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**INSTRUCTION MANUAL
OF
BLOTTING APPARATUS**

TECHNOBLOT-O

With Single Cassette

(CAT. NO. TBTBR-SC1010)

(CAT. No. 13-00001)

TECHNOBLOT

With Dual Cassette

(CAT. NO. TBTBR-DC1010)

(CAT. No. 13-00000)

TECHNOBLOT

With Four Cassettes

(NEW CAT. NO. TBTBR-FC1010)

(CAT. No. 13-00004)

For Western, Southern, Northern blotting

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Essential components of the TECHNOBLOT:

- a) One tank.
- b) One cathode and one anode plate.
- c) Two anode-facing blotting sandwich grid (BSG) frames.
- d) Two cathode-facing winged BSG frames, each bearing four tube nuts.
- e) Four fibre sponge pads.
- f) Eight push-screws.
- g) One pair of connecting cords.
- h) One lid.

Additional components of the TECHNOBLOT:

- a) Storage box for the entire unit.

CAUTION

Use the apparatus with care. It should be washed and rinsed with soft water, air dried at room temperature and stored covered.

It should **NOT BE**:

subjected to impact,

exposed continuously to temperatures outside the range of 0-40°C,

handled with abrasive or sharp objects,

exposed to organic solvents such as aromatic or halogenated hydrocarbons, ketones, esters, alcohols over 30% or concentrated acids over 25%,

exposed to other solutions which will attack plastics such as acrylic, acetal, Celcon, Noryl, Dacron and epoxy.

To avoid electrolytic corrosion, always dry the connecting pins on central electrode unit before attaching the electrical cords to them.

Step 1. Assemble all components of the apparatus nos 1-6

Step 2. Slide the anode and cathode plates in the respective slots of the tank.

Note: The anode/cathode plates will not slide into the tank if placed in the wrong slot.

Step 3. Adjust the stoppers fitted on the screws of the winged BSG frame (Gel side). It is important that all four stoppers are accurately levelled (see manual)

Step 4. Assembly of blotting sandwich.

You will require

- a) Borosilicate tray
- b) Filter papers cut to size of sponge pads
- c) Membrane cut to size of gel
- d) Buffer solution
- e) Membrane forceps
- f) Winged BSG cassette frames.

IMP: Assemble the blotting sandwich under buffer in a borosilicate tray in the order listed below. Ensure that no bubbles get trapped between any two layers.

- a) Place the winged BSG frame bearing the stoppers on the floor of the tray, flat surface upwards.
- b) Place a soaked sponge pad over the frame.
- c) Place a wet filter paper over the sponge pad.
- d) Gently place the gel on the filter paper
- e) With a membrane-forceps, carefully place the wet membrane on the gel.
- f) Place a wet filter paper over the membrane. (For northern/Southern blotting place three more wet filter papers over the gel.)
- g) Place a soaked sponge pad over the filter paper. (For Western Blotting)
- h) Place the BSG frame over the sandwich.

Secure the assembled blotting sandwich with the push-screws.

Step 5. Ensure that the tank is at least half filled with chilled buffer.

Step 6. Quickly transfer the assembled blotting sandwich to the tank, with winged cassette frame facing the cathode plate. Adjust buffer level so that the gel sandwich is completely submerged.

Step 7. Cover the unit with the lid, attach connecting cords and connect to power supply unit.

Step 8. Run the apparatus, in an ice water bath or refrigerator

Items needed to perform a Western blotting experiment:

- a) Four white filter paper sheets; $10 \times 11 \text{ cm}^2$ (thickness equivalent to Whatman # 3).
- b) Hydrophilic nitrocellulose membrane; $\leq 10 \times 11 \text{ cm}^2$ (pore size $0.22 \mu\text{m}$) for the transfer of proteins.
- c) Methanol-containing transfer buffer.

- d) Aluminium foil, gel slicer.
- e) A constant voltage (CV) or constant current (CC) power supply.
- f) Gel containing the separated proteins.
- g) Heavy-walled borosilicate glass tray (15×15×4.5 cm), a two-litre beaker, 10 mL pipettes.
- h) Ice-water bath/ refrigerator shelf-space.
- i) Blunt-edged membrane-forceps.

Items needed to perform a Northern/Southern blotting experiment:

- a) Six white filter paper sheets; 10×11 cm² (thickness equivalent to Whatman # 3).
- b) Positively charged/uncharged nylon membrane; ≤ 10×11 cm² (pore size 0.22 μm) for the transfer of nucleic acids.
- c) Transfer buffer.
- d) Aluminium foil, gel slicer.
- e) A constant voltage (CV) or constant current (CC) power supply.
- f) Gel containing the separated nucleic acids.
- g) Heavy-walled borosilicate glass tray (15×15×4.5 cm), a two-litre beaker, 10 mL pipettes.
- h) Ice-water bath/ refrigerator shelf-space.
- i) Blunt-edged membrane-forceps.

ASSEMBLING THE TECHNOBLOT- A SUMMARY

- 1) Prepare transfer buffer and, if desired, chill it to 4°C.
- 2) Before use, pour buffer into the tray. Immerse the fibre-sponge pads in buffer and squeeze out all the entrapped air.
- 3) Slide the electrode plates into their respective slots.
- 4) Assemble the blotting sandwich under buffer in a borosilicate tray in the order listed below. Ensure that no bubbles get trapped between any two layers.
 - a) Place the winged BSG frame bearing the tube nuts on the floor of the tray, flat surface upwards.
 - b) Place a soaked sponge pad over it.
 - c) Place a wet filter paper over it.
 - d) Gently place the gel on the filter paper
 - e) With a membrane-forceps, carefully place the wet membrane on the gel.
 - f) Place a wet filter paper over it.

For western blotting, follow steps 'g' and 'k'. For Northern/Southern blotting, follow steps 'h' and 'k'.

 - g) For western blotting, place a soaked sponge pad over it.
 - h) For northern/Southern blotting, place three more wet filter papers over it.
 - k) Place the BSG frame over the sandwich.
- 5) Secure the assembled blotting sandwich with the push-screws.
- 6) Pour chilled buffer into the tank so as to fill half of it.
- 7) Quickly transfer the entire assembly to the tank with the winged cassette facing the cathode plate. The buffer level should be adjusted so as to ensure that the gel is fully submerged.
- 8) Cover the unit with the lid and place it in an ice-water bath/refrigerator.
- 9) Perform the transfer at a voltage gradient of 2–4 V/cm overnight or at 10 V/cm in 1.5 hr.

DETAILED PROCEDURE FOR ASSEMBLING THE TECHNOBLOT

I Preparatory tasks:

- 1) Prepare 1.5 L of transfer buffer. If methanol is a component, use the analytical grade. Lower purity methanol contains aldehydes which impede transfer. Add the methanol to the buffer and cover it with foil. Cool it to 4°C. (The dissolution of methanol in water liberates heat. Hence, it is best to add it first and then cool the mixture.)
- 2) Cut four pieces of filter paper (thickness equivalent to Whatman # 3) to the size of the sponge pad (10×11 cm²). Also cut the 0.22 µm nitrocellulose membrane to the size of the gel (size should be ≤ 10×11 cm²) that is to be blotted. Mark the corners with a pencil.
- 3) Clean all the parts of the Technoblot (except sponges) and the heavy- walled tray with water. If necessary, use detergent. Ensure that all the parts are finally rinsed with tap (soft) water. Drain off the excess water.
- 4) The assembly has been fitted with threaded stoppers on the guide pins to ensure that the gel does not suffer distortion due to excessive pressure within the sandwich. To adjust the correct position of the stoppers, place the broader frame with its flat surface upwards. Place two rectangles of filter paper and two sponges over the ribs. Place the narrower BSG frame over the sponges such that all the four guide-pins slide into their corresponding holes. Move the stoppers up or down to allow the BSG frame to rest lightly on the sponge. The stoppers may then be further raised or lowered to the desired height depending on the thickness of the gel, number of filter paper sheets employed and the pressure that can be tolerated by the gel (a function of its composition such as % agarose, or %T and %C of poly-acrylamide).

II Assembling the sandwich.

- 1) After the electrophoresis has been terminated, pour chilled buffer into the borosilicate tray and immerse the fibre-sponges in it. The presence of methanol facilitates wetting of the sponges and most of the entrapped air can be easily squeezed out. If the buffer does not contain methanol, squeeze the sponges repeatedly till all the air is removed. Transfer the wet sponges to the beaker containing the buffer.
- 2) Remove the gel-sandwich from the MINIKIN and prise the plates apart. Sprinkle some buffer on the gel to prevent it from drying. Detach the gel from the bottom plate and trim away the unnecessary gel portions. The gel should not be larger than 10×11 cm². Mark the orientation of the gel.
- 3) For the transfer of anionic proteins, assemble the blotting sandwich under buffer in the sequence listed below. Ensure that no bubbles are trapped between the layers.
 - a) Place the broader black winged BSG frame bearing the tube nuts on the floor of the tray with its flat surface upwards. Note that the engraving 'GEL SIDE' should be at the top.

- b) Place a wet sponge pad over the ribs of the BSG frame. The tube of the nuts that protrude through the BSG frame will prevent the sponge from moving.
- c) Wet a filter paper with buffer, and beginning from one edge, gently lower it on to the sponge. Push the fine bubbles to the side.
- d) Gently lift the gel and place it on the filter paper. Position the gel appropriately. It is difficult to see the gel against the white background. The ink spots help delineate the boundaries.
- e) Wet the nitrocellulose filter paper in methanol-containing transfer buffer to remove the bubbles. (In case the hydrophobic PVDF membranes are used, they should be immersed in 100% methanol.) With a membrane-forceps, carefully place the membrane on the gel. Begin at one edge and proceed to the other whilst lowering the membrane on to the gel.
- f) Place a buffer-soaked filter paper over the membrane. To do so, repeat step II-3-c.
- g) Then place a wet sponge pad over it.
- h) Finally place the narrower BSG frame (flat surface downwards) over the sandwich such that the guide pins, protruding from the winged frame, slide into the smaller holes in the narrower frame.
- i) Place one's palm over the narrower frame and push it down such that it comes to rest on the stoppers.
- j) Secure the assembled blotting sandwich with the push-screws. To do so, insert the push-screw such that the threaded portion slides through the unthreaded arc of the tube-nut and its head rests on the upper surface of the frame. Turn the screw in a clock-wise direction through ninety degrees to lock it. Repeat the process for the diagonally opposite push-nut and then for the other two push-nuts.
- k) Orient the tray such that the thickened portion of the wing is on the top right.
 - 4) Pour chilled buffer into the tank to fill half of it.
- 5) Slide the cathode plate into the tank. It being larger than the anode plate, will fit in only one pair of channels. Ensure that the connector faces inwards.
- 6) To put the blotting sandwich into the tank, lift it out of the tray and slide the wings into the central channels provided. Ensure that the black-winged frame faces the cathode (black connector) and that the thickened portion of the wing is on the top. The sandwich will not fit properly in any other orientation
- 7) Repeat step 3 for assembling the other sandwich, if desired.
- 8) Slide the anode (red connector) plate into the tank. Ensure that the connector faces inwards.
- 9) Adjust the buffer level so as to ensure that the gel is completely immersed in it.
- 10) Cover the unit with its lid.
- 11) Connect the black electrical cord to the cathode plate and the red cord to the other plate.
- 12) Lift the unit with the help of its handles and place it in the refrigerator/ice-water bath.
- 13) Connect the male end of the cords to the power supply. Perform the transfer at constant voltage or constant current. (See note below.)
- 14) The time for transfer depends on gel thickness, molecular mass of biomolecule(s), ionic strength of

transfer buffer, presence of ionic detergent and amperage used during transfer.

- 15) The resistance of the system with the commonly used chilled Tris-glycine-methanol buffer (pH 8.3) is around 400 ohms. The transfer can be done using a voltage gradient of 2-4 V/cm (20 - 40 V or 50–100 mA in a refrigerator) overnight or at 10 V/cm (100 V or 250 mA in an ice-water bath) in 1.5 hr.

Note: If your power supply has a CC feature, then it is safest (especially for rapid transfers) to perform the blotting using this mode. When blotting is done at CV, and the joule heat produced during blotting is greater than that removed from the system, the temperature of the buffer will keep on rising. In such cases, the resistance of the system decreases as the buffer gets warmer. If the voltage is maintained at a constant value, more current flows through the system which further heats up the buffer, setting up a dangerous positive- feedback loop. In the CC mode, the voltage will drop as the temperature of the buffer rises. Consequently, less heat will be generated and the system will stabilize at a steady-state value, where the heat extracted from the system will be equal to that produced due to the passage of the current.

- 16) After the selected time-interval has elapsed, switch off the power supply, remove the Technoblot unit from the ice-water bath/ refrigerator. Disconnect the cords by holding the lid down and pulling the connectors up.
- 17) Remove the lid.
- 18) Pull out the blotting-sandwich and unbolt the frames by turning the push-screw counter-clockwise through 90 degrees.
- 19) Remove the narrower BSG frame, sponge pad and filter papers.
- 20) With the help of a blunt-edged membrane-forceps, carefully peel off the nitrocellulose membrane from the gel.
- 21) Transfer the membrane to a dish and process it for protein staining or immunoanalysis.
- 22) Transfer the gel to a staining tray and stain for proteins to ascertain the effectiveness of the transfer.
- 23) Wash the sponge pads with copious amounts of water, rinse them finally with soft water and allow to drip dry.
- 24) Clean all the parts of the Technoblot with water. Use detergent if necessary. Ensure that all the parts are finally rinsed with tap (soft) water. Drain off the excess water.
- 25) Dry the parts, fit them into the tank and store at room temperature in the box provided.

TIPS AND NOTES

- 1) Proteins can be fixed on nitrocellulose paper by either drying the paper at 37°C, or exposing the moist paper to fumes of paraformaldehyde, or by soaking the paper for 5 min (20°C) in 1% KOH, followed by rinsing, 2×10 min with TBS.
- 2) Binding capacity of nitrocellulose (NC) membranes is 250 $\mu\text{g}/\text{cm}^2$ and that of PVDF membranes is 172 $\mu\text{g}/\text{cm}^2$.
- 3) Methanol retards transfer of high ($>10^5$) M_r proteins.
- 4) NC membranes are not compatible with reagents used in micro-sequencing procedures. Hence, use PVDF membranes when the blotted proteins have to be sequenced. Also, amino buffers such as Tris and glycine should be avoided as they interfere with the sequencing.
- 5) The maximum recommended amperage for blotting is 0.8 mA/cm^2 of electroblotting buffer cross-sectional area, in the direction of transfer.
- 6) Binding of nucleic acids to nitrocellulose requires high salt concentrations. Such buffers will have a high conductivity and hence, will not result in efficient electrotransfers. Therefore, nitrocellulose membranes are incompatible with Northern/Southern electroblotting.
- 7) Nylon membranes, which can bind nucleic acids even in low ionic strength buffers, are recommended for the electroblotting of nucleic acids.

BUFFERS FOR ELECTROTRANSFER

Towbin's electroblotting buffer [25 mM Tris, 192 mM Gly]

(Most widely used buffer system with nitrocellulose membranes)

Tris	6.0 g
Glycine	28.8 g
Distilled Water (D/W)	1570 mL
Dissolve	
Methanol	400 mL
Use chilled	

Phosphate electroblotting buffer

(Useful for transfer of membrane receptor protein on to nitrocellulose membranes)

NaH ₂ PO ₄ ·2H ₂ O	3.86 g
Na ₂ HPO ₄	1.70 g
Distilled Water (D/W)	1.50 L
Dissolve	
Adjust pH to 6.8 with either 1 N NaOH or 1 N H ₃ PO ₄	
Methanol	400 mL
Make volume to 2.0 L with DISTILLED WATER	
SDS	1.0 g
Dissolve	
Chill and use	

High ionic strength electroblotting buffer [0.25 M Tris, 1.92 M Gly]

(Useful for efficient and reproducible transfer of protein onto nitrocellulose and PVDF membranes)

Tris	60 g
Glycine	288 g
Distilled Water	1650 mL
Dissolve	
Make volume to 2.0 L with D/W	
Use chilled	

CAPS electroblotting buffer [10 mM CAPS]

[Used with nitrocellulose and PVDF (Immobilon-P) membranes. Blotted proteins on PVDF membranes can be sequenced directly]

3-(cyclohexylamino)-1-propanesulfonic acid	4.4 g
Distilled Water	1670 mL
Dissolve	
Adjust pH to 11.0 with 1 N NaOH	
Methanol	200 mL
Use chilled	

Tris-borate Electroblothing buffer [0.05 M Tris, 0.05 M Borate pH 8.5]

[Used with nitrocellulose and PVDF (Immobilon-P) membranes. Useful to blot glyco-proteins. Blotted proteins on PVDF membranes can be analyzed for N-terminal sequences]

Tris	12.11	g
Boric Acid	6.18	g
Distilled Water	1550	mL
Methanol	400	mL
Dissolve		
Make volume to 2.0 L with DISTILLED WATER		
Use chilled		

BUFFERS FOR IMMUNODETECTION**Tris buffered saline** (10× stock) [TBS]

NaCl	76	g
KCl	3.72	g
Tris	12.1	g
Distilled Water	890	mL
Dissolve		

6 N HCl to pH 8.0. Approx 10 mL

Store in stoppered (borosilicate) glass bottles.

Add DISTILLED WATER to make vol to 1000 mL.

DAB buffer [water and alcohol insoluble brown, blue/black product]

(Used to detect horse-radish peroxidase immobilized on membrane)

NaCl	0.76	g
Tris	0.121	g
Distilled Water	97	mL
3,3'-Diamino benzidine.4HCl	0.05	g

Dissolve

1 N HCl to pH 7.5

Filter

H₂O₂ (100 vol) 67 μ L

Use within 15 min.

Phosphate buffered saline (pH 7.5) can be used instead of Tris-NaCl.

With the above buffer horse-radish peroxidase produces a brown reaction product. If Cobalt or Nickel Chloride (0.03% w/v) is added to the above buffer a blue/black water and alcohol insoluble reaction product will be formed.

AEC buffer [water insoluble, alcohol soluble red product]

(Used to detect horse-radish peroxidase immobilized on membrane)

Sodium acetate trihydrate	0.517	g
Distilled Water	98	mL
3-Amino-9-ethyl carbazole.HCl	24.6	mg
Dissolve		
1 N acetic acid to pH 5.0		
Filter		
H ₂ O ₂ (100 vol)	33	μL

4C1N buffer [water insoluble, alcohol soluble blue/black product]

(Used to detect horse-radish peroxidase immobilized on membrane)

NaCl	0.58	g
NaH ₂ PO ₄ .2H ₂ O	0.78	g
Distilled Water	80	mL
4-Chloro-1-naphthol	40	mg
Dissolve		
1 N NaOH to pH 7.5		
Add DISTILLED WATER to make vol to 100 mL.		
Filter		
H ₂ O ₂ (100 vol)	67	μL
Use within 15 min.		

Tris-buffered saline (pH 7.5) can be used instead of phosphate- buffered saline.

BLOCKING AND ANTIBODY DILUTION BUFFERS**Tween TBS**

Spread 1 mL of Tween 20 on the base of a 1 L conical flask

Distilled Water	400	mL
Dissolve Tween 20		
Glass Distilled Water (DISTILLED WATER)	500	mL
10× TBS	100	mL
Mix		

Serum TBS

10× TBS	50	mL
Distilled Water	400	mL
Serum	50	mL
Mix		

Tween 20 (0.5 mL) can be added to obtain Tween Serum TBS.

The serum used should be from the same species from which the antibody that is bound to the enzyme is raised.

BLOTTO

Distilled Water	440	mL
10× TBS	50	mL
Fat free milk powder	15	g
Dissolve milk powder		

Carbohydrates present can prevent binding of antibodies recognizing glyco-epitopes.

If nylon membranes are used instead of nitrocellulose then add 50 g of fat free milk powder.

Tween 20 (0.5 mL/15 g milk powder) can be added to obtain Tween BLOTTO.

Bovine serum albumin (BSA) TBS

10× TBS	50	mL
Distilled Water	450	mL
BSA	5	g
Dissolve the BSA		

Tween 20 (0.5 mL) can be added to obtain Tween BSA TBS.

SOLUTIONS FOR STAINING PROTEINS ON MEMBRANES

India Ink staining. [Used to stain proteins electroblotted on NC or PVDF membranes.]

Solution:

Tween 20	150	μL
Spread on base of flask		
TBS	50	mL
Dissolve Tween 20		
India Ink	50	μL
Mix.		

Method:

Immerse the membrane in 0.3% Tween TBS for 5 min and then in India Ink staining solution for 3 hr or more (discard stain after use). Destain with 0.3% Tween TBS. Black bands on light grey background.

Sensitivity as low as 0.08 μg for some proteins.

Ponceau S staining. [Used to reversibly stain proteins electroblotted on NC or PVDF membranes.]

Stock Solution:

Distilled Water	40	mL
Ponceau S	2	g
Dissolve		
TCA	30	g
Dissolve		
Sulfosalicylic acid	30	g
Dissolve		
Add D/W to make vol to 100 mL.		

Working Solution:

Distilled Water	90	mL
Ponceau S stock solution:	10	mL

Method:

Immerse the membrane in Ponceau S working solution for about 5-10 min (discard stain after use). Destain with D/W till desired contrast is obtained. Dye can be completely removed by washing with D/W. Sensitivity 0.1-1 μ g.

Amido Black 10 B staining. [Used to stain proteins electroblotted on NC or PVDF membranes.]**Solution:**

Methanol	250	mL
Amido Black 10 B	1	g
Dissolve		
Glacial acetic acid	100	mL
Distilled Water	650	mL

Filter through Whatman #1 and store in a glass-stoppered / plastic-capped bottle.

Keep away from metals.

Method:

Immerse the membrane in Amido Black 10 B staining solution for 5

Sensitivity is 0.02

Prussian blue staining. [Used to stain proteins electroblotted on NC or PVDF membranes. Staining is compatible with immunoblotting, N-terminal and micro-sequencing procedures.]

Solution:

Sodium acetate trihydrate	1.259	g
Distilled Water	450	mL
Glacial acetic acid	2.20	mL
K ₄ Fe(CN) ₆	18.42	g
FeCl ₃ soln (sp.gr. 1.793)	3.712	mL

Dissolve and adjust pH to 4.0 with (approx. 2 mL) 1 N acetic acid. Make volume upto 500 mL with Distilled Water. Can be stored for long periods in amber-coloured bottles. Any precipitate formed can be dissolved by vigorous shaking or sonication. [To make FeCl₃ solution, dissolve the hexahydrate salt in warm Distilled Water (30°C). Collect the filtrate/supernatant in another flask and ascertain its density. Adjust the density of the solution to 1.793 g/cc with water. Store at >25°C.]

Method: Immerse the membrane in above staining solution (Staining solution can be reused)

Destain with Distilled Water till desired contrast is obtained. Dye can be stripped off the proteins by treating membrane with 20 mM EDTA, 100 mM Tris pH 10 for 10–15 min.

Sensitivity 0.01–0.02 μ g. Variability in protein staining is minimal.

Sensitivity ($\mu\text{g protein}$) of different protein stains for select proteins

electroblotted on nitrocellulose or PVDF membranes:

	Myosin	β -Galactosidase	Phosphorylase B	Bovine Serum Albumin	Ovalbumin
Ponceau S	0.1	0.1	0.1	0.25	1.0
Amido Black 10B	0.02	0.05	0.1	0.02	0.25
Fast Green	0.015	0.05	0.05	0.05	0.25
Colloidal Gold	0.01	0.015	0.01	0.01	0.015
Prussian blue	0.01	0.01	0.01	0.015	0.02

Property	Manufacturer	Pure Nitrocellulose (Reinforced)			PVDF		Neutral Nylon (Plus)	
Pore size		0.45 μm	0.2 μm	0.1 μm	0.45 μm	0.2 μm	0.45 μm	0.2 μm
Binding interaction		Hydrophobic & Electrostatic			Hydrophobic (soak in methanol before transfer)		Electrostatic Covalent (when UV cross linked)	
Binding Capacity ($\mu\text{g}/\text{cm}^2$)	Schleicher & Schuell	75-85 (ditto)	80-90 (ditto)	140-150 (ditto)	NA	50-150	400 (600)	400 (NA)
	Sartorius	115 (120)	168 (170)	NA (NA)	NA	NA	105 (NA)	145 (NA)
Thickness (μm)	Sartorius	130 (160)	130 (160)	NA	NA	NA	130 (NA)	130 (NA)