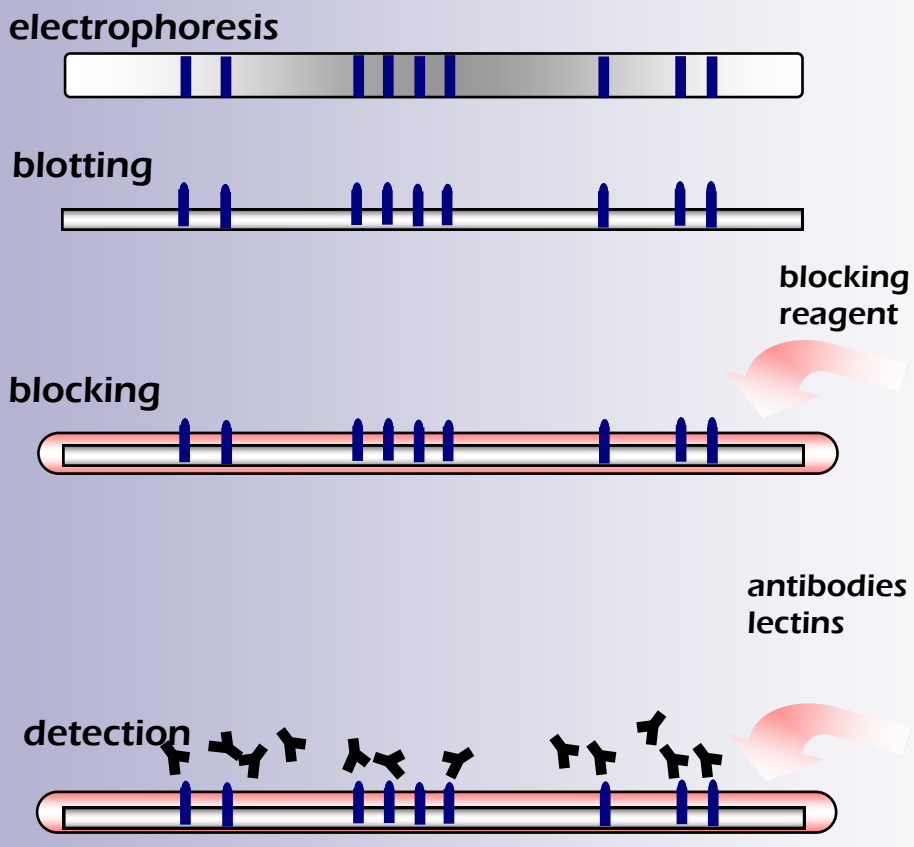
Blotting of Horizontal Electrophoresis Gels

Adapted and slightly changed from: Reiner Westermeier:."Electrophoresis in Practice"
VCH Weinheim, FRG 1997. EDC: http://www.electrophoresis-development-consulting.de/index.html

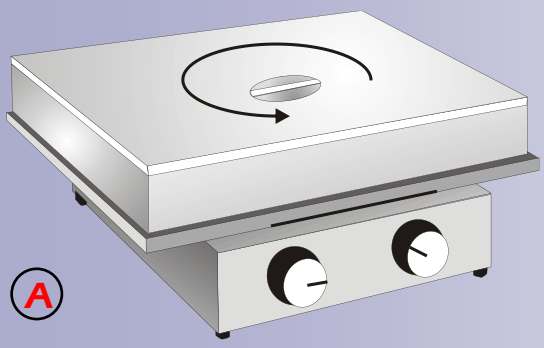
Principle

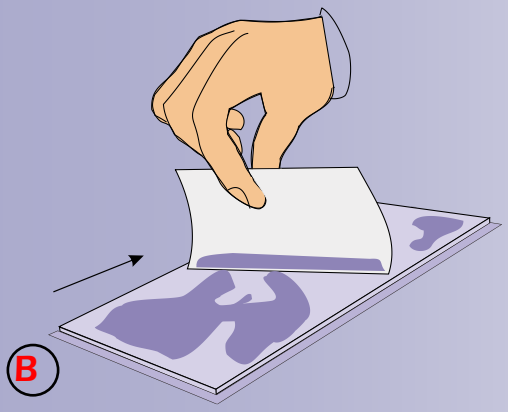
Blotting is the transfer of large molecules on to the surface of an immobilizing membrane. This method broadens the possibility of detection for electrophoretically separated fractions because the molecules adsorbed on the membrane surface are freely available for macromolecular ligands, for example antigens, antibodies, lectins or nucleic acids. Before the specific detection the free binding sites must be blocked with substrates which do not take the part in the ensuing reaction.

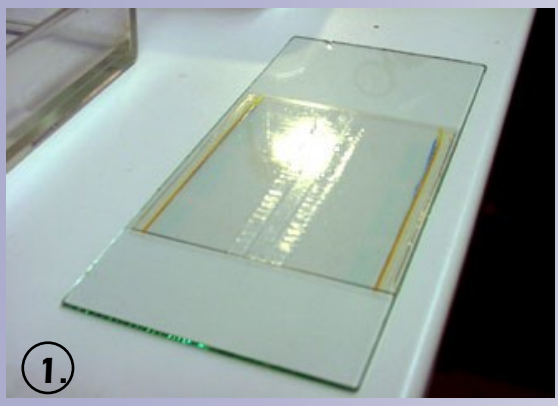


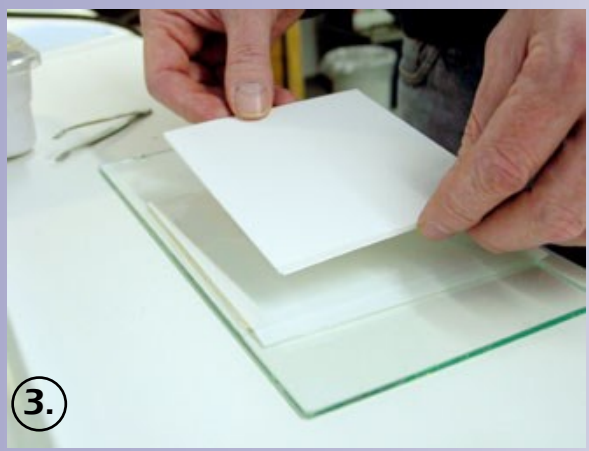
Blotting membranes

Nitrocellulose is the most commonly used membrane. It is available in pore sizes from 0.05 µm to 0.45 µm. The pore size is a measure of the specific surface: the smaller the pores the higher the binding capacity. Recommended pore-size is 0.2 µm for Semi-Dry and Contact-blot.
Proteins adsorbed on nitrocellulose can be reversibly stained so that the total protein can be estimated before specific detection .
Polyvinylidenedifluoride (PVDF) membranes on a Teflon base possess a high binding capacity and a high mechanical stability like nylon membranes. PVDF membranes can also be used for direct protein sequencing.
In our experiments the PVDF-membranes and the 0.2 µm Nitrocellulose membranes are used.









Contact Blot

The most simple blotting method after a IEF, SDS– or 2D electrophoresis is the "Contact Blot". Using this transfer-method the plastic-support of the SDS-gel must not be removed.
Because water must pass the membrane, a hydrophilic material is recommended: Nitrocellulose, 0.2 µm pore size (page Alternatively: PVDF-membranes (Teflon) will work also.

Please note that SDS– and 2D-gels - run longer than 2 - hours - have to be equilibrated in a buffer solution to amend the loss of water during electrophoresis.

Surely the effectivity of the protein-transfer is not as good as with electrophoretic blotting methods.

After the electrophoretic procedure the blotting membrane is directly layed on the gel-surface. With the help of dry filter paper and a weight the SDS-proteins are forced to migrate onto the membrane.
For SDS-proteins run in a 12.5% T gel nice results are achieved up to 70 kD. Bigger proteins should be blotted overnight and/or run in a 7.5% gel.
This blotting method is no quantitative technique, but sufficient for many qualitative tests.
Cut the necessary filter papers (5 x Whatman´s No.1) and the blotting membrane to the size of the gel (25.5 cm x 11.5 cm).

⇒ <u>For SDS– and 2D-gels:</u>
A) The gel should be first equilibrated 30 min in 50 mM Tris/Cl pH 8.0, 0.05% SDS to amend the gel's lost water content.
B) Then dry the gel's surface with a Drying Cardboard.

<u>IEF-gels:</u>
Blot of IEF-gels can be started right here.

⇒ The PVDV-membrane is activated in 100% methanol for 10 minutes. This step is not necessary for Nitrocellulose-membranes. The membrane is then placed in dist. Water for 5 min.
⇒ The SDS-gel is placed on a glas-plate with the support-film down. Fig.1.
⇒ The membrane is layed on the gel-surface, avoid air-bubbles! Fig.2.
⇒ Five dry and clean filter-papers are layed onto the membrane. Fig.3.

Further steps see next page.

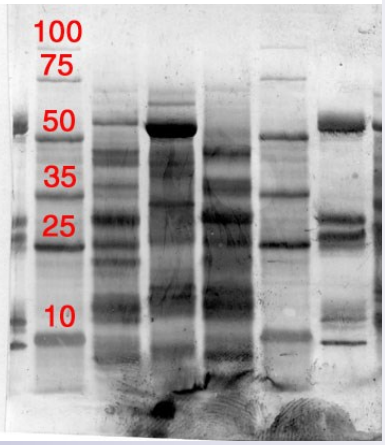
⇒ An additional glas-plate is applied on the stack. Fig.4.

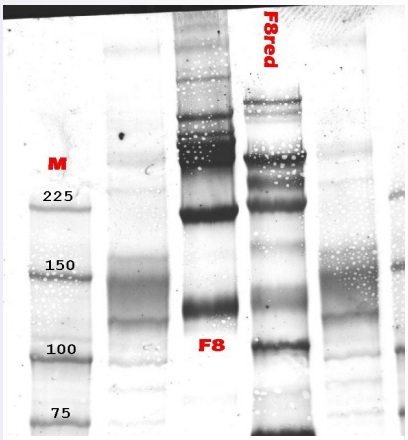
⇒ A 2-3 kg weight is standing on the contact-blot for 2 hours (IEF) and 4h – overnight for SDS– 2D- and native-gels. Fig. 5.

Staining: Indian Ink see below and also page 9.

Contactblot using a 5 liter beaker and a glas-plate.
Alternative: See below the BEO-Blotter

Contactblot out of a 12.5 % T gel, transfer: 5-75 kD



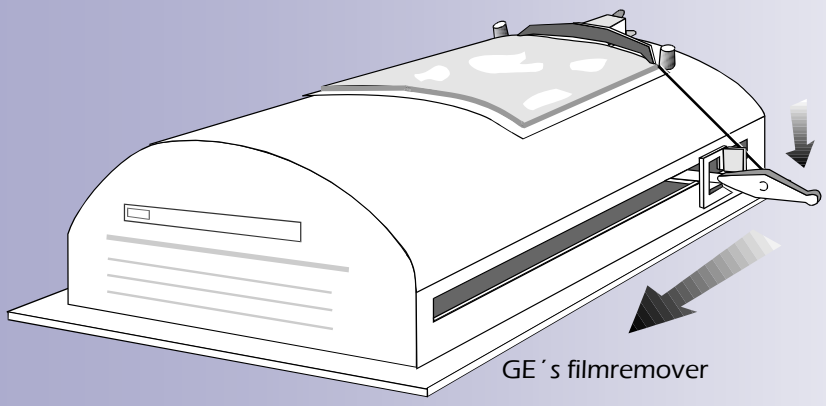


Contactblot out of a 7.5 % T gel, transfer up to 800 kD

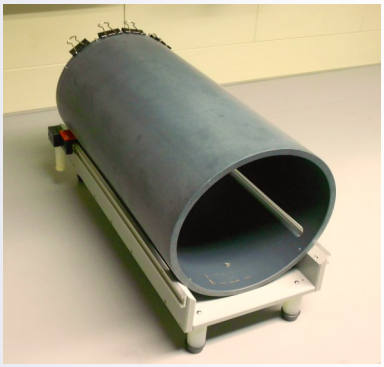
Dyeagnostics BEO-Dry-Blotter

Electro-Blot: Semi-dry blotting

When gel are bound to backing materials, such as polyester sheets, these materials must be removed before electrophoretic blotting. This is done by an instrument called "film-remover".
Semi dry blotting between two horizontal graphite plates has gained more and more acceptance in the last few years.

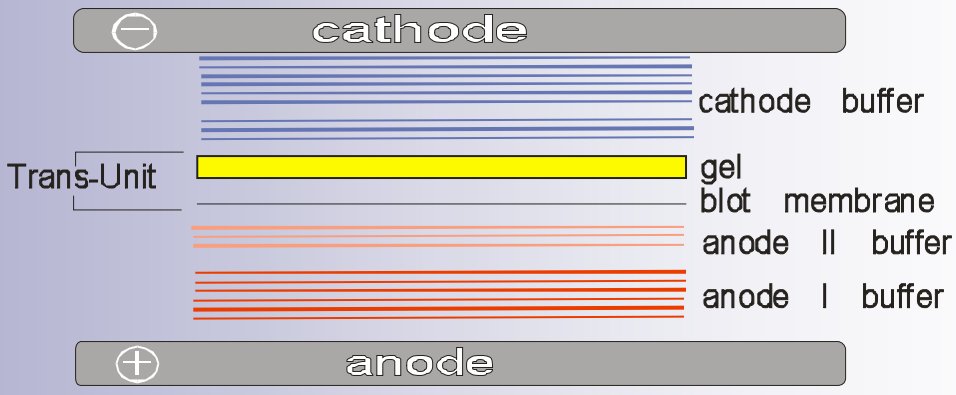


GE´s filmremover



EDC´s filmremover

Semi dry blotting between two horizontal graphite plates has gained more and more acceptance in the last few years. Only a limited volume of buffer, in which a couple of sheets of filter paper are soaked, is necessary. This technique is simpler, cheaper and faster and a discontinuous buffer system can be used. Kyhse-Andersen J. J Biochem Biophys Methods. 10 (1984) 203-209. Tovey ER, Baldo BA. Electrophoresis. 8 (1987) 384–387.
Graphite is the best material for electrodes in semi dry blotting because it conducts well. does not overheat and does not catalyze oxidation products.



The transfer time is approximately one hour and depends on the thickness and the concentration of the gel.
Ready-made or self-made gels backed by support films are used more and more often for electrophoresis of protein" .and electrofocusing. These films which are inpermeable to current and buffer must be separated from the gels so that electrophoretic, transfers or capillary blotting can be carried out. To separate the gel and the film without damage an apparatus exists with a taut thin steel wire which is pulled between them.

Most blotting procedures can also performed with an continuous buffer system:
Anode I, Anode II and cathode are the same buffer.
Addition of 0.01%SDS can perhaps help, see next page.

compound	concentration	weigh in - ***1000 ml***
TRIS	25 mM	3.03 g
Glycine	192 mM	14.41 g

.....gives a pH of 8.4; do not titrate!

Semi-dry blotting buffer system
Continuous buffer system (single buffer system: Anode I = Anode II = Cathode)
25 mM Tris (3.03g), 192 mM Glycine (14.41g), make up to 1000 ml with H_2O bidest, see last page.
Described here in detail:
Discontinuous buffer system (three buffer system)
Anode I: 0.3 mol/l Tris (18.15 g), 20 % methanol (100 ml), make up to 500 mL with H_2O bidest.
Anode II: 25 mmol/l Tris (3.03g), 20 % methanol (200 ml). make up to 1000 mL with H_2O bidest
Cathode: 40mmol/l 6-aminohexanoic acid (ε–aminocaproic acid) (2.6g), 20 %methanol (100 ml) (when a native gel *or IEF(should be blotted 0.01 % SDS can help, but check the following biological reaction!), make up to 500 mL with distilled water.

If the transfer efficiency of high molecular weight proteins (80 kDa) is not satisfactory, the gel can be equilibrated in the cathode buffer for 5 to 10 min before blotting.
For enzyme detection the buffer must not contain any methanol, otherwise biological activity is lost. Brief contact with a small amount of SDS does not denature the proteins.For transfers from urea IEF gels the urea should first be allowed to elute out of the gel by soaking it in cathode buffer. Otherwise the proteins, which possess no charge after IEF cannot bind to SDS as required for electrophoretic transfer.

Technical procedure, Practical tips
The filter paper and the blotting membrane must be cut to the size of the gel so that the current does not flow around the sides of the actual blot sandwich. Small blot sandwiches can be placed beside one another.

The transfer buffers contain 10% - 20% methanol (for %T-value up to 7.5%T, take 35% for a 12.5% T-gel), so that the gels do not swell during the transfer and the binding capacity of the nitrocellulose is increased.

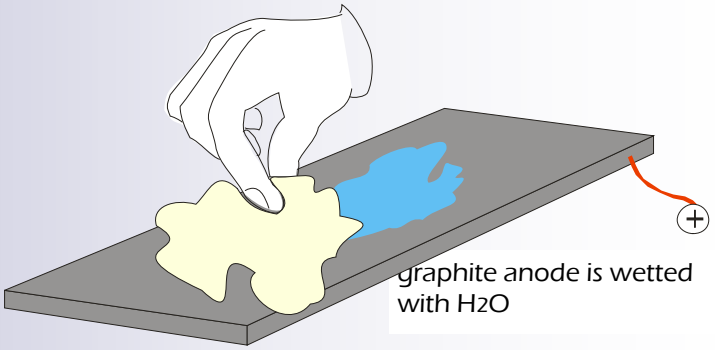
When gels are bound to a plastic film, the gel and the support film should first be separated with the GelRemover.A thin wire is pulled between the gel and support film.

In discontinuous buffer systems the speed of migration of the proteins changes during the transfer because of the different ionic strengths of both the anode buffers (0.3 mol/L and 0.025 mol/L). This means that fewer proteins are transferred. The slow terminating ion in the cathode buffer compensates for the differences in speed of migration of the leading ions (here the proteins): a more regular transfer is obtained. In this case, SDS is only added in the cathode buffer.

What you need for Semy-Dry blotting: Tray 1 –3, Tray for methanol
In case of Tris/ Glycin only one buffer-tray is necessary

Performance
To avoid contaminating the buffer, blotting membranes and filter papers, rubber gloves should be worn to carry out the following operations. 3 staining trays and 1 methonol-tray are necessary!

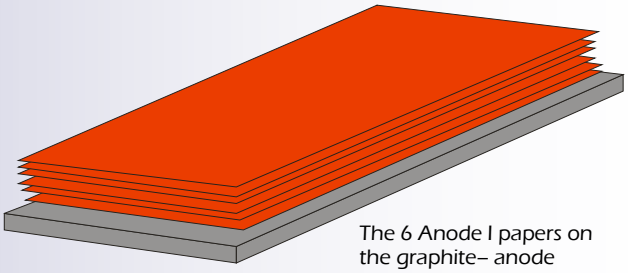
⇒ Wet the graphite anode plate (with the red cable) with distilled water, remove the excess water with absorbent paper.


graphite anode is wetted with H_2O

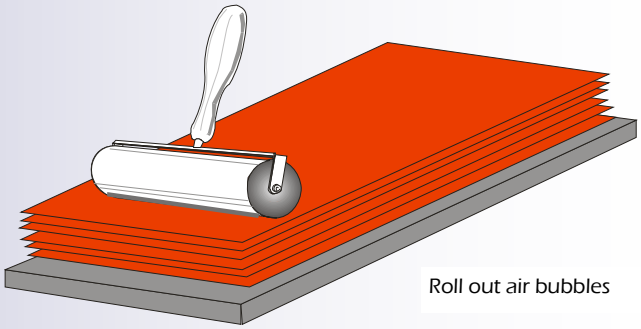
⇒ Cut the necessary filter papers (6 for the anode I, 3 for the anode II, 9 for the cathode) and the blotting membrane to the size of the gel (25 cm x 11.5 cm).

Only PVDF membranes must be activated for 10 minutes in 100% methanol. See next page: „Blot-Sandwich and Cutting with PVDF-membranes".
For Nitrocellulose-membranes this step is not necessary and should also not done yet.
See next page: „Blot-Sandwich and Cutting with Nitrocellulose-membranes"

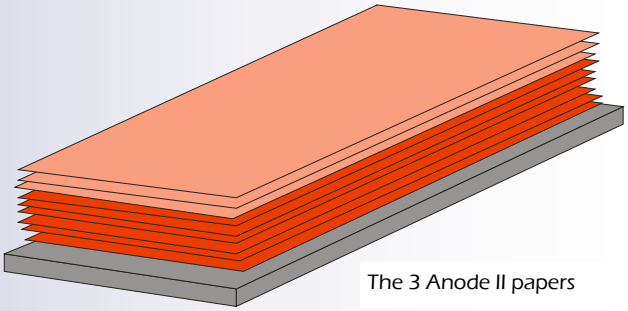
⇒ Pour 200 ml of Anode II solution in the staining tray #1.
⇒ The blot-membrane and the gel must be equilibrated in 400 ml Anode II buffer: membrane for 5 minutes and the gel for 20 minutes. Gels on support films are left to swim on the top of the buffer with the gel surface down. The membrane can swim below the gel. Place this staining tray on a rocking platform and shake slowly.
Do not equilibrate EDC's BlotGel-Membrane sandwich! It will fall apart!
⇒ Now pour 200 ml of anode I solution into the staining tray #2;
⇒ slowly impregnate 6 filter papers in Anode I and place them on the anodal graphite plate.
⇒ When building a blot sandwich as described here, it is difficult to completely avoid air bubbles. They must therefore be pressed out with a roller: Start in the middle and roll out in all four directions.
Press in such a way that the buffer in the sandwich oozes out but is not completely pressed out. When the roller is removed, the buffer should be "drawn" back in;
⇒ Slowly impregnate 3 filter papers in the Anode II solution and place them on the anode I batch;
⇒ Roll out air bubbles, see above
⇒ Take the cooling plate from the electrophoresis chamber:
⇒ Place the anode plate in the electrophoresis chamber, plug in the cables.
Take out the equilibrated gel (20-30 min!) and place it on a paper. Remove exceed buffer volume using a drying cardboard.
⇒ Place gels with the film side down on the Gelremover; so that a short side is in contact with the gel stoppers.
⇒ Attache the clamps on the short side of the gel's film support
⇒ Hook the first handle on the one side into its grip. Place the wire over the edge of the gel



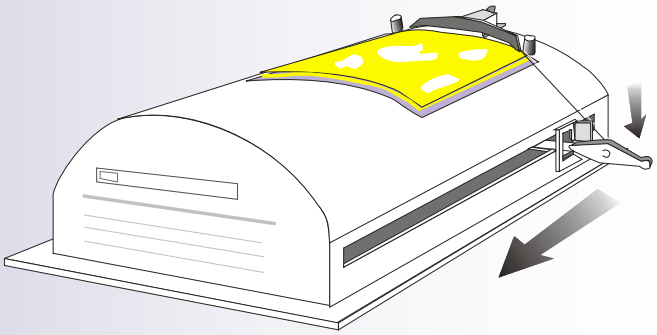
The 6 Anode I papers on the graphite– anode



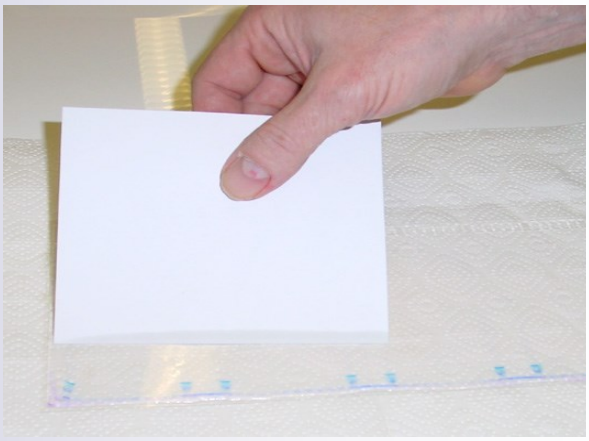
Roll out air bubbles



The 3 Anode II papers



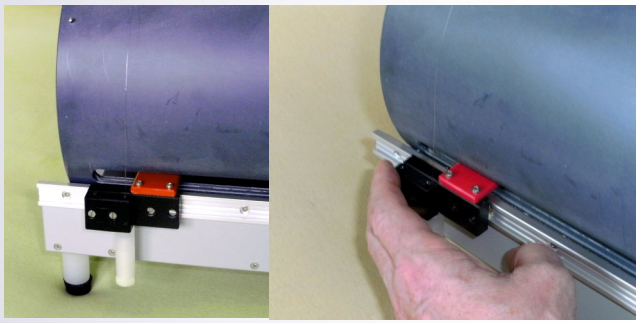
The gel and its plastic support is placed on the Gelremover



The exceed buffer volume is removed from the gel surface.

beside the clamps and tear the handle down to the grip of the other side.
Now the file has tension enough to be drawn over the film/support

⇒ The wire now has the mechanical tension necessary to separate the gel completely from the support film.



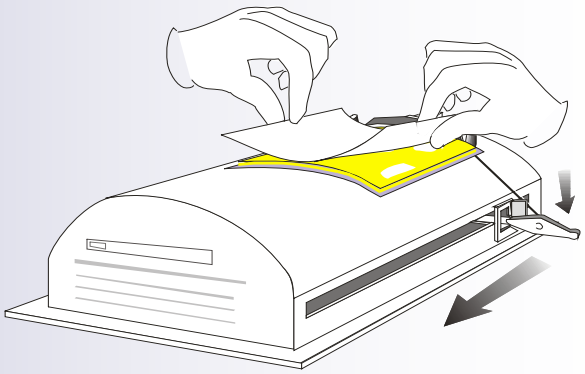
Lever position off in left picture, lever position on in the right picture

The methods of building up the sandwiches for nitrocellulose-membranes (NC-membranes) and PVDF-membranes are different. Due to the fine adhesive capacity of a dry NC-membrane (to the gel-surface) it should be not equilibrated before cutting.
The PVDF-membranes cannot be wetted directly with water – they should be first wetted with MeOH then with the transfer-buffer.

<u>Blot-Sandwich and Cutting with Nitrocellulose-membranes</u>

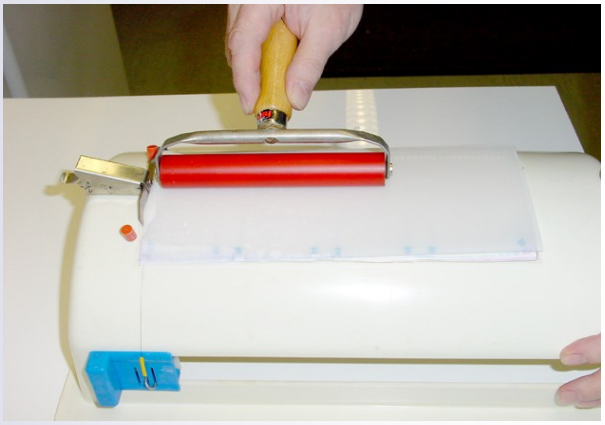
This membranes are used dry so the membrane can stick to the gel's surface

⇒ The dry Nitrocellulose-membrane is pressed slightly on the gel with a roller. Now the transfer-unit (gel + blot-membrane) sticks together forming a „transfer-sandwich".



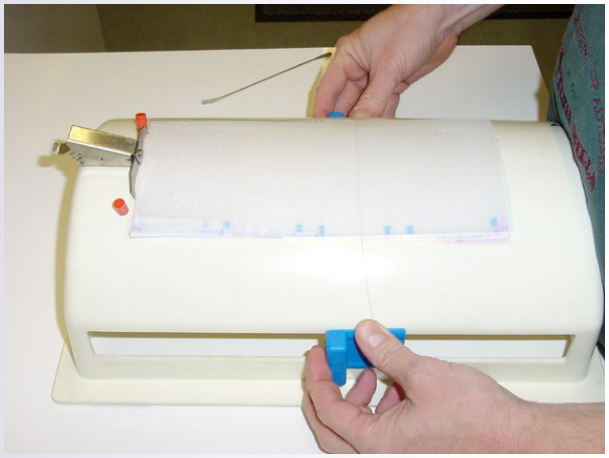
The dry NC-membrane is placed on the gel surface.

⇒ Roll out any air-bubbles with a roller



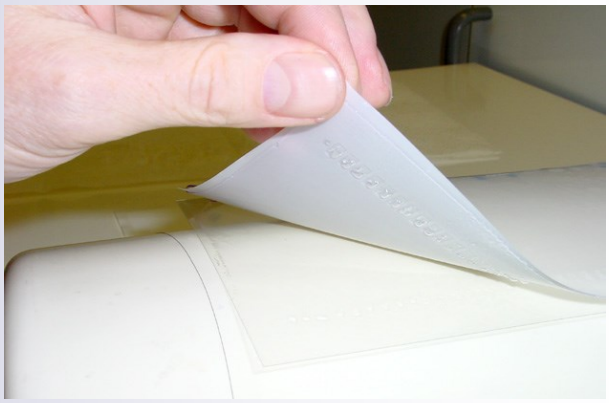
The air bubbles are rolled out the trans-unit

⇒ Grasp the handles with both hands and smoothly pull the wire towards you.



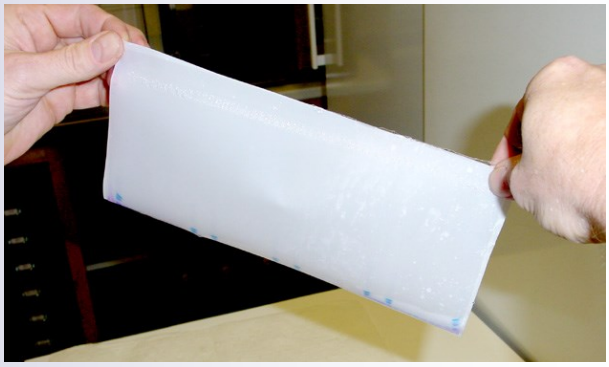
The wire cuts below the gel.

⇒ Lift the blot-foil at one end while holding the gel with a spatula towards the membrane.



The membrane together with the gel is lift off the support film.

⇒ Carefully lift the transfer-unit totally using now both hands.

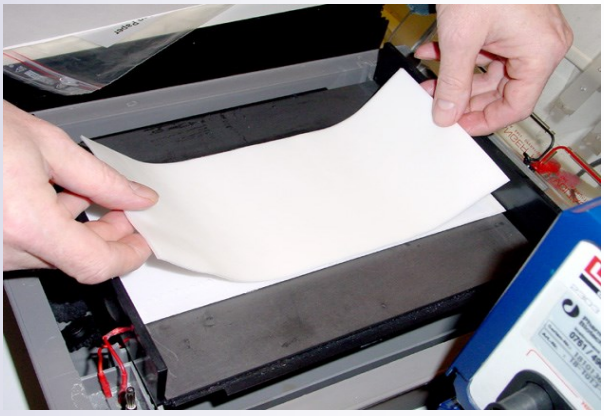


The membrane together with the gel is tran-ported to the blot-stack.

⇒ The transfer-unit can now be taken to the blot chamber. It is layed down on the paper-stack with the blotting membrane down. Avoid air bubbles and foldings.

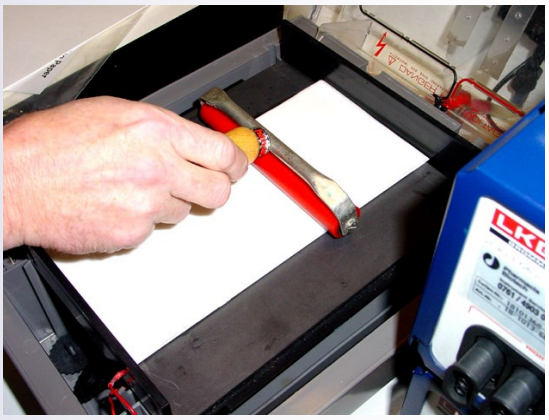
The trans-unit is layed on the anodal blot-stack with the membrane down.

⇒ Pour 200 ml of the cathode solution in the staining tray #3.
⇒ Soak 9 filter papers in the cathode buffer and place them on top of the stack



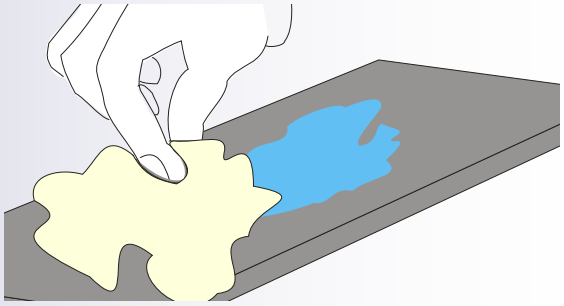
The cathodal paper stack is layed on the gel.

⇒ Roll out the air bubbles. Start in the middle and roll out in all four directions,



The air bubbles are rolled out of the blot-stack.

⇒ Wet the graphite cathode (black cable) with distilled water; remove the excess water with absorbent paper;
⇒ Place the cathode plate on the stack, plug in the cable:
⇒ place the safety lid on the electrophoresis chamber and connect the cable to the power supply.



Graphite cathode

Power Supply settings
see page 11

Method #2 for PVDF-membranes

⇒ PVDF-membranes cannot be wetted directly with water - they have to be first wetted with MeOH then with the transfer-buffer/water.

⇒ The gels with 10-15% T have to be equilibrated with their transfer buffer including 30 - 35% MeOH.
IEF-gels (4 - 5% T) should have 20% MeOH for 20 minutes.

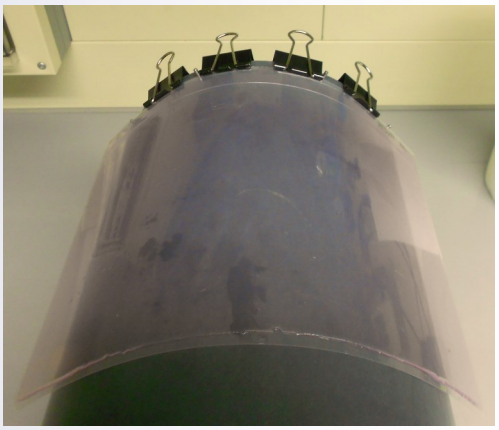
IEF-gels (4-5%T)	20% MeOH	10 min
SDS-gels (10-15%T)	30-35% MeOH	20 min

The exceed buffer volume is removed from the membrane

⇒ The blot-membrane is taken out of the transfer-buffer (Anode II buffer). Remove the exceed buffer volume using a drying cardboard.M
Lay the gel on the gelremover and push it forward until it touches the stoppers.

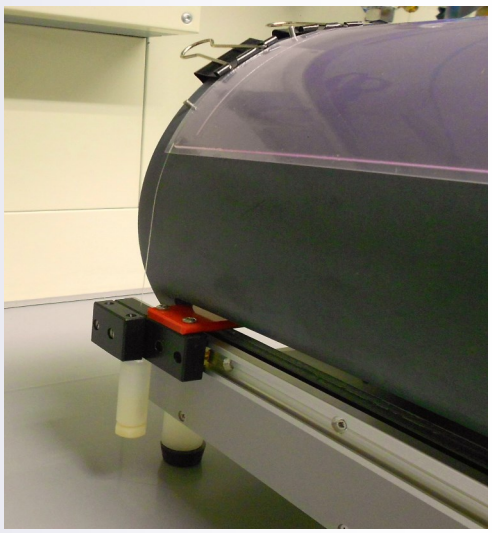
The gel is set on the Gelremover, support-side down

⇒ The clamps are clipped to the support-film to hold it while later pulling the wire.



The clamps are holding the support-film

⇒ Hook the wire to the drawing bar with its handles



The file is hooked to the sliding bar with its handles

⇒ Pull the wire slowly but without stopping through the

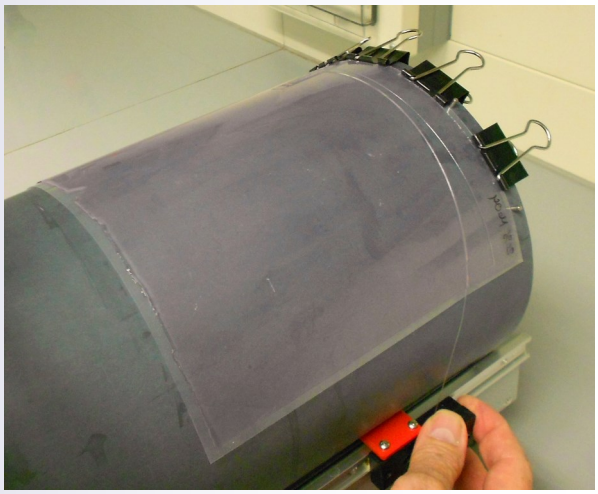
layer of the gel and the film:
Below the gel and on the support-film!

⇒ Take the sandwich, turn it upside down an place the gel - hanging still at the support-film– on the PVDF-membran that is already waiting on its anodal paper-stack

⇒ The support-film is now on top of the blotting stack: With the help of a spatula the sandwich is splitted so that the support film can be lifted upward and the gel stays on the blot-membrane.

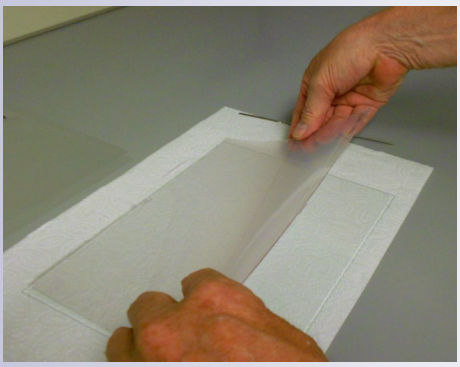
⇒ Bend the support-film to an angle of about 90°: This is the best way to finish the removal of the support

The gel should lay bubble-free on the anodal blotting stack
Finish the blot-sandwich like on page 8, 9.

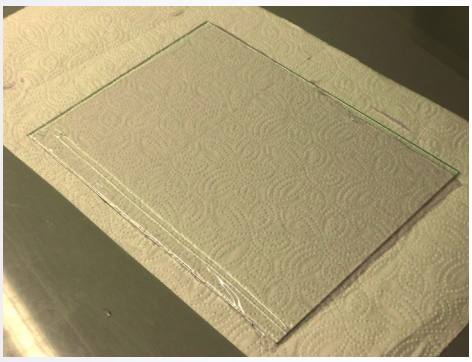


The wire is pulled through the layer:
Under the gel and on the support-film!

The sandwich is splitted with a spatula. The support is lifted upward and the gel stays on the membrane



With the angle of ~90° the support is lifted away from the gel



The gel should lay bubble-free on its anodal stack

Electrical Transfer conditions:

Blot at a constant current: 0.8 mA/cm^2 =235 mA 1 hour or better 235 mAh for whole blot.
Higher currents result in a rise of the temperature of the gel and are not recommended.

0.5 mm thick gel (11.5 cm x 25.5 cm = 295 cm ^{2}/

Set:	235 mA const	max	30 V	max	10 W
Stop	235 mAh = approx. 1 hour				
Read:	235 mA	4 - 15 V (Cont.Buffer), 1-2 W			
	235 mA	10 -30 V (Disc.Buffer). 2-7 W			

The blot does not warm up under these moderate conditions. If thicker or more concentrated gels are blotted, the blotting time can be increased up to two hours; it is then recommended to press down the cathode plate with a 1 to 2 kg weight so that no electrolytic gas pockets form.
These gels have enough mechanical stability that they are not crushed.

⇒ Switch off the power supply, unplug the cable:
⇒ remove the safety lid and the cathode plate;
⇒ take the blot sandwich apart;
⇒ stain the gel with Coomassie Blue to check.

Before visualization, either dry the gel overnight or for 3 to 4 h at 60 °C in a heating cabinet.

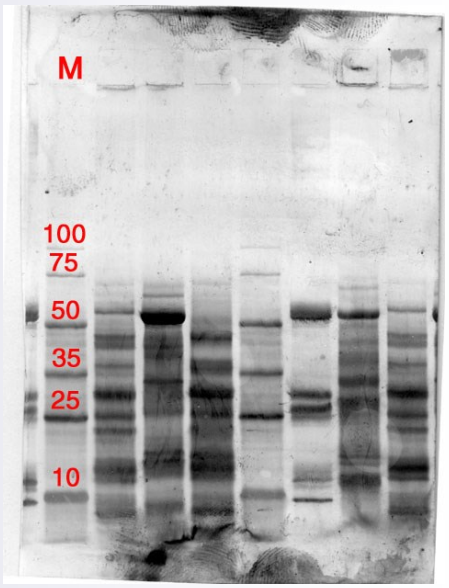
Visualization

Indian Ink staining

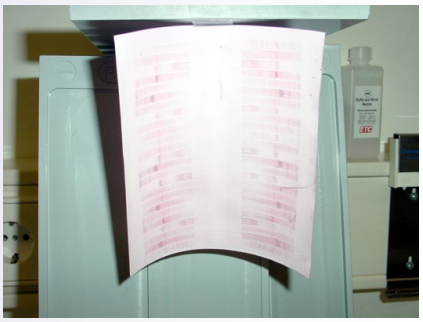
Unfortunately Indian Ink is no longer produced. However, the description of this fine method is no longer removed from this issue of the book for the case it would again be produced, or an ink with comparable performance would be found. The sensitivity comes close to the silver staining procedure.

PBS-Tween: 48.8 g of NaCl + 14.5 g of Na_2HPO_4 + 1.17 g of NaH_2PO_4 + 2.5 ml of Tween 20, fill up to 5 L with distilled water (pH 7.3).

⇒ Dunk PVDF-membranes in 100% methanol
⇒ Staining: 2 h or overnight with 250 ml of PBS Tween + 2.5 ml of acetic acid + 250 µl of fountain pen ink ("Pelikan Fount India"). Use a rocking platform, Filter the staining solution first.
⇒ Washing : 2 x2 min with water;
⇒ Drying: air-dry



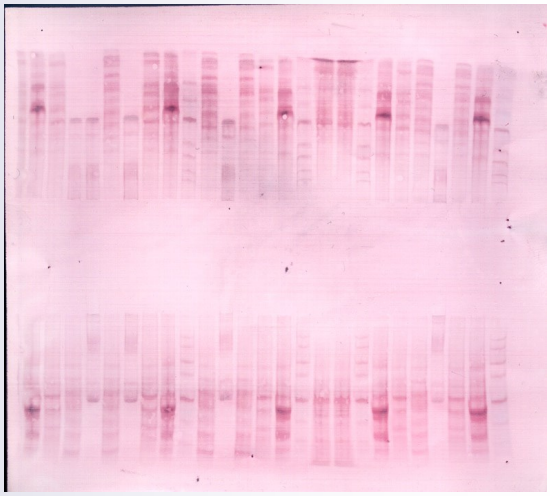
Indian Ink Staining overnight on Nitrocel-lulose


Hanging up the membra-ne for drying

Ponceau Staining

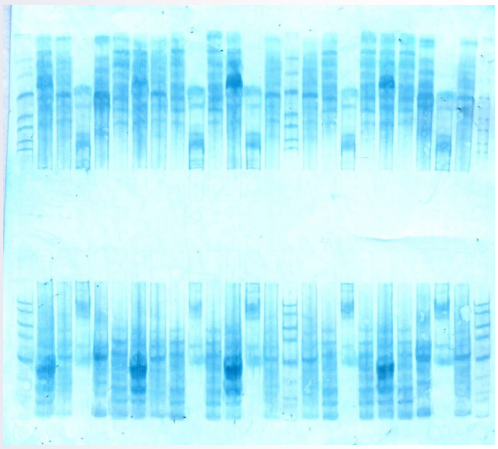
This staining is less sensitive, but reversible: A so-called soft stain. Difficult to scan.

⇒ Dunk PVDF-membranes in 100% methanol for 3 minutes after the blot-procedure
⇒ Stain blot in 0.5% (w/v) Ponceau S red / 1% acetic acid/ 3% NaH_2PO_3 (add short before staining) / 0.05% Triton X 100, for 5 minutes.
⇒ Destain membrane submarine in hand-warm water for about 2 - 3 minutes. Cau-tion: This can remove stain completely.
⇒ Drying: Hang the membrane in the air (see right picture) to dry for 25 minutes. This will develop fully the colour-contrast.


Ponceau S Staining PVDF

Fast Green Staining

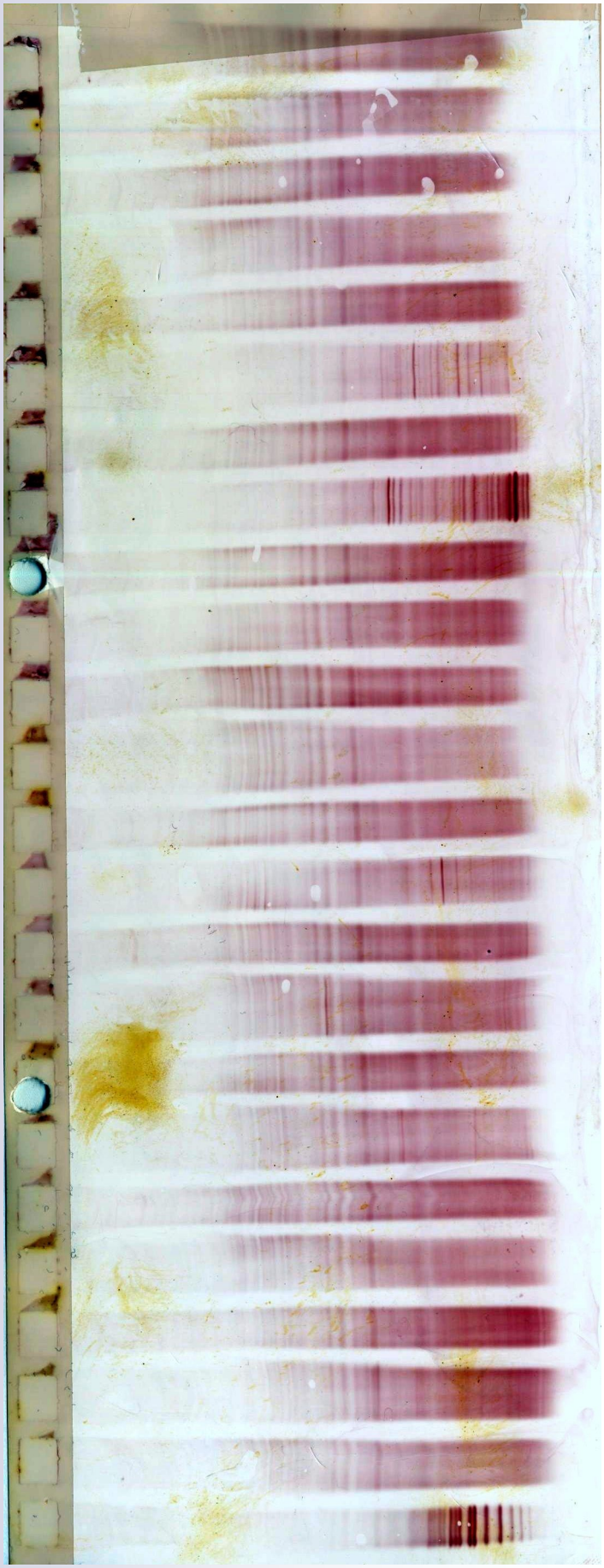
⇒ Dunk PVDF-membranes in 100% methanol
⇒ Stain blot in 0.2 % (w/v) Fast Green / 1% acetic / 3% NaH_2PO_3 (add shortly before use)/ 0.05% Triton x 100 for 5 minutes.
⇒ First destain: move membrane submarine in water for about 5 minutes.
⇒ Second destain in methanol for about 2 minutes, bands will occur
⇒ Drying: Hang the membrane in the air to dry for 25 minutes. This will develop fully the colour-contrast.
⇒ Total (band) destain: Dilute 0.4 M NaOH 1 + 1 with methanol. Destain for 10 min..


Fast Green Staining on PVDF

Comparison: Effectiveness of transfer in contact blot

Silver staining *(E.Dubois, Neurology Tübingen, DE)*

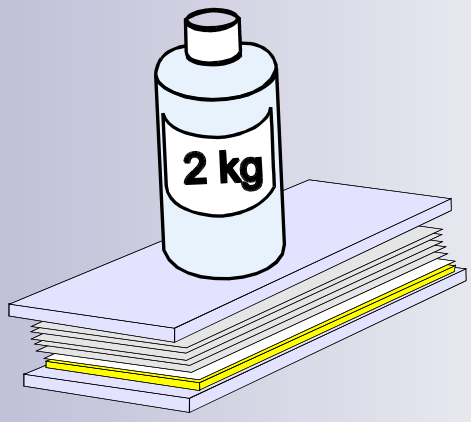
Contact blot and Immunostaining
(E.Dubois, Neurology Tübingen, DE)



Bottom lines show the BioRad IgG standard

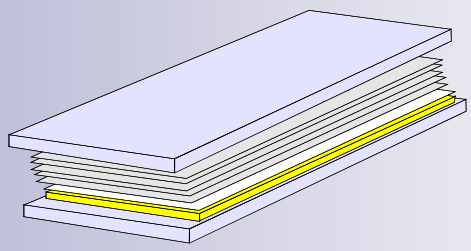
Steps for Contact Blotting

6



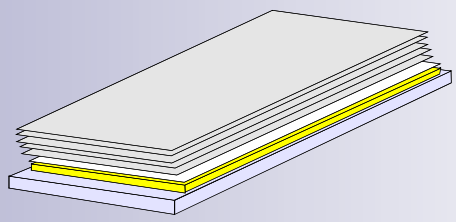
A weight stands on top

5



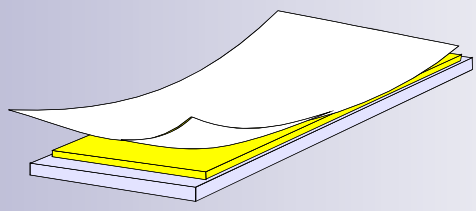
The upper glasplate

4



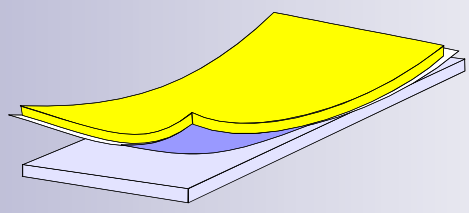
The filter papers on the blotting-foil

3



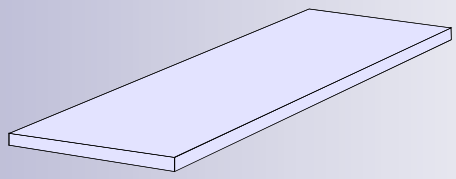
The blotting-foil is placed on the gel-surface

2



The gel with the backing foil down on the glasplate

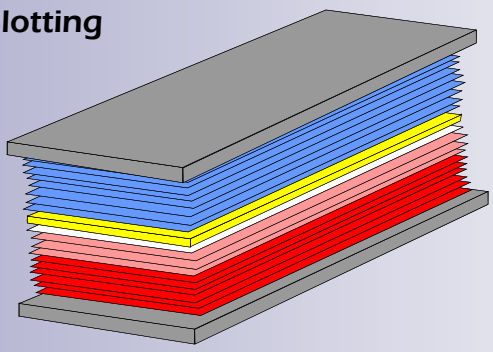
1



The lower glasplate

Steps for Semi-dry Blotting

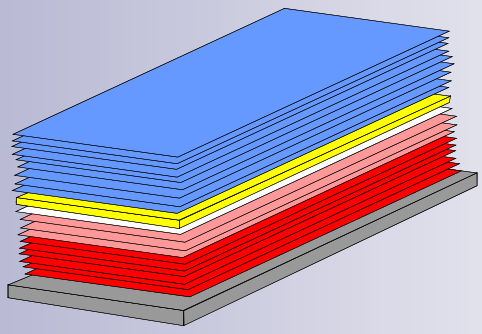
7



graphite cathode

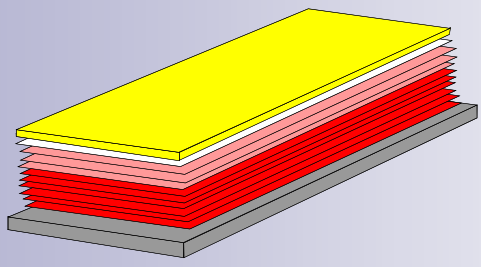
cathode buffer

6



gel on the blot foil

5



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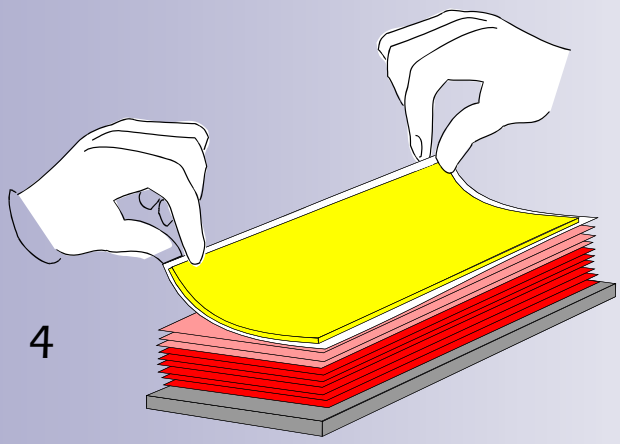
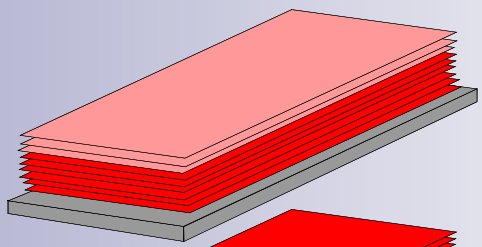


blot membrane together with the gel, blot membrane down

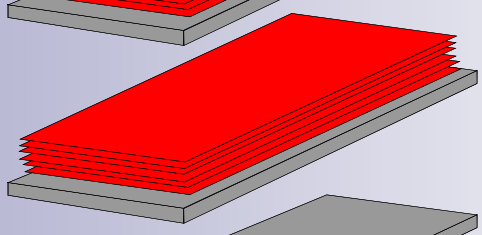
anode buffer II

3



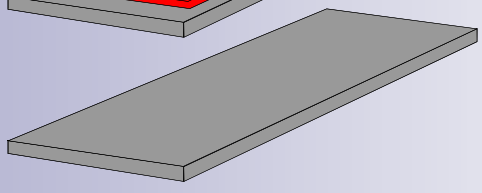
anode buffer I

2



graphite Anode

1

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