SDS-PAGE PROTOCOL: NCBS MS-FACILITY

Chemicals:
H2O (HPLC grade-Spectrochem)
Methanol (HPLC grade-Spectrochem)
Acetic Acid (HPLC grade-Spectrochem)
10% Ammonium per Sulphate (sigma-161-0700)
10% SDS (biorad-161-0416)
TEMED (161-0801: Bio-Rad)
Laemmile Buffer-2X (Bio-Rad - 161-07-37)
30% Acryl amide (Bio-Rad - 161-0156)
10% Ammonium Per sulphate (Bio-Rad - 161-0700)
Tris 1.5M and 0.5M (Bio-Rad 161-0716)
10X Tris Glycine Buffer (Bio-Rad- LC2675)
Loading Buffer (2X) (Bio-Rad -161-0737)
CBB stain (Biorad-161-0436)
MW Marker Broad range (Bio-Rad-161-0317)
Destaining solution: 50 % methanol in water with 10 % acetic Acid.
Instrument: Amersham Biosciences SE 250

10% SDS PAGE Cocktail

Sr No.	10% Separating Gel (ml)	Stacking gel (ml)
H2O (HPLC grade)	4	1.36
30% Acryl amide	3.3	0.34
Tris	2.5(1.5M)	0.25(0.5M)
10% SDS	0.1	0.02
10% APS	0.1	0.02
TEMED	0.004	0.002
Total volume (ml)	10	2

Casting the gel

1. Before and after experiment clean both notch plate and glass plate with HPLC water followed by 70% Alcohol. wipe it with Kim wipe or air dry it use for experiment(don't use detergents and tape water for washing)
   As per above table make separating gel cocktail and pour into cassette, Overlay the poured gels with several drops of water. Leave the gels for about 30 min to polymerize.
2. Overlay the polymerized separating gel with (stacking) gel (As per Table)

Loading Protein solution:

3. Adjust volume of protein samples and marker solution to load the sample with Loading Buffer (2X).
4. Heat the above mixture at 95 ^{0}C for 5min.(Use heating block)
5. Always heat Molecular weight marker along with proteins (for quantitative scanning)
6. After 5 min spin it down so that everything will settle down (Only a short spin)
7. Let the protein mix cool down to room temperature. Mix the protein sample in the tube for 3 times before loading the gel. Make sure that you don't induce foam formation.
8. Load same volume in each well (10 µl) (No well should be empty –In case small sample set load same volume of 1X loading Buffer in remaining wells)

Electrophoresis conditions

10. Start electrophoresis with an initial voltage of 30 V and maintain at this voltage until the sample has completely entered the stacking gel followed by 105V.

Protein visualization: Coomassie staining

9. To remove excess of SDS wash the gel with water 5-7 min (3-4 times)
10. Stain the gel with CBB 250 (Bio-Rad) for 30 min.
11. Destain the gel with destaining solution for 1- 2 hour., followed by water washes still no blue background.(make sure no blue back ground as it affects in the Quantitation)

Critical Steps:

*Make sure you were hand glove in each and every step; also try not to touch gel (to avoid keratin contamination)

References:

1. Laemmli, U.K. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 227, 680–685 (1970)
2. http://www.changbioscience.com/calculator/sdspc.htm
3. H Schägger, Tricine–SDS-PAGE *Nat. Protocols* **1**, 16–22 (2006)

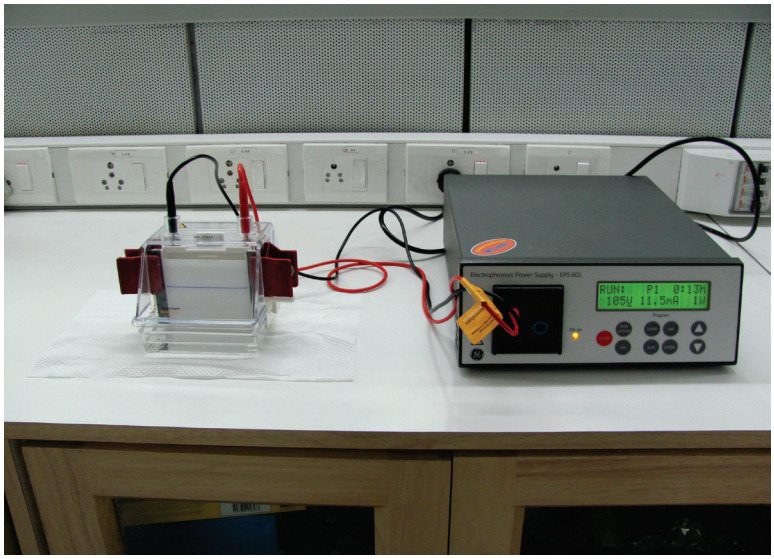

Fig 1) Experimental setup of SDS PAGE at MS Facility NCBS Bangalore

Scanning a Gel and Quantification with Image J

1. The gel was interphased between two transparencies and scanned with HP scanner: Hp scan Jet G4010 at 200 ppi with millions of color
2. The brightness and contrast was adjusted according to gel (But generally used 50-55)

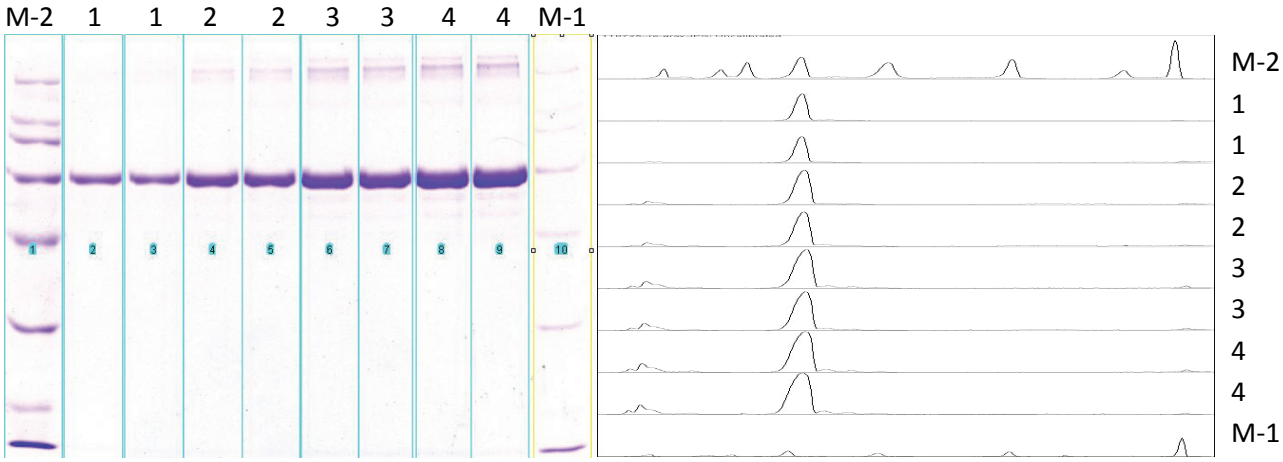

Fig 2) Gel Image of CBB 250 stained gel and Image J Analysis
Right panel) Different amounts of BSA (1, 2, 3, 4 micrograms each in duplicate- from right to left) were loaded of which Quantification was done with respect to Molecular weight marker of know amount. M-1, M-2 are 4.2 µg and 0.84 µg. respectively.
Left panel) Image J Pseudo Chromatogram representation of the lanes

Absolute Quantification of Protein Bands

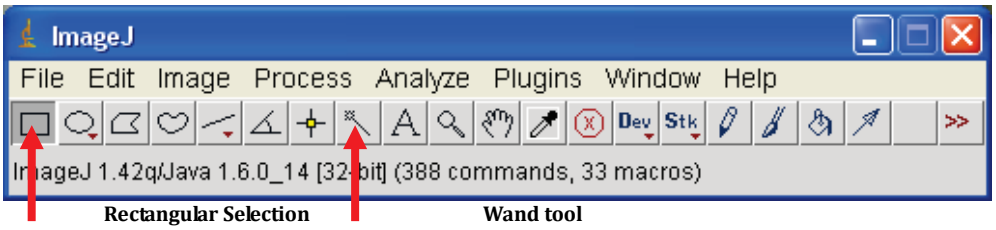

Fig 3) screenshot of Image J software Tool

1. ImageJ is a free program that was originally written at NIH (http://rsbweb.nih.gov/ij/). ImageJ is used to analyze/process all sorts of images in biology-related research.
2. Do a tight selection (rectangular selection – Fig2) around the lane of interest. Press "Ctrl+1" to designate the first lane.(as given in fig.1)
3. Press "Ctrl+2" to designate the second lane, then if you select a third lane, you press "Ctrl+2" for any additional lane. Once you're done, press "Ctrl+3" to plot (Fig-2)
4. Now you can identify each band by using the "Wand Tool"(see fig-3)
5. Click with the Wand Tool inside each selection corresponding to a band.
6. A "Results" window that lists the results will pop up containing area values. Use the "Area" values for further analysis; in our case we use it for the relative protein amount calculation from gel. (See Table 1)

Table 1) Example Results for Quantitation of BSA

Theoretical amount (µg)	Peak area From Image J	Estimated Amount (µg)	% Error
4.2	12486.17	**4.2**	-
1	2838.376	0.99	-0.5
1	2926.397	1.02	2.4
2	5338.024	1.83	-8.7
2	5803.581	1.98	-1.0
3	7947.036	2.69	-10.3
3	8038.157	2.72	-9.2
4	9431.007	3.19	-20.4
4	10228.54	3.45	-13.7
0.84	2371.5	**0.84**	-