Protocol for protein SDS –PAGE and Transfer

According to Laemmli, (1970)

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Selection of a protein source

Serum

cell cultures (bacteria, yeast, mammalian, etc.)

Tissue

genetically engineered - tagged proteins, over-expression

Liquid (hemolymph, milk)

Plant leave

Plant seed

must be fresh or at least reserved at -80ºC

Extraction and Analysis of Diagnostically Useful Proteins from Formalin-fixed, Paraffin-embedded Tissue Sections (Morito etal 1998)

Composition of lysing buffer:

100ml 0.625M (0.706 g/100ml) Tris-HCl (pH 6.8) – 2% SDS (w/v) - 10% glycerol (v/v) - 5% 2-mercaptoethanol (v/v)

Note: RIPA buffer (Tris-Hcl, Na Cl, Triton X100, protease inhibitors and phosphatase inhibitors) is recommended as lysis buffer for western blot

Isolation of protein procedures

first method

(~o.1 gm fresh weight) of the sample was suspended in 1.0 ml lysing buffer. Heated at 100 ˚C for 5 min., centrifuged at 10,000 rpm for 30 min., and 100 µl of each supernatant as extracted protein used for protein analysis.

the second method

1. Label all tubes. Prepare solutions and have ready at hand.
2. Remove the tissue from the –80oC freezer and thaw on ice. If the tissue is fresh, keep on ice (or alternatively work in a cold room).
3. Place tissue in a mortar and pestle.
4. Add ~ 1ml of lysing buffer per ~1g tissue.
5. Grind tissue until no more chunks are visible.
6. Remove ~1ml of the liquid grindate into a microfuge tube.

7. Place on ice.
8. Rinse mortar and pestle to remove all traces of sample and proceed to the protein isolation of the next tissue sample.
9. Spin samples at top speed in the microfuge
10. Transfer the liquid supernatant into a second (new) microfuge tube.
11. Store samples in the –80ºC.

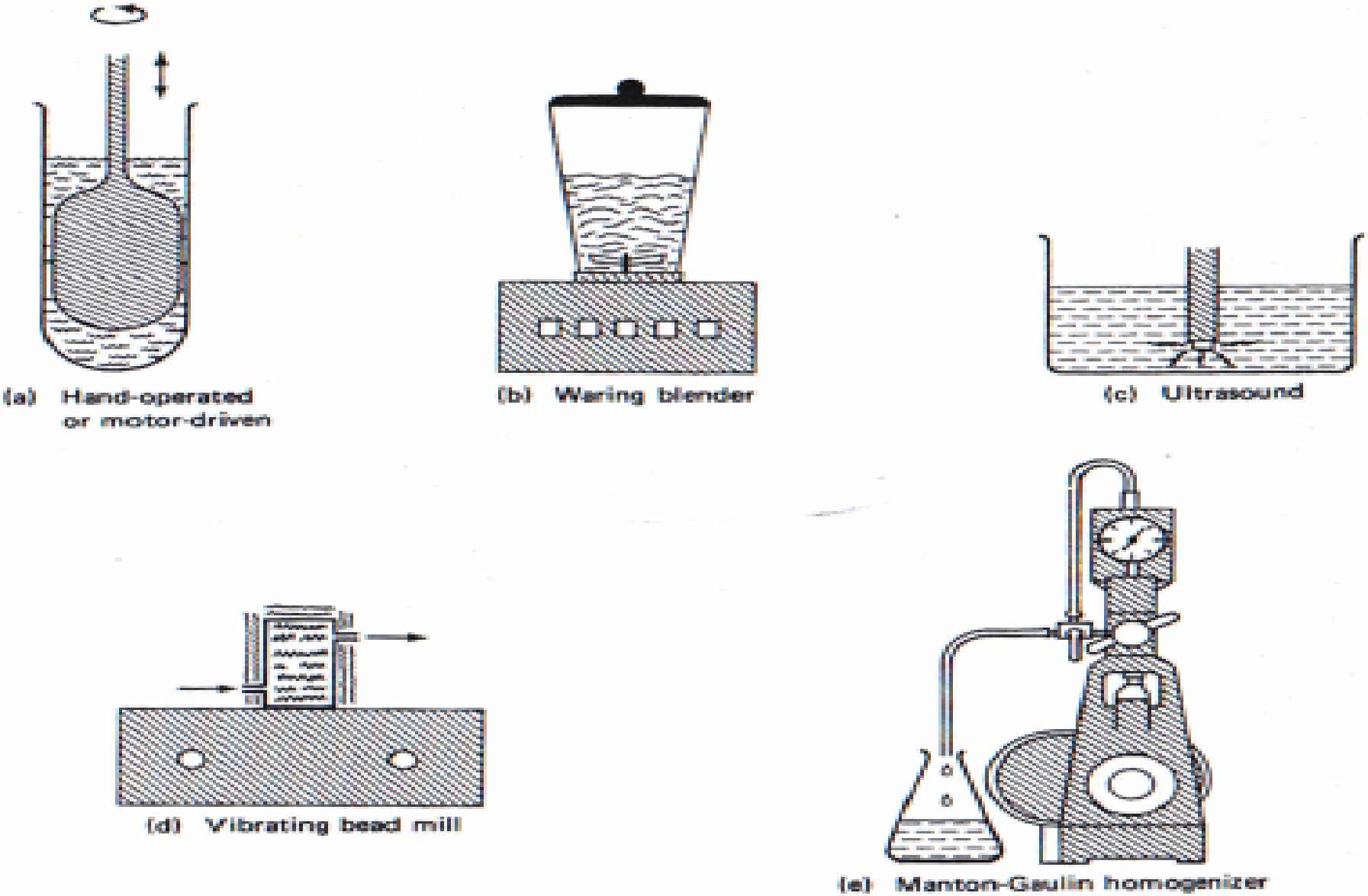


Figure 2.2. Equipment used for breaking up cells to obtain an extract. (a) Hand-operated or motor-driven glass homogenizer. (b) Waring blade-blender (food processor). (c) Ultrasonic probe. (d) Vibrating glass bead mill. (e) Manton-Gaulin cell disintegrator.

SDS-PAGE and Western blot solutions

Stock (1)

Acrylamide (30 grams) and 0.8 gram bis N.N. methylene bisacrylamide were dissolved in 100 ml distilled water (each component dissolved separately). The solution was then filtered through glass filter under vacuum and kept at 4 ˚C in a dark bottle.

Stock (2)

Tris-HCl (18.2 grams) was dissolved in 50ml distilled water. The pH was adjusted with HCl to 8.8 after that the volume was completed to 100 ml distilled water and the solution stored in the refrigerator.

Stock (3)

Tris-HCl (6 grams) dissolved in 50ml distilled water. The pH was then adjusted with HCl to 6.8 after that the volume was completed to 100 ml distilled water and the solution was stored in the refrigerator

Caution: Acrylamide and Bisacrylamide are potant neurotoxins and are absorbed through the skin

Sodium dodecyl sulfate solution (SDS 10% W/V):
Stock solution was prepared by dissolving 1.0 grams SDS in 10ml distilled water.

Ammonium persulfate solution (APS 10% W/V): **(freshly prepared)**
A solution of ammonium persulfate was prepared by dissolving 1.0 gram in 10ml distilled water, just before use in gel preparation.

Electrode buffer solution (2x):
Tris-HCl (6 grams), Glycine (28.8 grams) and 2 grams SDS were dissolved in 1000 ml distilled water. pH 8.8

Loading buffer (2x) (double concentration)

100 ml 0.125M (1.512g/100) Tris-HCl (pH 6.8) – 4% SDS (W/V) – 20% glycerol (V/V) -10% 2- mercaptoethanol (V/V) – 0.002% (W/V) bromophenol blue.

Staining solution

0.5 gm Commassie R-250 brilliant blue was dissolved in 250 ml methanol, 50 ml glacial acetic acid, completed by distilled water to 500ml mixed and kept at room temperature.

Fixation & Distaining solution:

Composed of 250 ml methanol, 50 ml glacial acetic acid and 200 ml distilled water

Reagents for Transfer to Nitrocellulose Membrane

Transfer buffer (prepared one day before use, store at 4 C)

3.03 g Tris base
14.4 g Glycine
200 ml methanol
Adjust pH to 8.3 and add dH2O to 1 L

Blot staining solution

2% Panceau S (2g)
30% trichloroacetic acid (30g)
30% sulfosalycilic acid (30g)
Complete to 100 ml dist water (dilute 1:10 for use)

Washing buffer

1X Tris buffer saline (TSB)
0.1 % tween-20

Blocking buffer

1X Tris buffer saline (TSB)
0.1 % tween-20
5 % non fat dry Milk

To prepare 10X TBS, Dissolve 24.2 g Tris base in 700 ml DW, Adjust pH to 7.4 then dissolve 80 g Na cl and comlete to 1000 ml DW

Procedure for gel preparations

1- The gel components were mixed in beaker using sterrir

2- The acrylamide solutions were quickly poured between the plates glass (see table)

2- The meniscus of the acrylamide solution should be far enough below the top of the notched plate to allow for the length of the comb plus 1cm.

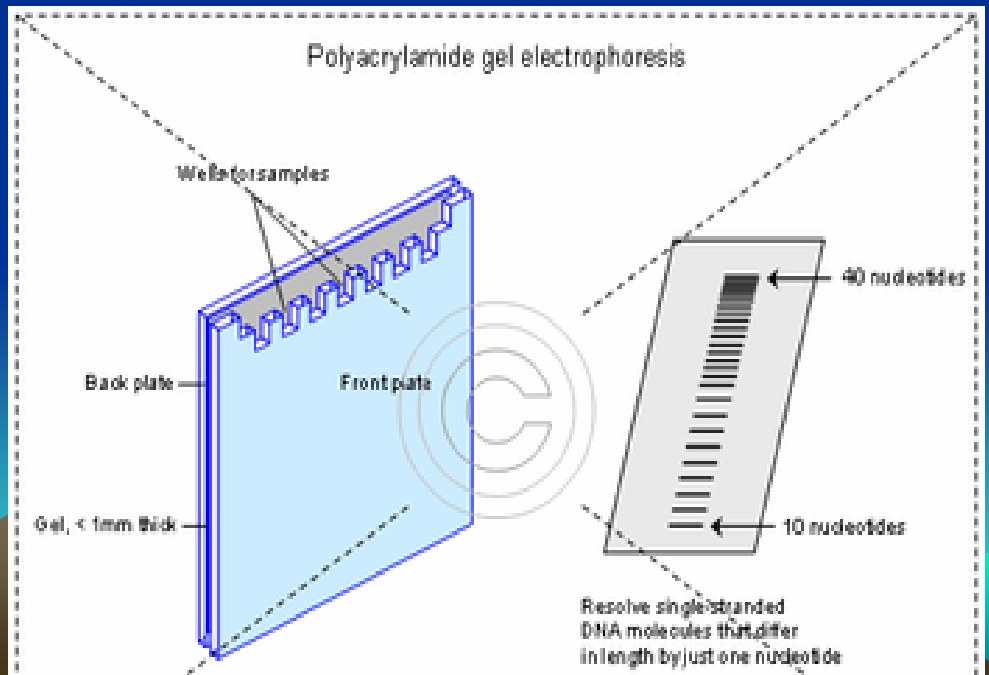
3- add 0.5 ml of butanol or iso-propanoal

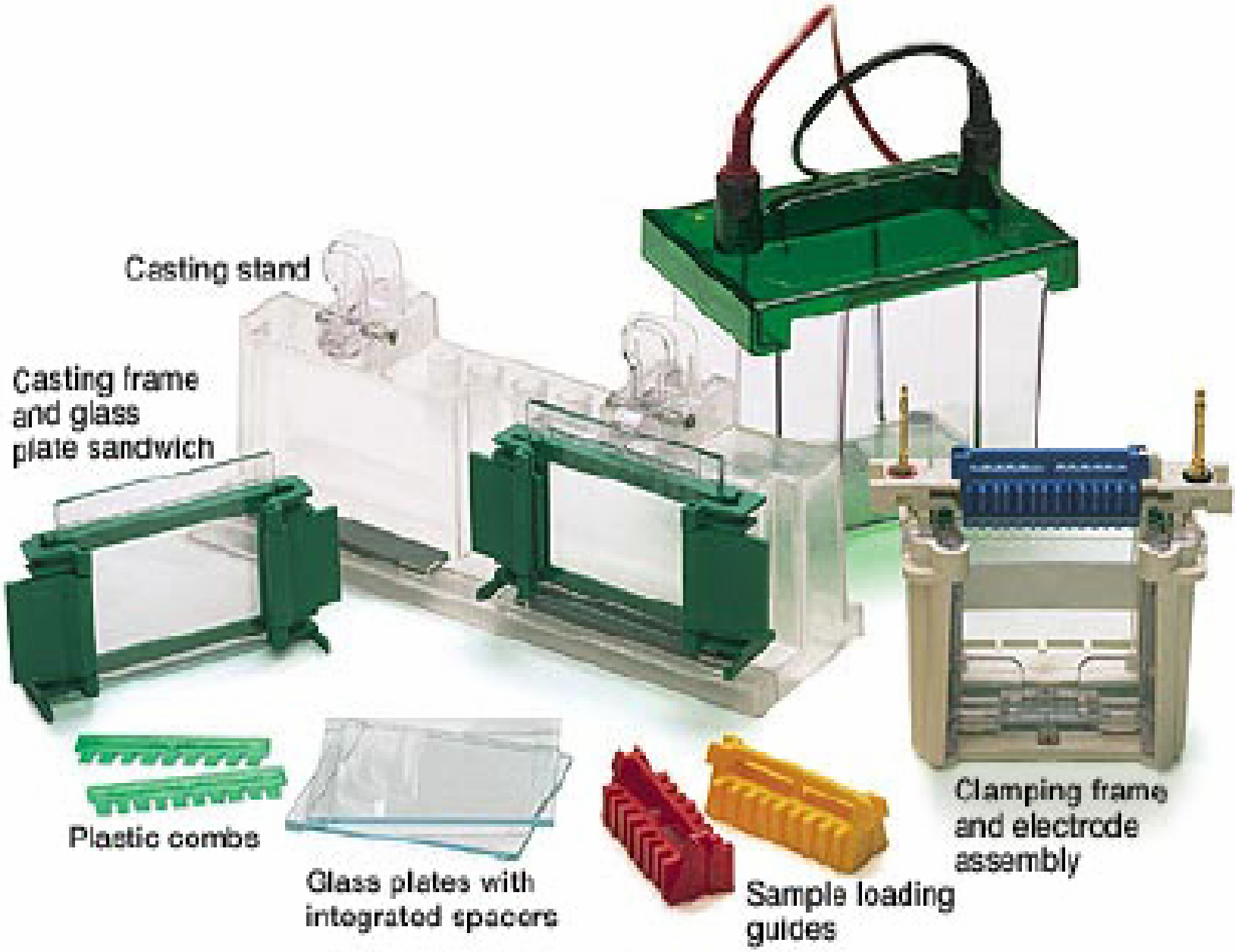
4- After the gel has been set about 20-30 min, the overlay water poured off and the top of the separating gel was washed several times with distilled water. Excess water was drained with the edge of filter paper.

5- The stacking gel solution was prepared as shown in the table and poured directly into the polymerized separating gel.

6-The appropriate comb was placed into the gel solution being careful not to make any bubbles. The comb was cleaned by washing with distilled water.

7- The gel was placed in a vertical position at room temperature. The stacking gel would set in approximately 15 min.




Casting stand
Casting frame and glass plate sandwich
Plastic combs
Glass plates with integrated spacers
Sample loading guides
Clamping frame and electrode assembly

Preparation of 12% and 5% SDS-PAGE

The gel was prepared as shown in the following table:

Acrylamide concentration (%)	Linear range of separation (KDa)
15	12-43
12	18-52
10	16-68
7.8	36-94
5	57-212

Reagents	12% separating gel	5% stacking gel	**For Mini-BioRad cell**	
			12% separating gel	5% stacking gel
Distilled H2O	9.9 ml	11 ml	6.6 ml	7.3 ml
Stock (1)	12.0 ml	2.6 ml	4 ml	1.7 ml
Stock (2)	7.5 ml	….	5 ml	….
Stock (3)	…..	2.0 ml	…..	1.3 ml
10% SDS	300 µl	100 µl	200 µl	66 µl
10% APS*	300 µl	100 µl	200 µl	66 µl
TEMED	12 µl	6 µl	16 µl	4 µl
Volume	30.012	15.806	16.016	10.0156

Preparation of samples for injection

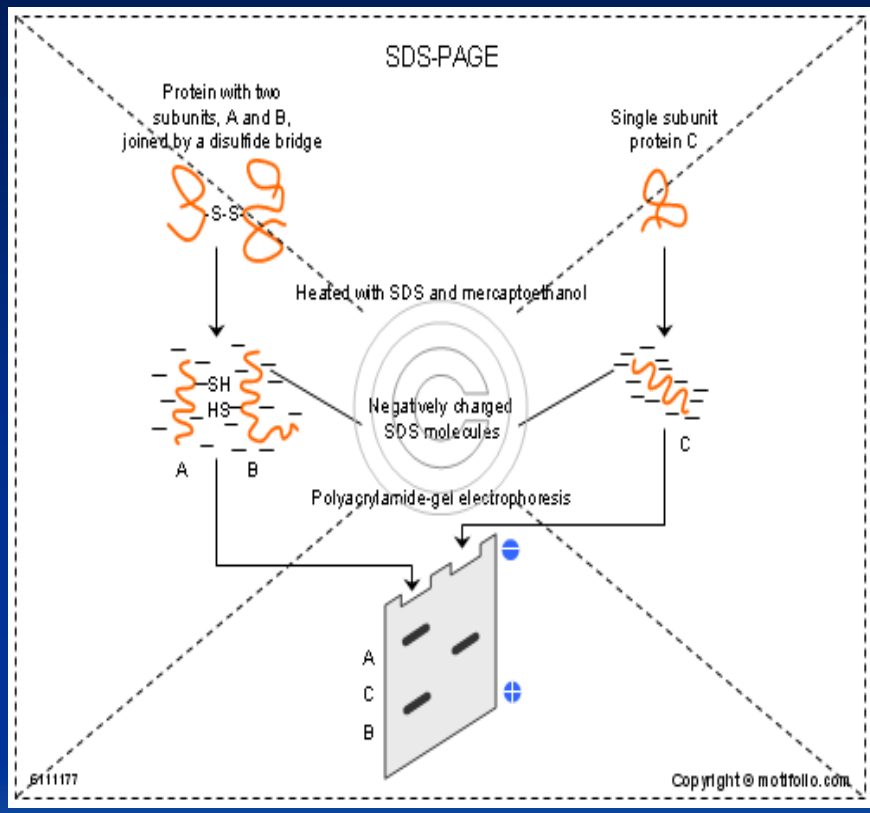
Get the samples from -80 ºC

Add v/v from the lading buffer to the samples

Heat at 50 ºC to denaturate the proteins

Centrifugation at 10.000 rpm

Now the samples are ready for injection



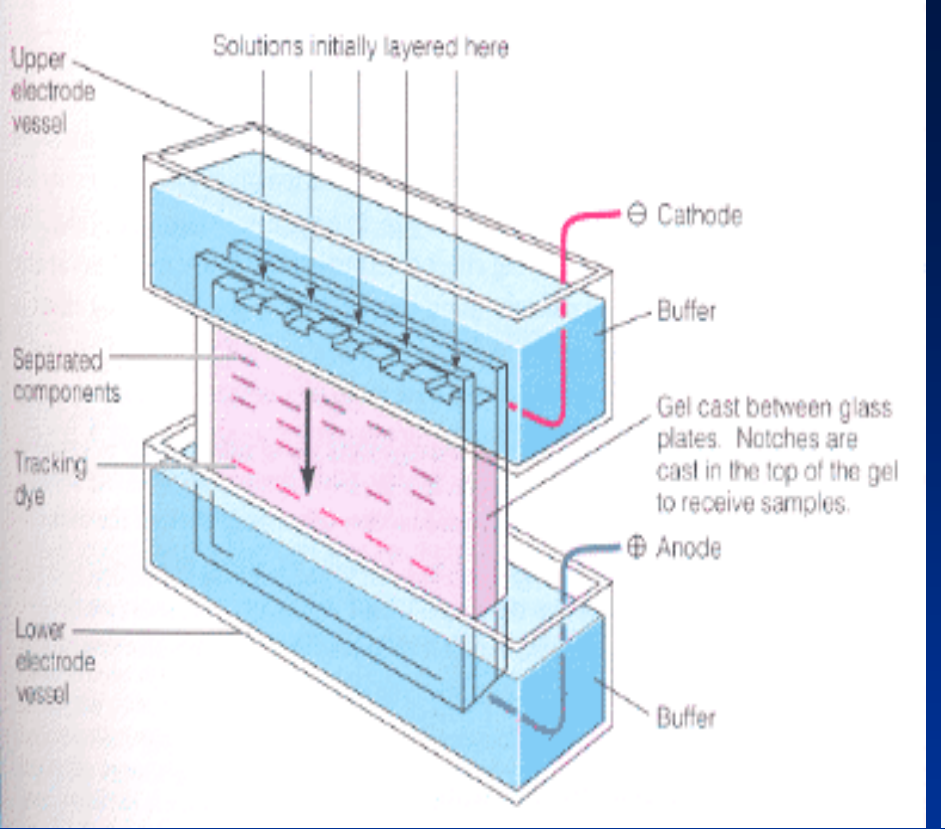
Sample loading and application

1- After the stacking gel has set, carefully remove the comb. Wash the wells immediately with distilled water to remove unpolymerized acrylamide.

2- 20µl of each sample was loaded directly into the bottom of the wells, using a microliter syringe washed by electrode buffer after each sample.

Electrophoresis

After sample loading, the molded gel was completed with electrode. Electrophoresis was carried out at (140V) for each well for 120 min.



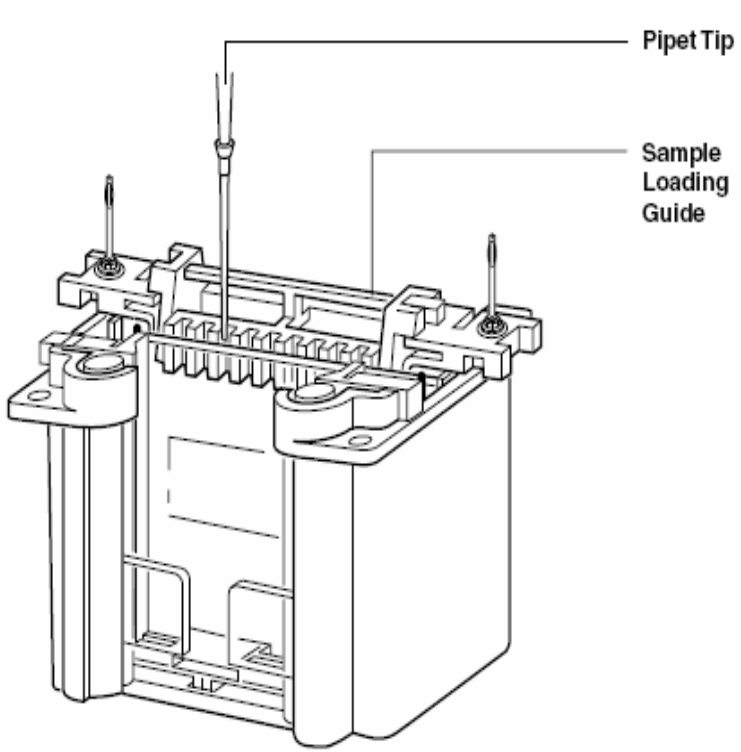


Fig. 6. Using the Sample Loading Guide (patent #5,656,145).

What happens after electrophoresis???????

1. Fix the proteins in the gel and them stain them.
2. Electrophorectic transfer to a membrane (Western blotting) and then probe with antibodies.

Fixation

Gels were fixed in 200 ml of fixation solution for less than 1 hour

- A more stable fixative is 25% isopropanol, 65% water and 10% acetic acid, which can be stored at 4oC for up to 4 months.

Staining

Gels were stained overnight in 200 ml of Commassie brilliant blue R-250 solution.

Destaining

in 200 ml of destaining solution with gentle shaking. The destaining solution was changed several times until background colour was removed.

Scan or photo the Gel

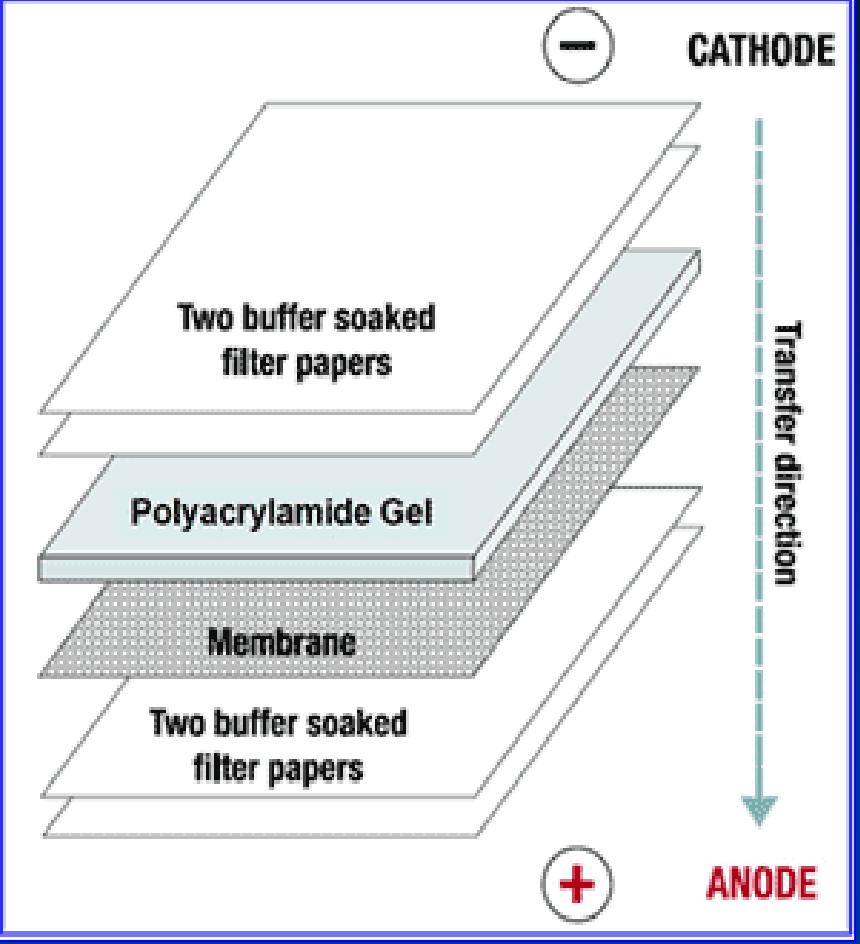
Determination of molecular weight and percentage of proteins using available program (labimage 2006, Gelpro analyzer)

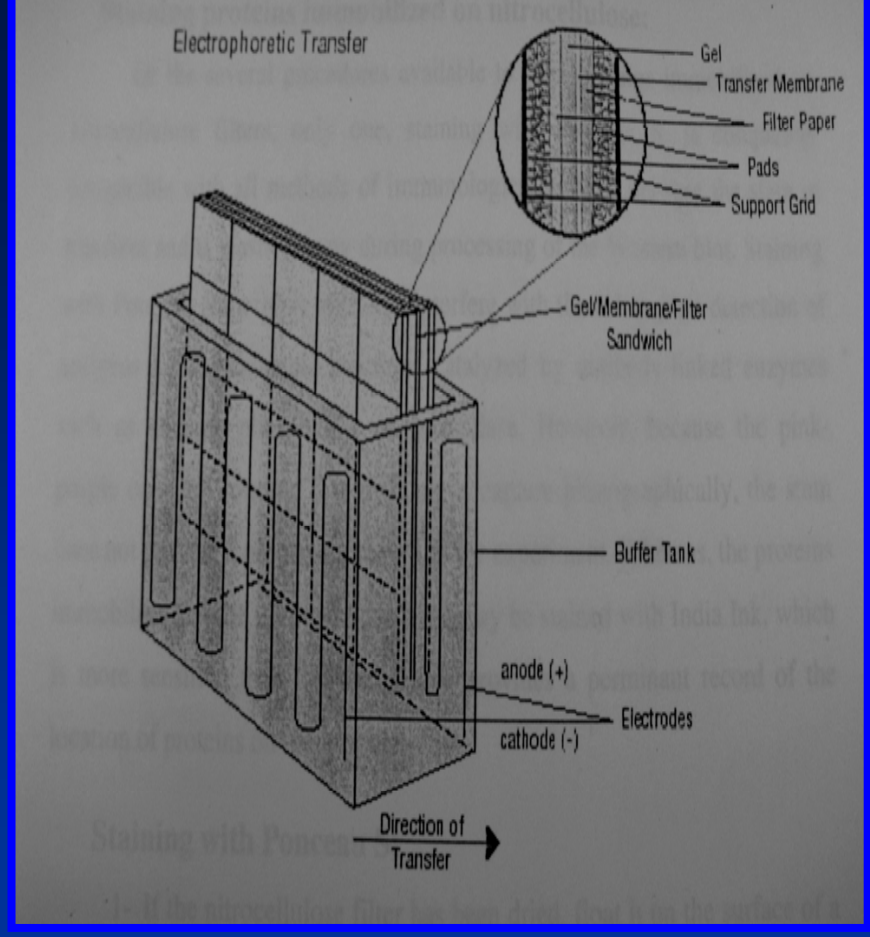
before Transfer

1-30 min before the end of electrophoresis , wear gloves and cut a nitrocellolose membrane and filter paper or thick Whatman paper

2- Place gel in dish containing Transfer Buffer. Allow to soak for ~10 min.

3- Per gel, cut to size: 1 piece of nitrocellulose and 6 pieces of Whatman
paper or 2 thick blot pads. Place all in Transfer Buffer.





Dry blotting

Wet blotting

Transfer to Nitrocellulose

1. Layer onto Trans-Blot Electrophoretic Transfer cell from **bottom to top**:

a. 3 pieces Whatman (or 1 thick blot pad)

b. nitrocellulose

c. gel

d. 3 pieces of Whatman. (or 1 thick blot pad)

2.In this procedure, a sandwich of gel and solid support membrane (Nitrocellulose) is compressed in a cassette and immersed in buffer between two parallel electrodes.

3. Make sure there are no air bubbles.

4. A current is passed at right angles to the gel, which causes the separated proteins to electrophorese out of the gel and onto the solid support membrane

at 15V (small gels) for ~30-45 min.

5. Stain nitrocellulose with Ponceau for (5-10) min to ensure protein transfer.
6.On a rocker, wash the unstained nitocellulose membrane in DW for 5 min
7.Rinse with washing buffer for 10 min.
8. Block the non-specific binding site by rocking the membrane in blocking buffer for 1 hour at room temperature.
9. Wash the membrane in washing buffer for one min
10. Incubate the membrane with diuted antibody for overnight on a rocker or shaker at 4 C
(the primary should be diluted to their optimal concentration in 1X TBS+ 0.1 % tween-20+ 5% Bovine Serum Albumin)

•http://video.search.yahoo.com/search/video;_ylt=A0geuo8BkoVMBB4BustXNyoA?ei=UTF-8&p=SDS%20page&fr2=tab-web&fr=yfp-t-701


Questions
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