurate and helpful information to researchers and scientists seeking to master the techniques of Western blotting for the betterment of scientific research and societal progress.

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ANNEXURE-1

Table 1 Solutions for preparing Resolving Gels for SDS-Polyacrylamide Gel Electrophoresis

		(Vol. of components required to cast gels of indicated vol. & conc.)							
↓Components	**Gel Volume →**	**5 ml**	**10 ml**	**15 ml**	**20 ml**	**25 ml**	**30 ml**	**40 ml**	**50 ml**
6 % Gel									
H_2O		2.600	5.300	7.900	10.60	13.20	15.90	21.20	26.50

30 % acrylamide mix	1.000	2.000	3.000	4.000	5.000	6.000	8.000	10.00
1.5 M Tris (pH 8.8)	1.300	2.500	3.800	5.000	6.300	7.500	10.00	12.50
10 % SDS	0.050	0.100	0.150	0.200	0.250	0.300	0.400	0.500
10 % ammonium persulfate	0.050	0.100	0.150	0.200	0.250	0.300	0.400	0.500
TEMED	0.004	0.008	0.012	0.016	0.020	0.024	0.032	0.040
8 % Gel								
H_2O	2.300	4.600	6.900	9.300	11.50	13.90	18.50	23.20
30 % acrylamide mix	1.300	2.700	4.000	5.300	6.700	8.000	10.70	13.30
1.5 M Tris (pH 8.8)	1.300	2.500	3.800	5.000	6.300	7.500	10.00	12.50
10 % SDS	0.050	0.100	0.150	0.200	0.250	0.300	0.400	0.500
10 % ammonium persulfate	0.050	0.100	0.150	0.200	0.250	0.300	0.400	0.500
TEMED	0.003	0.006	0.009	0.012	0.015	0.018	0.024	0.030
10 % Gel								
H_2O	1.900	4.000	5.900	7.900	9.900	11.90	15.90	19.80
30 % acrylamide mix	1.700	3.300	5.000	6.700	8.300	10.00	13.30	16.70
1.5 M Tris (pH 8.8)	1.300	2.500	3.800	5.000	6.300	7.500	10.00	12.50
10 % SDS	0.050	0.100	0.150	0.200	0.250	0.300	0.400	0.500
10 % ammonium persulfate	0.050	0.100	0.150	0.200	0.250	0.300	0.400	0.500
TEMED	0.002	0.004	0.006	0.008	0.010	0.012	0.016	0.020
12 % Gel								
H_2O	1.600	3.300	4.900	6.600	8.200	9.900	13.20	16.50
30 % acrylamide mix	2.000	4.000	6.000	8.000	10.00	12.00	16.00	20.00
1.5 M Tris (pH 8.8)	1.300	2.500	3.800	5.000	6.300	7.500	10.00	12.50
10 % SDS	0.050	0.100	0.150	0.200	0.250	0.300	0.400	0.500
10 % ammonium persulfate	0.050	0.100	0.150	0.200	0.250	0.300	0.400	0.500
TEMED	0.002	0.004	0.006	0.008	0.010	0.012	0.016	0.020
15 % Gel								
H_2O	1.100	2.300	3.400	4.600	5.700	6.900	9.200	11.50
30 % acrylamide mix	2.500	5.000	7.500	10.00	12.50	15.00	20.00	25.00
1.5 M Tris (pH 8.8)	1.300	2.500	3.800	5.000	6.300	7.500	10.00	12.50
10 % SDS	0.050	0.100	0.150	0.200	0.250	0.300	0.400	0.500
10 % ammonium persulfate	0.050	0.100	0.150	0.200	0.250	0.300	0.400	0.500
TEMED	0.002	0.004	0.006	0.008	0.010	0.012	0.016	0.020

Modified from Harlow and lane (1988)

Table 2 Solutions for preparing 5 % Stacking Gels for Tris-glycine SDS-Polyacrylamide Gel Electrophoresis

		(Vol. of components required to cast gels of indicated vol.)							
↓Components	**Gel Volume →**	**1 ml**	**2 ml**	**3 ml**	**4 ml**	**5 ml**	**6 ml**	**8 ml**	**10 ml**
H_2O		0.680	1.400	2.100	2.700	3.400	4.100	5.500	6.800
30 % acrylamide mix		0.170	0.330	0.500	0.670	0.830	1.000	1.300	1.700
1.0 M Tris (pH 6.8)		0.130	0.250	0.380	0.500	0.630	0.700	1.000	1.250
10 % SDS		0.010	0.020	0.030	0.040	0.050	0.060	0.080	0.100
10 % ammonium persulfate		0.010	0.020	0.030	0.040	0.050	0.060	0.080	0.100
TEMED		0.001	0.002	0.003	0.004	0.005	0.006	0.008	0.010

Modified from Harlow and Lane (1988)