

SDS-PAGE

(Laemmli, U.K., 1970 Nature 227:680-685)

M. IEF buffer supplement A:

The preceding protocol was devised for 10 cm long gels with 2 mm diameters. The second dimension gels are intended to be the larger format gels with 0.75 mm spacers. These gels are run as described in the following protocol for SDS-PAGE with a few modifications and do not exceed 50 µl load on 1st dimension.

The following protocol is for preparation of IEF samples from SDS-PAGE samples:

10% (W/V) NP-40	4.00 ml.
pH 4-6 ampholines	0.16 ml.
pH 5-7 ampholines	0.16 ml.
+22 µl NP-40	0.80 ml.
+12 µl Ampholines	
+133.8 mg urea	

C. stock grind:

SDS	0.10 gr.
2-mercaptoethanol	1.00 ml.
urea	5.70 gr.
ddH ₂ O	4.00 ml.

SDS-PAGE Gel and Electrical Conditions

GELS:

A. 7.0% light running gel acrylamide:

glycerol	2.0 gr.
SDS running gel buffer	6.0 ml.
dHOH	10.0 ml.
acrylamide (light, G2)	5.6 ml.
10% ammonium persulfate	80 µl
<u>TEMED</u>	<u>24 µl.</u>
total	24 ml.

B. 10.0 light running gel acrylamide:

glycerol	1.6 gr.
SDS running gel buffer	6.0 ml.
dHOH	8.0 ml.
acrylamide (light, G2)	8.0 ml.
10% ammonium persulfate	80 µl
<u>TEMED</u>	<u>24 µl.</u>
total	24 ml.

C. 17.5% heavy running gel acrylamide:

SDS running gel buffer	3.0 ml
dHOH	2.0 ml.
acrylamide (heavy, G3)	7.2 ml.
10% ammonium persulfate	40 µl
<u>TEMED</u>	<u>12 µl.</u>
total	12 ml.

D. overlay solution:

SDS running gel buffer	2.5 ml.
dHOH	7.5 ml.

E. 5% acrylamide stacking gel:

stacking gel buffer	4.5 ml.
dHOH	10.5 ml.
acrylamide (stack, G1)	3.0 ml.
10% ammonium persulfate	60 µl.
<u>TEMED</u>	<u>23 µl.</u>
total	18 ml.

ELECTRICAL CONDITIONS:

CONSTANT VOLTAGE MODE

<u>voltage/current (gel)</u>	<u>time</u>	<u>mode</u>
75V/8-9mA	0 hr.	constant voltage start stacking
75V/4-5mA	2 hr.	constant voltage end stacking
150/11-12mA	2 hr.	constant voltage start resolving
150/4-6mA	8-10 hr.	constant voltage end run

CONSTANT POWER MODE

<u>voltage/current (gel)</u>	<u>time</u>	<u>mode</u>
75V/8-9mA	0 hr.	constant voltage start stacking
75V/4-5mA	2 hr.	constant voltage end stacking
150/11-12mA	2 hr.	constant power start resolving
150/7-8mA	6-7 hr.	constant power end run

Voltage used is for 12-14 cm long gels (50%).

Loading gel:

Sample can be loaded by underlayering in a well already filled with electrode buffer or overlayering the sample with electrode buffer.

Unloading gels:

Particular care should be taken when unloading the gels from the glassplates as to not break glass or gels. First take out side spacers then pry the glass apart at the sides gently.

SOLUTIONS:

A. SDS running (resolving) gel buffer:

tris	18.17 gr.
3M HCl	8.20 ml.
SDS	0.40 gr.
dHOH	to 100 ml.

Dissolve tris and SDS in 73 ml. HOH and then titrate with 3M HCl, then bring to volume. Adjust the pH to 8.6-8.8 (optimal 8.8). Store at 4°C.

3M HCl= 24.75 ml (conc. HCl 14.6M) + 95.875 HOH

B. SDS stacking gel buffer:

tris	6.05 gr.
SDS	0.40 gr.
2M HCl	29.1 ml. ca.
dHOH	to 100 ml.

Titrate this buffer to 6.8-7.0 (optimal 6.8) Store at 4°C.

C. SDS-sample buffer:

glycerol	12.5 gr.
tris	0.76 gr.
2-mercaptoethanol	5.00 ml.
SDS	2.30 gr.
0.5M HCl	12.3 ml.
dHOH	71.0 ml.

Titrate this buffer to pH 6.8-7.0 (optimal is 6.8).

D. embedding gel (for 2-D):

agarose	1.0 gr.
SDS-sample buffer	100 ml.

E. SDS electrode buffer:

glycine	28.84 gr.
tris	6.05 gr.
SDS	2.00 gr.
dHOH	to 2000 ml.

Do Not titrate this buffer or add small ions (e.g. Cl⁻). The pH should be between 8.0 and 8.5 (optimal 8.3).

F. tracking dye:

bromophenol blue	10 mg.
SDS electrode buffer	10 ml.

G. SDS Acrylamide Stocks:

1. stacking gel (29.6-0.4)

acrylamide	7.4 gr.
bis-acrylamide	0.1 gr.
dHOH	18 ml.
total	25 ml.

2. light running gel (29.2-0.8)

acrylamide	14.6 gr.
bis-acrylamide	0.4 gr.
dHOH	36.0 ml.
total	50.0 ml.

3. heavy running gel (29.2-0.8)

acrylamide	14.6 gr.
bis-acrylamide	0.4 gr.
75% (W/V) glycerol	41.5 gr.
total	50.0 ml.

75% glycerol=150 gr. glycerol +80 ml. HOH

Filter all stocks with 0.22 µm filters and store at 4°C.

COOMASSIE STAIN PROTOCOL

A. Coomassie Brilliant Blue stain 0.05%:

Coomassie blue R-250	0.5 gr.
ethanol or methanol	250 ml.
glacial acetic acid	100 ml.
dHOH	650 ml.

If precipitate is present filter.

B. destain solution:

ethanol or methanol	350 ml.
glacial acetic acid	100 ml.
dHOH	550 ml.

FLUOROGRAPHY

A. 50% (W/V) Trichloroacetic Acid:

TCA	500 gr.
dHOH	1000 ml.

B. 18% (W/W) PPO/DMSO:

PPO	80.0 gr.
<u>DMSO</u>	<u>364.0 ml.</u>
total	400.0 ml.

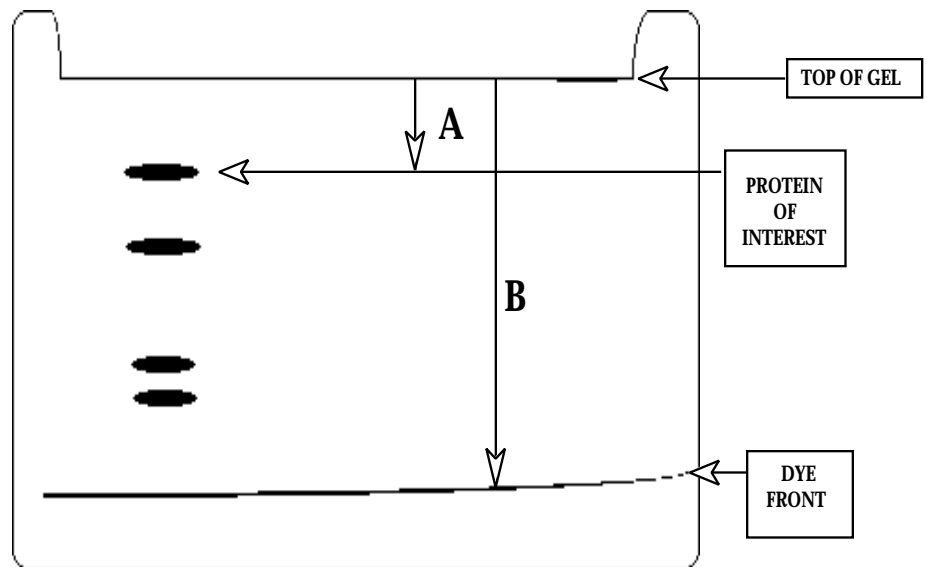
Gels can be fixed in TCA (optional) for 30 mins. Gels are then de-hydrated by washing in DMSO 3X for 1 hr. each. Then the gels are washed with PPO/DMSO for two hrs. The PPO is then precipitated by washing the gel in water 5X briefly to remove surface precipitate. Then the gel is washed in water for 1hr. and dried.

Molecular weight determination can be made from SDS PAGE gel with the use of relative mobility calculations (R_f) and comparing R_f values of molecular weight standards to unknowns. A more accurate way of using R_f in determination of molecular weight is by use of Ferguson plot (K.A. Ferguson, 1964, Metabolism 13,21), but I will not describe this here. Simple plotting of R_f values of a range of standards run on the same gel as the unknown should suffice in calculating approximate molecular weight.

(formula for relative mobility)

$$R_f = \frac{\text{distance protein travels (A)}}{\text{distance dye front travels (B)}}$$

(gel diagram)



IEF and NEpHGE Electrophoresis

(O'Farrell, 1975, J. Biol. Chem. 250:4007-4021)

(O'Farrell, 1977, Cell 12:1133-1142)

IEF & NEpHGE METHODS:

The following protocol for isoelectric focusing (IEF) and non-equilibrium pH gel electrophoresis (NEpHGE) was devised for 11 cm long gels with 2 or 3 mm diameters. The second dimension gels are intended to be the larger format gels with 0.75 mm spacers. These gels are run as described in the following protocol for SDS-PAGE with a few modifications described below. Do not exceed 50 μ l load on 1st. dimension.

A. sample preparation:

(1) The following protocol is for preparation of IEF samples from SDS-PAGE samples.

100 μ l SDS-PAGE sample
+22 μ l NP-40
+12 μ l Ampholines
+133.8 mg urea

(2) The following is the procedure from cytoskeletal sample preparation.

Dissolve in stock grind 3 vol.
+ IEF buffer supplement A 4 vol.
+ IEF buffer supplement B 1 vol.

B. gel preparation:

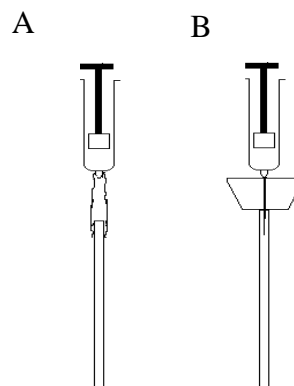
Gels are poured using a 10 ml. syringe with Tygon tubing fitted to the end and 13 cm. x 3 mm long glass tubes mark at 11 cm. The bottom ends of these tubes are sealed with parafilm. Gels are filled with acrylamide by running tubing to the bottom of the glass tubes and filling to 1/4 cm. above 11 cm mark, withdrawing the tubing as the tubes fill. The gels are overlaid with 20 μ l of ddwater. and allowed to polymerize for 2 hrs.

C. running conditions:

Gel recipes, electrical conditions and physical configuration for both IEF and NEpHGE are described on the following page.

D. worm (first dimension gel) preparation:

The gel is extruded from the tube in one of two ways. Method A involves connecting a syringe to the tube via Tygon tubing and applying pressure. method B involves placing the tube over a needle that is through a rubber stopper which forms a seal. The needle is attached to a syringe which pressure can be applied.



Each worm is then equilibrated in SDS sample buffer by incubating in two changes of 5 ml. SDS sample buffer for 1 hrs. each. The gels are then stored at -80°C or loaded on second dimension gel.

E. second dimension gel:

The second dimension gels are modifications of SDS-PAGE Laemmli gels. The stacking gel is poured all the way to the top of the plates with a glass rod placed on top over the slit to insure even polymerization across the gel. Also, one should make sure that air bubbles are not drawn under the glass rod.

After gel polymerization the residual fluid is removed from the surface of the gel by a kimwipe. The first dimension is mounted on top of the stacking and embedded in agarose (see SDS-PAGE protocol)

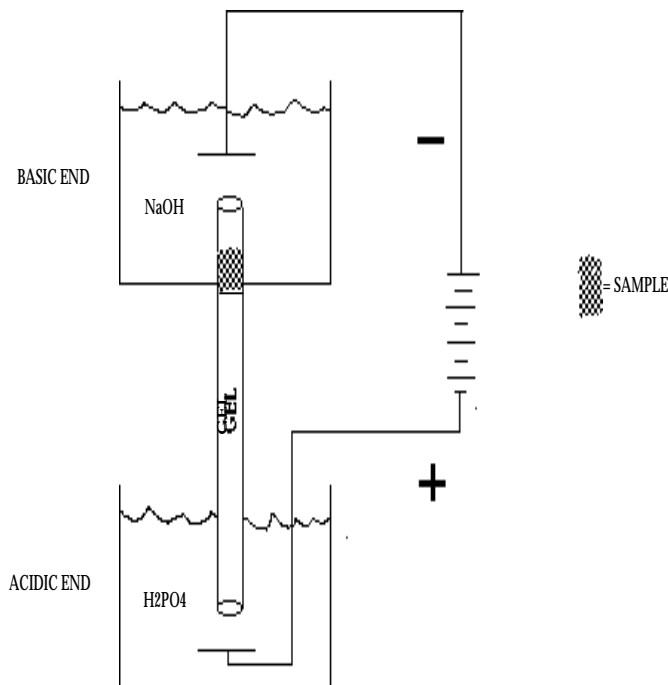
IEF Gel and Electrical Conditions

GEL:

urea	5.50 gr.
acrylamide stock	1.33 ml.
NP-40 stock	2.00 ml.
pH 5-7 ampholines	0.40 ml.
pH 3.5-10 ampholines	0.10 ml.
10% ammonium persulfate	20.0 μ l.
TEMED	14.0 μ l.
<u>dHOH</u>	<u>1.97 ml.</u>
total	10.0 ml.

ELECTRICAL CONDITIONS:

prefocus	200 V for 15 mins.
	300 V for 30 mins.
	400 V for 30 mins.
main focus	4800 V/HRs.
fine focus	800 V for 60 mins.



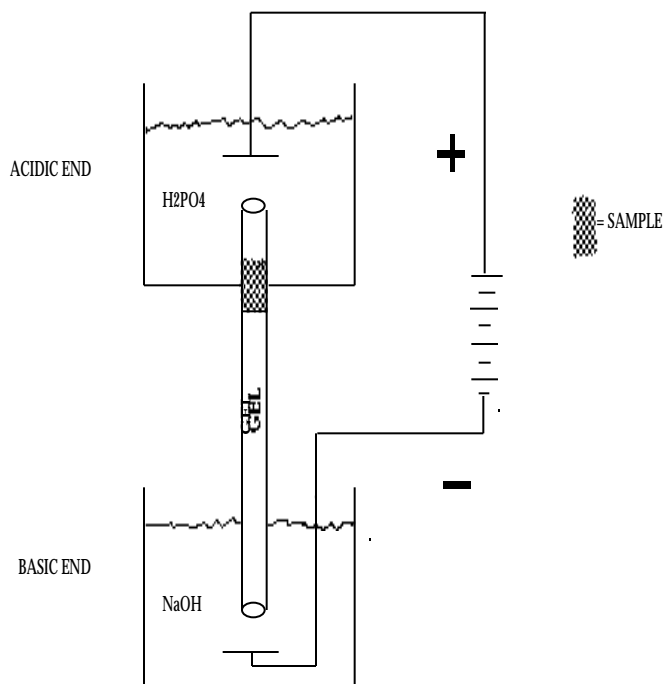
NEpHGE Gel and Electrical Conditions

GEL:

urea	5.50 gr.
acrylamide stock	1.33 ml.
NP-40	2.00 ml.
pH 3.5-10 ampholines	0.50 ml.
(or 9-10 or 7-10)	
dHOH	1.97 ml.
10 % ammonium persulfate	20.0 μ l.
<u>TEMED</u>	<u>14.0 μl.</u>
total	10 ml.

ELECTRICAL CONDITIONS:

Electrophoresis is carried out for 1600-2000 V/HRs. (no prefocusing).
 400 V for 4-5 hrs is standard. 2000 V/HRs recommended,
 1600 V/HRs is common, and 1200 V/HRs is used for very basic proteins.



G. TEMED: Used as supplied. Store at room temperature or 4°C.

IEF & NEpHGE SOLUTIONS:

A. lysis buffer:

urea	5.70 gr.
dHOH	2.66 ml.
10% (W/V) NP-40	2.00 ml.
2-mercaptoethanol	0.50 ml.
pH 5-7 ampholines	0.40 ml.
pH 3.5-10 ampholines	0.10 ml.

Store at -70°C.

B. sonication buffer:

Stock #1 MgCl ₂ /6H ₂ O	0.10 gr.
10 mM Tris-HCl	100 ml.

Working Buffer

Pancreatic DNase I	0.50 ml. (1mg/ml)
pancreatic RNase A	0.50 ml. (1mg/ml)
stock #1	9.00 ml.

C. IEF acrylamide stock:

acrylamide	7.09 gr.
bis-acrylamide	0.41 gr.
<u>dHOH</u>	<u>17.8 ml.</u>
total	25.0 ml.

Filter and then store at 4°C.

D. 10% NP-40:

NP-40	5.0 gr.
dHOH	45 ml.

Refrigerate

E. Ampholines either LKB or Biorad:

F. 10% ammonium persulfate:

ammonium persulfate	0.10 gr.
dHOH	1.00 ml.

May be stored for several days at room temperature or weeks at 4°C.

H. gel overlay solution:

urea	2.75 gr.
<u>dHOH</u>	<u>2.83 ml.</u>
total	5.00 ml.

This is used only if gels are going to be stored overnight.

I. anode solution, 0.01M phosphoric acid:

H ₃ PO ₄ (85%)	1.69 ml.
dHOH	2500 ml.

Use immediately or store overnight.

J. cathode solution, 0.02M NaOH:

NaOH	1.60 grs.
dHOH	2000 ml.

This solution must be thoroughly de-gassed before use, otherwise carbonate ions will distort the shape of the pH gradient and precipitate the urea in the acidic region of the gel. The following procedure is recommended:

Bring 2100 mls. of dHOH to a full boil for 5 mins. Cool at room temperature for 1 hr. Add the NaOH and de-gas under vacuum. Use good vacuum (20-25 in. Hg) and solution should snap when hit with hand.

K. sample overlay solution:

urea	13.0 gr.
dHOH	14.3 ml.
pH5-7 ampholines	0.5 ml.
<u>pH3.5-10 ampholines</u>	<u>0.125 ml.</u>
total	25.0 ml.

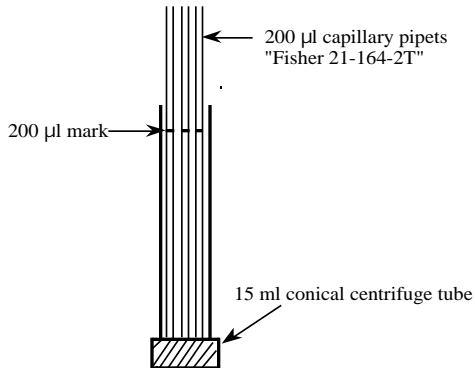
This solution is stored at -70°C. in 1 ml aliquots.

L. tube cleaning solution:

KOH	190 gr.
ethanol (95%)	500 ml.

Soak tubes in this solution in a graduated cylinder for 2 hrs. then rinse extensively with dHOH and dry.

Appendix 2: Mini IEF Tube Gels (O'Farrell)

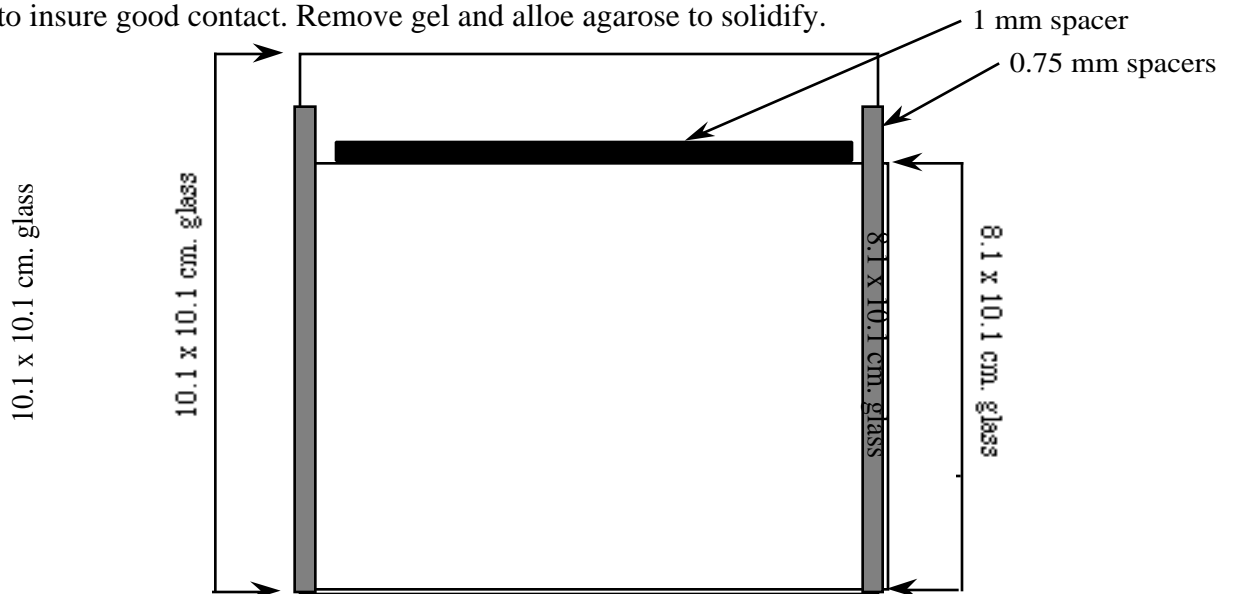


Pouring 1st. dimension IEF gels; 15 tubes are placed in a 15 ml conical centrifuge tube which is inverted and has the tip cut off. 4 mls. of IEF gel is prepared and placed in the tube. This is then overlayered with distilled water, pushing the gel up into the tubes. Overlayer until gel reaches the 200 µl mark. Allow gel to polymerize.

Running 1ST dimension gels; Gels are removed and mounted in gel apparatus. 20 µl samples are loaded. Gels are run at 334 V for 19 hours. Note, no pre-focusing is used. After the run, gels are extruded by syringe fitted with a rubber tube and pressure being applied.

Pouring stacking gel of 2nd dimension IEF/SDS gels and loading; Slab gels poured using a large plate (10.1 x 10.1 cm.), a small plate (8.1 x 10.1 cm.) and 0.75 mm spacers are placed horizontal with large plate side down. Stacking gel is prepared and used to fill the remaining space to the top of the small plate. A 1mm spacer is layed down on top of this sandwich, as shown in diagram, as an overlayer to facilitate an even straight gel surface. Allow gel to polymerize.

Get worm form 1st. dimension ready, stretched out on parafilm with excess fluid removed, and embedding agarose melted. Remove 1 mm spacer from gel and arrange worm on to the large plate parallel to the stacking gel surface and positioned approximately 0.25 cm from the gel surface. Place slab gel with worm on slide warmer for 1 minute pour agarose over surface of gel and push worm on to the surface of the gel. run blunt probe over the worm pushing slightly towards the gel surface to insure good contact. Remove gel and allow agarose to solidify.



Western Blot Analysis

(Towbin, H., Staehelin, T. and Gordon, J. (1979): Electrophoretic Transfer of Proteins from Polyacrylamide Gels to Nitrocellulose Sheets: Procedure and Some Applications. Proceedings of the National Academy of Sciences 76:4350-4354.)

TRANSFER METHOD:

Gels are run according to Laemmli. The transfer is accomplished using a GenieBlotting apparatus. (assembly of the system is described in the diagram below). The gels are then briefly incubated in Transfer Buffer (see below) and a piece of 3mm filter paper cut to slightly larger than the gel is floated under the gel. The gel is then lifted out of the buffer from one edge. This is then placed on the scotch Brit pad than is soaked in tranfer buffer inside the genie tough. The a piece of pre-soaked support membrane ,either Immobilon (polyvinylidene difluoride, PVDF) or nitrocellulose, is applied to the gel starting from one edge. A glass rod is then rolled over the top of this to insure close contact between gel and paper. Next, a second piece of pre-soaked 3mm filter paper is placed over the support membrane Care should be taken at every step to remove bubbles trapped between layers. Then more Scotch Brit pads, a bubble screen , electrode and plexiglas sheet are placed on top so that it requires slight compression to make plexiglass flush with top of the tray. The tray is inserted into the genie chamber.

Electrical running conditions are generally as described here. Most transferes are done at 24 volts. for between 10 and 45 minutes. Some variation might be required for different proteins and gels used.

TRANSFER SOLUTIONS:

A. transfer buffer:

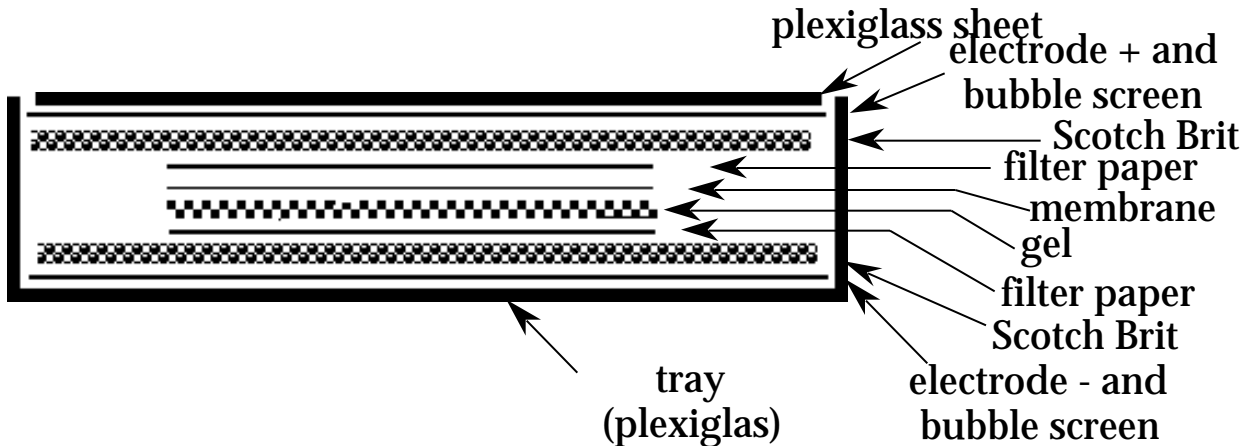
tris-base	12.1 gr.
glycine	57.6 gr.
methanol	800 ml.
dHOH	to 4 l.

Do not titrate. This should be right pH
Optional 0.1% SDS can be added

B. 10x transfer buffer:

tris-base	30.2 gr.
glycine	144 gr.
dHOH	to 1 l.iter

to make 1 x; 1 part 10x buffer
2 parts methanol
7 parts H₂O



IMMUNO-DETECTION METHOD:

After proteins have been transferred to support membranes the membranes are blocked for immuno detection. This is done by emmersion of membrane into TBS containing 3-5% non-fat dry milk and 0.2% Tween-20 (detergent) for 1 hour at room temperature with agitation.

The blot is then emmersed in TBS containing 1% non-fat dry milk, 0.04% tween 20 and a primary antibody of interest. Amount of antibody added varies with antibody used, but they are used at dilutions of 1/200-1/1000, generally. Blots can be incubated with primary for 1 hour at room temperature or 4° C. overnight. After primary antibody incubation the blot is washed with TBS containing 0.2% Tween-20 four times for 5-10 minutes each.

IMMUNO-DETECTION SOLUTIONS

A. TBS:

TRIS	2.422 gr.
NaCl	8.766 gr.
dHOH	1000 ml.

B. washing buffer:

TBS	1000 ml.
Tween-20	2 ml.

C. blocking buffer:

washing buffer	250 ml.
non-fat dry milk	12.5 gr.

C. antibody buffer:

blocking buffer	50 ml.
TBS	200 ml.