Western Blotting

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1 Works for me dx.doi.org/10.17504/protocols.io.6rmhd46

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ABSTRACT

From growth to isolation and lysis, to quantification, and running the gel, transfer, blotting... the whole bit.

Protein Lysis Buffer.docx Protein Isolation.docx Western Blot Protocol.docx

GUIDELINES

Keep your proteins cold, on ice at all times.
Be sure of the type of gel needed for the size of the proteins you are interested in.

STEPS MATERIALS

NAME	CATALOG #	VENDOR
100X Protease/Phosphatase Inhibitor	5872	Cell Signaling Technology
Pierce BCA Protein Assay Kit	23225	Thermo Fisher Scientific
10X Tris-Glycine buffer	1610771	Bio-rad Laboratories
Immobilon-P PVDF Membrane, 0.45um, roll	IPVH00010	Millipore Sigma
Whatman Grade 3MM Chr Cellulose Western Blotting Membranes, GE Healthcare, Grade 3MM Chr Blotting Paper, roll, 10 cm × 100 m,	21427-546	VWR Scientific
Anti-Mouse IgG (H L) Goat Polyclonal Antibody (HRP (Horseradish Peroxidase))	115-035-003	Jackson Immunoresearch
SuperSignal™ West Femto Maximum Sensitivity Substrate	34095	Thermo Fisher Scientific
Restore™ Western Blot Stripping Buffer	21059	
Anti-Mouse IgG (H L) Goat Polyclonal Antibody (HRP (Horseradish Peroxidase))	115-035-003	Jackson Immunoresearch
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Before Starting:

1 **Before Starting:**

Prepare fresh Lysis Buffer for cells. Use 5X stock stored at 4C.
Stock solution consists of

Component	1X	5X
Tris	25 mM	125 mM
NaCl	150 mM	750 mM
EGTA	1 mM	5 mM
EDTA	1 mM	5 mM
NaF	10 mM	50 mM

Remaining ingredients to be added fresh to each sample:
Final concentration
1 mM DTT
1% Triton X-100
1X Protease/Phosphatase Inhibitor (Cell Signalling, Cat #5872)

Per 1 mL 1X buffer:
200 uL 5X stock
100 uL 10% Triton X-100
10 uL 0.1M DTT
10 uL 100X Protease/Phosphatase Inhibitor
680 uL ddH2O

Lysis buffer must be made fresh daily and stored during use on ice.

2 Grow Cells in culture and treat with whatever desired checmicals/drugs/siRNA/plasmid/etc are desired. Generally, protein is collected 72 hours post treatment. Occasionally, the proteins being monitored may show optimal change at 48 or 96 hours, dependent on cell cycle and protein cycling.

3 Remove media and wash cells with DPBS.

Collecting Cells

4 Collect Cells and either stored as a pellet for lysis later, or lyse directly now,

100X Protease/Phosphatase Inhibitor
by Cell Signaling Technology
Catalog #: 5872

step case

Direct lysis, adherent only

If desiring to lyse cells directly in the dish, be certain to remove ***all*** PBS from the dish so as not to dilute the sample. Place the dish on ice, add an appropriate amount of lysis buffer to collect the cells. (300 uL/10 cm dish, for example) Use a cell scraper (Corning, 3008) to scrape cells from the surface and displace them. Once detached, cells can be collected in an eppendorf tube for processing.

5 Lyse cells on ice, 15-30 min 2m

6 Sonicate samples, (Probe sonicator, Brodeur Lab, setting 6) 5 seconds

7 Spin samples, 4C, 15', at least 15000g. 1m
4 °C

8 Without disturbing the pellet, collect supernatant and transfer to clean labeled tube.
Can be stored at -80C. Avoid freeze/thaw cycles. Aliquot if necessary.

Quantification

9 Must quantify protein levels to know how much to load on a gel.
Run Pierce BCA assay

9.1

BCA Assay for protein quantification PREVIEW RUN
by Karina Conkrite

Ensure your samples are somewhere on the curve. If they are outside the curve, you will need to redo the assay with different dilution of sample:lysis buffer.

9.1.1 Use provided 0.2% BSA for creating a standard curve: Dilute amount necessary for the assay 1:2 using lysis buffer

9.1.2 Label tubes (1.5 mL Eppendorf) for standards.

Final Conc ug/mL Volume 0.1% BSA Volume buffer

Standard (ug)	Volume 0.1% BSA (uL)	Volume buffer(uL)
0	0	20
2	2	18
4	4	16
6	6	14
8	8	12
10	10	10
15	15	5
20	20	0

9.1.3 Add appropriate amount of Lysis Buffer to all tubes for the standard curve, following chart above.

9.1.4 Label tubes for samples.

9.1.5 Keeping all protein on ice throughout, use 3 uL/ sample + 17 uL Lysis Buffer for a total volume of 20 uL.

9.1.6 Add diluted BSA to standard tubes last, before adding working reagent.

9.1.7 To each sample and all standards, add 1 mL Working Reagent (comprised of [1 mL Reagent A + 20 uL Reagent B] x number of samples + 10% extra for error). Mix well.

9.1.8 Place tubes in 37° C incubator for 30 minutes to react. 3m

9.1.9 Cool samples to room temp. 1m

9.1.10 Aliquot 200 uL from each tube to a well of a clear, 96-well plate. This can be done in triplicate if desired.

9.1.11 Read on BCA program on GloMax plate reader at λ = 562 nm

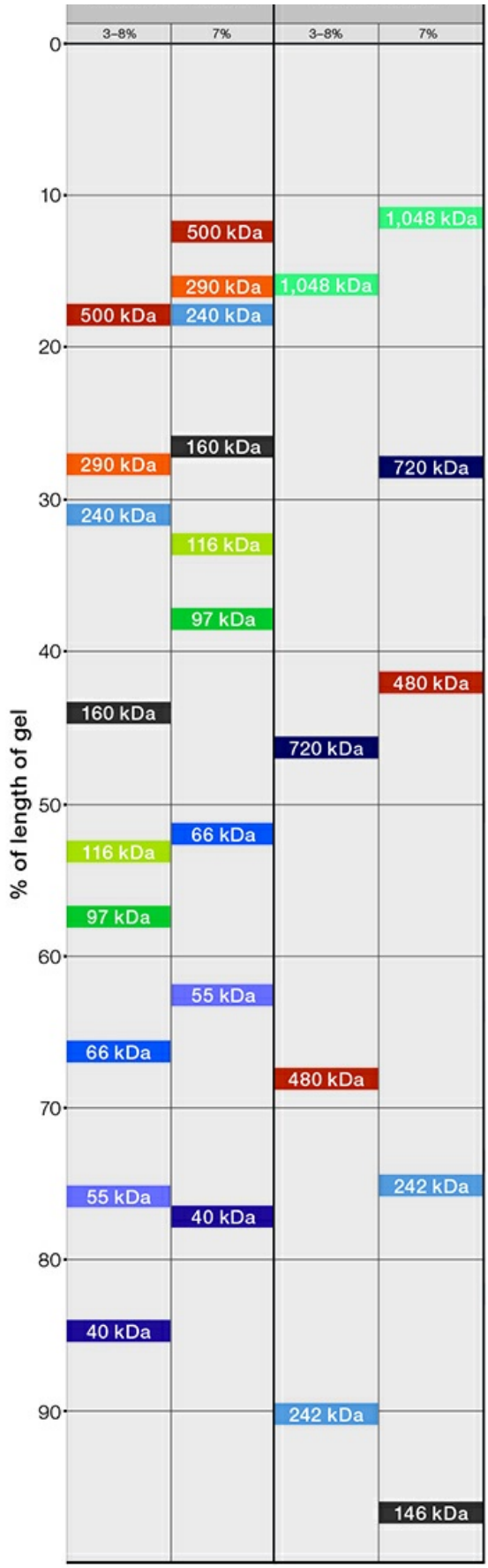
9.1.12 Use values from BSA standards to create standard curve. Can be done in Excel, Statmost, or GraphPrizm. Seeking R2 value as close to 1 as possible. Ensure all your samples are within the standard curve range. If not, need to be redone.

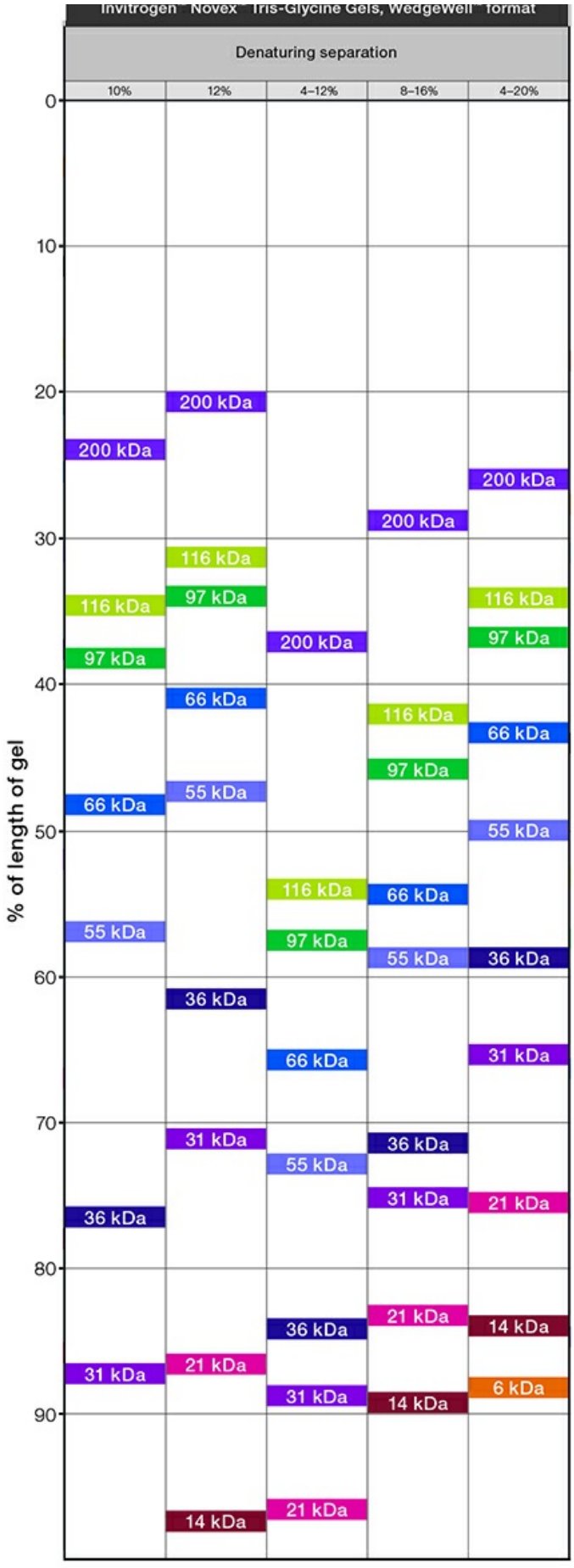
9.1.13 Use the resultant y=mx+b equation to quantify the amount of protein in each sample. Dividing by 3 will give you your protein concentration per uL.

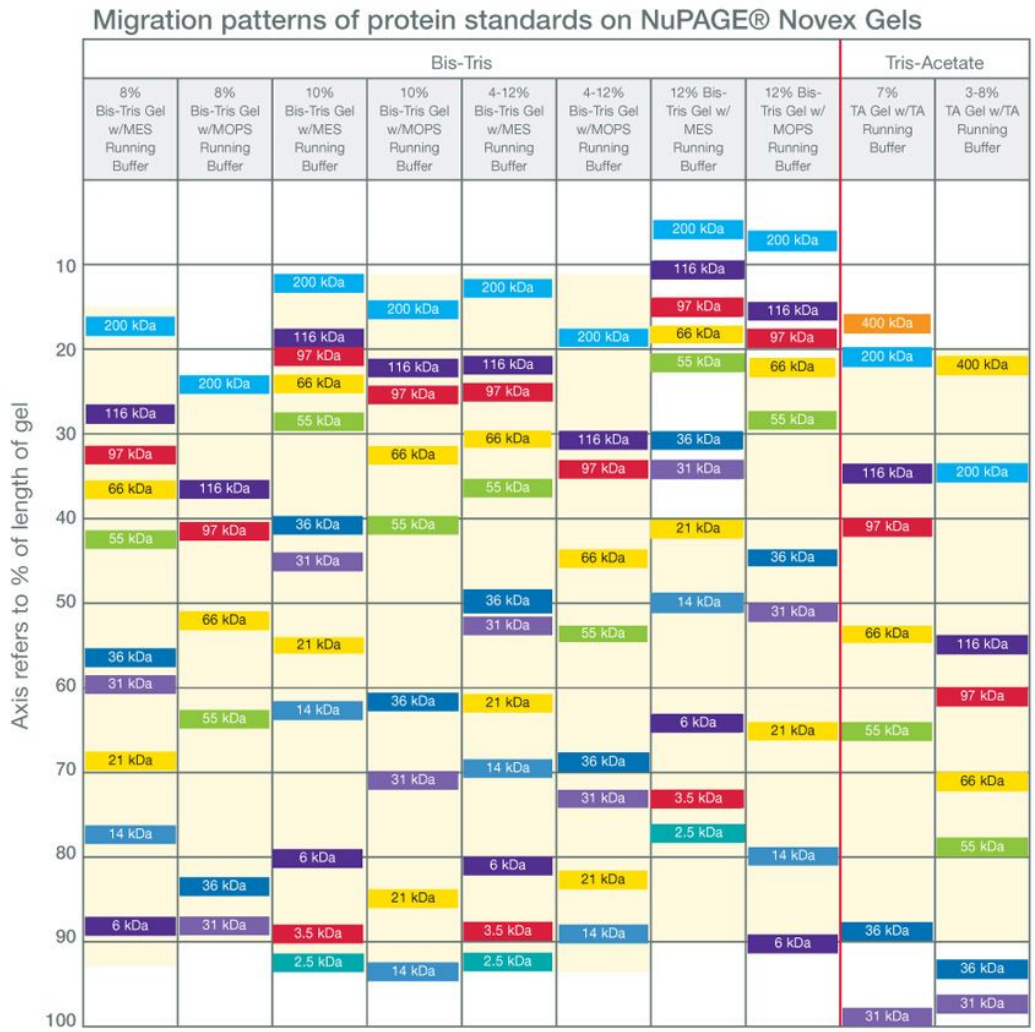
Prep the samples for running

10 Select the type and percentage gel you wish to run.
We tend to use Tris-Glycine gels. Percentage depends on your proteins of interest.

Invitrogen™ NuPAGE™ Tris-Acetate Gels	NuPAGE Tris-Acetate Gels
Denaturing separation	Native separation







11 Decide how much protein is necessary to run/sample (ug), 15ug? 30 ug?
Still keeping samples on ice, aliquot that amount, plus a suitable amount of 2X sample buffer (stored at -20C) and lysis buffer to make up to a standard volume for all the samples. Keep all samples on ice, 0 °C until the gel is ready to run.
Don't forget to prep your ladders at this time as well.

2X SDS Gel Loading Buffer/ Laemmli Buffer

Component	End Concentration	vol for 4 mL	vol for 40 mL
0.5 M Tris-HCl pH 6.8	125 mM	1 mL	10
Glycerol	20%	0.8 mL	8
20% SDS	5%	0.8 mL	8
b-Mercaptoethanol	10%	0.4 mL	4
0.5% Bromophenol Blue	0.025%	0.2 mL	2
H2O		0.8 mL	8

setting up the gel

12 Dependent on the gel type and transfer type you are doing, prep necessary buffers.
For example, if you are running 2 Tris-Glycine gels (Criterion or Novex, both take the same amount of buffer) you will use about 1L TG running buffer.

12.1 To make 1L of 1X TG Running buffer:

Component	Volume	end conc
10X Tris-Glycine buffer, BioRad 1610771	100 mL	1X
20% SDS, Technova S0293	5 mL	1%
H2O	895mL	

10X Tris-Glycine buffer
by Bio-rad Laboratories
Catalog #: 1610771

This buffer may be stored at 4 °C for a time. If using the Criterion set up, buffer may be reused in the tank, but should be fresh in the loading chamber.
If using the SureLock boxes, you will use the entire liter every time.

Also, prep for the transfer. This is very dependent on the size of your proteins of interest. If you are interested in proteins over 150 kDa, you will want to perform a fully wet transfer, possibly overnight in the cold room. In that case, for TG gels, you need to prep the buffer for the transfer, cut the membrane (1) and 3M filter papers (4) necessary to sandwich the transfer.

Immobilon-P PVDF Membrane, 0.45um, roll
by Millipore Sigma
Catalog #: IPVH00010

Whatman Grade 3MM Chr Cellulose Western Blotting Membranes, GE Healthcare, Grade 3MM Chr Blotting Paper, roll, 10 cm × 100 m,
by VWR Scientific
Catalog #: 21427-546

13 Remove plastic barriers on the bottom of pre-cast gels. If you forget to do this, your proteins will not migrate.
Assemble the gel/box cartridge, locking everything in place. Higher gel casing should be to the outside of the gel box, so you will load from behind. Fill buffer chambers with running buffer and remove combs from gels carefully, so as not to disturb the well edges and displace them.
Using running buffer and a p200 or p1000 pipet, flush the wells to remove the storage buffer.

14 While you are flushing the wells, heat samples according to the gel type. For example, on a TG gel, samples should be boiled at **95 °C** WITH LID LOCKS!!! for **00:10:00** **use lid locks**

Check the directions for the ladder you have selected. Different ladders have different instructions. Some need no boiling at all, some are 2 min, adjust accordingly.

15 Briefly spin down the tubes to collect samples. Load into gel according to your map using gel loading tips or Rainin p20 tips.

Run the gel

16 Run the gel according to gel type. For TG gels, we prefer to start slowly (75V) to get through the stacking gel (~20 min). Voltage can then be increased to run faster according to gel type/percentage. Run samples to the bottom of the gel. You can find the optimal voltage/amperage rates for your gels on the info sheets from their supplier.

Table 4. Gel running conditions in electrophoresis chamber systems.

	Running conditions in XCell Surelock Mini-Cell				Running conditions in Mini Gel Tank			
	Voltage (V)	Starting current (mA)*	End current (mA)*	Approximate run time (minutes)	Voltage (V)	Starting current (mA)*	End current (mA)*	Approximate run time (minutes)
Bolt 4–12% (MES)	NA	NA	NA	NA	200	160	70	20
Bolt 4–12% (MOPS)	NA	NA	NA	NA	200	160	50	35
NuPAGE 4–12% Bis-Tris (MES)	200	100 to 125	60 to 80	35	200	160	90	30
NuPAGE 4–12% Bis-Tris (MOPS)	200	100 to 125	60 to 80	50	200	140	50	42
Novex WedgeWell Tris-Glycine gels (denatured)	225	45 to 60	30 to 45	35 to 45	225	85 to 125	30 to 55	25 to 40
Novex WedgeWell Tris-Glycine gels (native)	125	25 to 30	13 to 15	1 to 2 hours	125	40 to 50	40 to 50	1 to 1.5 hours
NuPAGE 3–8% Tris-Acetate (denatured)	150	40 to 55	25 to 40	60	150	60	20	50
NuPAGE 3–8% Tris-Acetate (native)	150	18	7	2 to 3 hours	150	40	10	100
Novex 10–20% Tricine	125	80	40	90	125	110	40	65
NativePAGE 3–12%	150	12 to 16	2 to 4	90 to 115	150	10	<10	80
pH 3-10 IEF	100	7	NA	60	100	8	NA	60
	200	NA	NA	60	200	NA	NA	60
	500	NA	5	30	500	NA	5	30
10% Zymogram (gelatin)	125	30 to 40	8 to 12	90	125	40	10	90

* Per gel.
Note: Run times may vary depending on the power supply and gel percentage.

Novex Gels

Description | Specifications | Ordering | Accessories | Kits & Reagents | Download | Documents

Gel dimensions (W x L x thickness)	133 x 87 x 1 mm
Cassette dimensions (W x L x thickness)	150 x 106 x 5.3 mm
Cassette material	Styrene copolymer
Comb material	Polycarbonate
Gel storage conditions	Store flat at 4°C; do not freeze
Shelf life at 2–8°C*	12 months
Recommended sample buffer (Laemmli, dilute 1:1 with sample)	62.5 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 0.01% bromophenol blue
Recommended running buffer (Tris/Glycine/SDS)	25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3
Run times at constant voltage: 200 V	42–50 min
Run times at constant voltage: 300 V	20–26 min
* From date of manufacture.	

Criterion TGX gels

Set up the transfer

17 While gels are running, prep your transfer method.
This will depend on your proteins of interest. **Low to medium molecular weight proteins (20-150 kDa) can take advantage of the TransBlot Turbo system in the lab.**
If you are interested in higher molecular weight proteins, a more traditional wet transfer is called for. Recommended is overnight transfer in the cold room.
IN ALL CASES, prep your necessary buffer ahead of time so it is cold.

step case

Using TransBlot system

transfer occurs from top-down!!
https://www.bio-rad.com/webroot/web/pdf/lsr/literature/10016505E.pdf

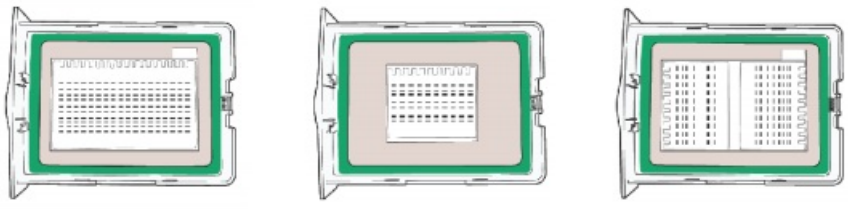


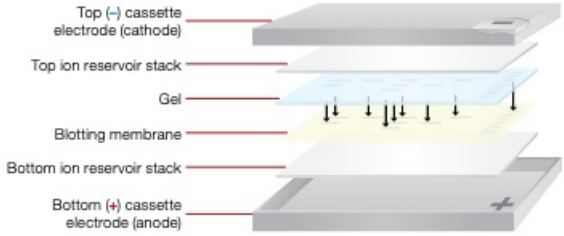
Fig. 12. Suggested placement of assembled transfer stacks in a cassette. A, midi stack and gel placement; **B,** mini stack and gel placement; **C,** two mini gels on a midi stack.

Stack arrangement in the Trans-Blot Turbo

Using RTA Transfer Kits [**1704273** Ready-to-assemble transfer kit includes 40 midi-sized PVDF membranes (8.5 x 13.5 cm), 80 transfer stacks, 2 L 5x transfer buffer, and 2 gel trays for wetting and equilibrating membranes and transfer stacks]:
1. Prepare Trans-Blot Turbo Transfer Buffer: 1 Part 5X trans-blot turbo buffer, 1 part 100% ethanol, 3 parts water. For 1 mini stack, make 100mL. For 2 minis or 1 midi, use 200 mL
2. Wet and equilibrate the membrane and transfer stacks:
PVDF membrane: immerse in 100% methanol or ethanol until the membrane is translucent. Transfer to a soaking tray with 30mL of 1X transfer buffer, submerge the membrane, equilibrate 2-3 minutes

Tranfer stacks: Midi stacks- immerse 2 stacks separated by blue sheet in two soaking trays, each containing 50-70 mL transfer buffer for 2-3 minutes

3. Assemble the sandwich according to figures below.



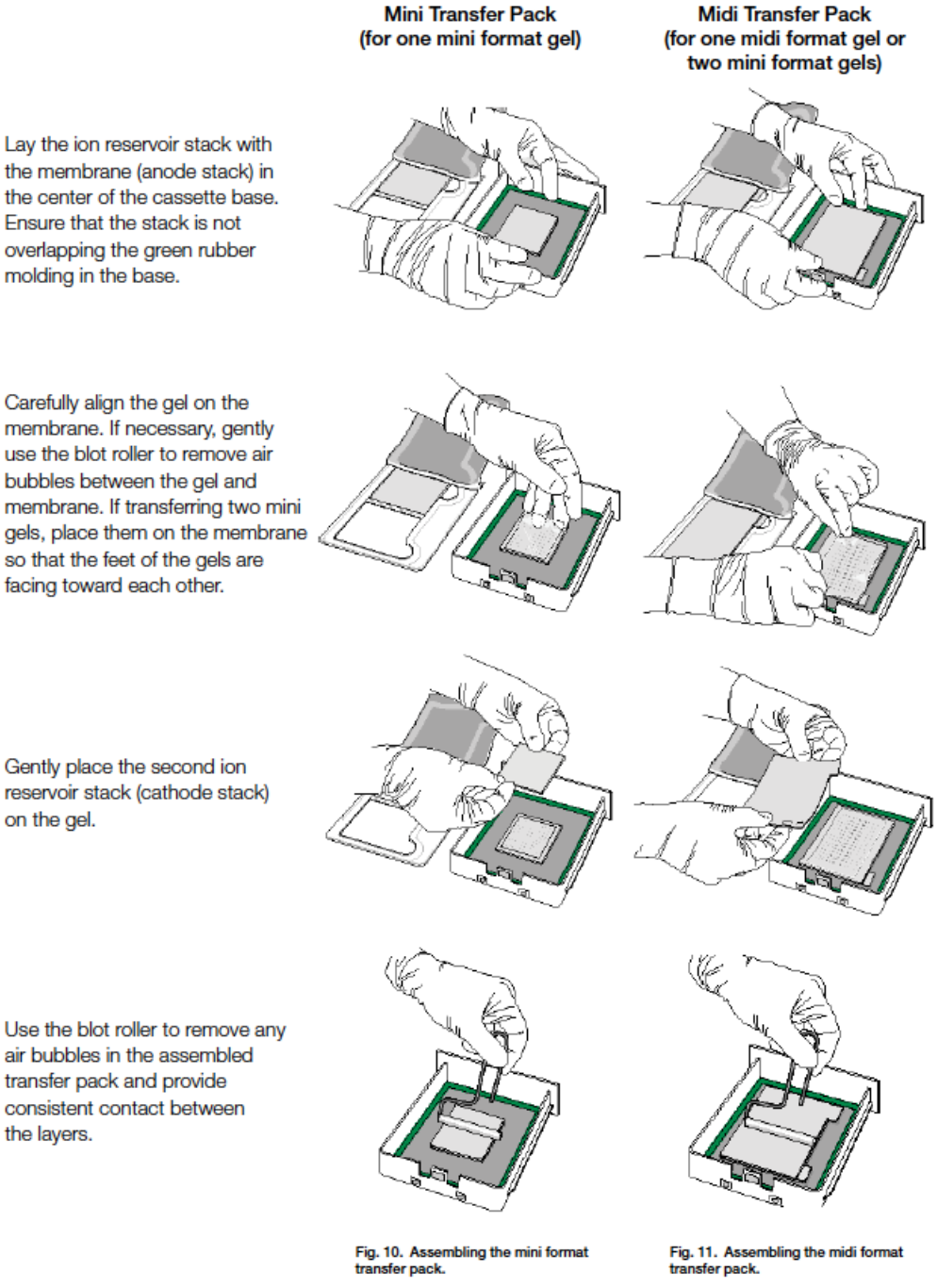


Fig. 10. Assembling the mini format transfer pack.

Fig. 11. Assembling the midi format transfer pack.

4. Once assembled, remove excess transfer buffer by inverting the cassette base with the assembled stack carefully held in place. Place the casette lid on and lock into place. Proceed with transfer step.

TransBlot Turbo

Recommended is the preprogrammed "mixed MW" setting, which will run at 1.3A, 25V for 7 minutes.
Insert cassettes and hit run!

3.4.3 Preprogrammed Protocols

Pressing the Bio-Rad preprogrammed protocols button accesses the protocols described in Table 3.

- **STANDARD SD** provides typical semi-dry transfer conditions for use with conventional semi-dry western blotting consumables (see section 3.7)
- **1.5 mm GEL** uses a longer transfer time (10 min) for more efficient transfer when using 1.5 mm thick gels
- **HIGH MW** is optimized for more efficient transfer of large proteins (>150 kD)
- **LOW MW** is optimized for more efficient transfer of small proteins (<30 kD)
- **MIXED MW** is for efficient transfer of proteins over a broad range of molecular weights (5–150 kD). This protocol is also accessed via the Turbo navigation button
- **1 Mini-TGX** is an ultrafast protocol that will transfer a single Mini-PROTEAN TGX Gel with mixed MW proteins (5–150 kD) in 3 min with excellent efficiency

Table 3. Bio-Rad preprogrammed protocols.

Protocol Name	MW, kD	Time, min	2 Mini Format Gels or 1 Midi Format Gel (per cassette)	1 Mini Format Gel (per cassette)
Standard SD	Any	30	Up to 1.0 A; 25 V	
1.5 mm GEL	Any	10	2.5 A, up to 25 V	1.3 A, up to 25 V
High MW	>150	10		
Low MW	<30	5		
Mixed MW (Turbo)	5–150	7		
1 Mini-TGX	5–150	3	–	2.5 A, up to 25 V

TRANSFERRING THE GEL TO THE MEMBRANE

18 Once you are set up with the appropriate sandwich/buffer/apparatus combo....
Check your current! And transfer...

Check the transfer efficiency

19 Transfer efficiency can be checked by staining and destaining the membrane with Ponceau stain.

0.1% Ponceau S (page 32) in 7% trichloroacetic acid (TCA) for 5 minutes. Rinse the membrane in deionized water to obtain transient staining or 10% acetic acid to obtain permanent staining, and air dry.

If planning to blot immediately, do not allow membrane to dry. Immediately apply blocking solution, or place in TBST until ready to continue.

Blotting

20 After confirming successful transfer, block membrane in 5% milk made in TBST for 1 hour prior to overnight incubation with primary antibody. This should be down with rocking.
For 1 mini gel (9X10cm) 8 mL milk is plenty to block in the Perfect Western boxes.
This will help cut down on non-specific binding of antibodies.

Per 100mL: add 5g powdered milk to 50 mL TBST in bottle with stir-bar. Shake. Add remaining 50 mL TBST and set on stir plate to mix until uniform.

21 If splitting membrane into sections, cut now.
This is most easily done with a clean scalpel, while the membrane is enclosed in a sheet protector. Recommended to line up the sections with ladder marks on both sides of the membrane and use a ruler for straight edges.
Splitting the membrane is only recommended when you are certain of the antibody's reactivity and patterns. This does allow for staining of multiple targets simultaneously, and the use of less antibody, but can disrupt patterns.

22 Mix antibody to the specified concentration in the same milk used for blocking buffer (alternately, some antibodies require PBS with serum).
For example, if preparing to stain the entire mini blot at 1:2500 with beta-Actin, you would mix 8 mL of milk with 3.2 uL beta-Actin.
Incubate overnight at **4 °C while rocking**

23 Remove blot from 4C, rinse quickly in TBST,
then wash 3 X 10' in TBST, with rocking.
During the last wash, prepare the secondary antibody

24 Secondary antibody:
whatever the primary antibody's host species was, you need an antibody against that.
We use HRP conjugated secondaries. So if you incubated against a beta-Actin antibody raised in mouse (ms anti- Beta-Actin) for a secondary you need an HRP conjugated anti-mouse, such as

Anti-Mouse IgG (H L) Goat Polyclonal Antibody (HRP (Horseradish Peroxidase))
by Jackson Immunoresearch
Catalog #: 115-035-003

at a 1:25000 dilution in 5% milk in TBST.
Apply and incubate with rocking, 1 hour

25 Discard the secondary antibody, rinse blot quickly in TBST,
then wash 3 X 10' in TBST, with rocking.

26 Develop the blot, using the ECL detection method of choice.
Lowly expressed proteins, recommend

SuperSignal™ West Femto Maximum Sensitivity Substrate
by Thermo Fisher Scientific
Catalog #: 34095

for other, more abundant proteins, less sensitive substrates may be used.
carefully, without touching anything other than the edges of the blot, remove from TBST and place in a sheet protector. Try to remove all extra TBST.
Apply ECL substrate uniformly to the blot with a pipet, then close the top half of the sheet protector to allow even distribution of the chemical.

27 Using the Fluor-Chem Q in CTRB 3300, expose the blot and optimize the images. Ensure things are focused prior to exposure and that the iris is open as far as possible. Get the best images you can without overexposing the blot.

Stripping/Reprobe

28 If you need to re-probe the blot for other targets, you can strip the antibodies from the blot and re-probe.
Carefully put the blot in a hybridization tube, with the protein side facing the inside of the tube. Apply 10 mL

Restore™ Western Blot Stripping Buffer
Catalog #: 21059

and incubate in the hyb oven at 37 °C while turning, for 15-20 min

29 Place blot back in TBST, rinse quickly, then wash 3 x 10' in TBST

30 Return to step 20 "Blocking" and block, then apply desired primaries at specified dilutions. Follow as before, as many times as desired.

Collecting Cells

step case 1m

Collecting cells without lysis

for suspension lines or adherent.
Treat cells with dissociation agent such as versene or trypsin until fully detached.
collect cells up and inactivate with media.
Spin cells down at 300x g, 5 min, to collect.
Carefully remove media and wash pellet with ice cold PBS and aspirate off PBS carefully, not disturbing the pellet.
Repeat wash step and aspiration without disrupting the pellet. Can be snap-frozen at this point, or continue on to lysis.

Add appropriate amount of lysis buffer and allow to react.

5 Lyse cells on ice for 15-30 minutes.

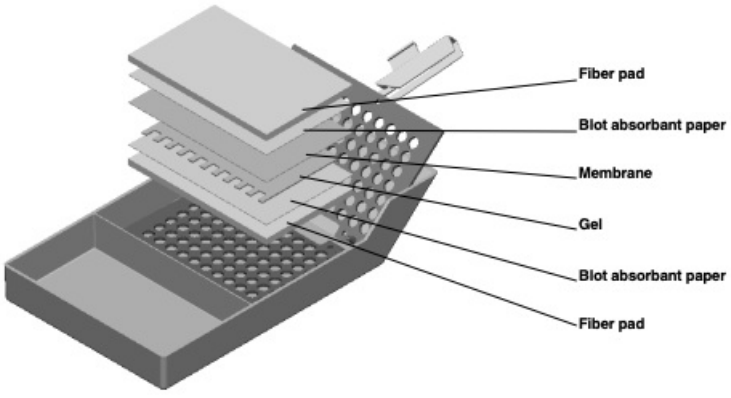
6 Sonicate with probe sonicator (Brodeur Lab, setting 6) 5 seconds to break membranes

TRANSFERRING THE GEL TO THE MEMBRANE

step case

Traditional Wet transfer

Criterion Blotter Cell Assembly 2.1 Preparation for Blotting
1. Freeze ice block prior to preparation of blot assembly.
2. Prepare the transfer buffer. (Towbin Buffer with 20% Methanol 25 mM Tris, 192 mM glycine, 20% v/v methanol, pH 8.3
a) Use Premixed buffer concentrate solution: 100 ml of 10X Tris/Glycine buffer (catalog number 161-0734 1L bottles or catalog number 161-0757 5L cube) 700 ml of dd H2 O 200 ml of methanol.)
3. Cut membrane and the filter paper to the dimensions of the gel if necessary. Suggest notching a corner and marking the front with pencil to help with orientation later.
4. Equilibrate gel in transfer buffer for 15 minutes. Note: Always wear clean gloves when handling the transfer cell, membranes, filter paper, or gels to prevent contamination.
5. Set up transfer apparatus. • Fill the Criterion Blotter tank with transfer buffer to about 50% of the fill volume. • Place a magnetic stir bar inside the tank. (0.8–10 mm) • Place the ice block in the ice block pocket in the back of the cell. Flip down the lever to hold the ice block down.
6. Prepare the membrane by submerging in 100% methanol until translucent (2-3 min). Transfer to water bath until it stops "dancing," then to transfer buffer to equilibrate. Set the filter papers in transfer buffer as well.
7. Assemble the "sandwich" for transfer. Note*** Direction of transfer is from bottom to top as you build.



a. Pour chilled transfer buffer into each compartment of the gel/blot assembly tray.
b. Place the membrane (nitrocellulose, PVDF, etc.) in the front/small compartment of the tray. Let it soak while you set up steps c–g.
c. Place the cassette in the back/large compartment of the tray: Open the cassette so that the red side with the handle is vertical (anode) and the black side (cathode) is laying horizontal and submerged in transfer buffer.
d. Place a fiber pad on top of the black side of the cassette, submerged in buffer. Push on the fiber pad with gloved finger tips to thoroughly soak the pad.
e. Place a piece of filter paper on top of the fiber pad (it will wet immediately).
f. Gently place the pre-equilibrated gel on top of the filter paper. Use the roller to remove any air bubbles that may be trapped underneath the gel.
g. Take the membrane from the front compartment and place it on top of the gel taking care not to trap any air bubbles. The membrane should not be moved or adjusted once it touches the gel because this can cause data ghost prints and artifacts. If you feel that you must adjust the membrane placement, use a fresh pre-wetted membrane. Use the roller to roll out bubbles (see figure).
h. Place a piece of filter paper on top of the membrane. Run the roller gently over the top of the filter paper to remove any air bubbles trapped in the sandwich.
i. Wet a second fiber pad in the front compartment of the tray (where the membrane was soaking) again using finger tips to completely saturate the pad with transfer buffer. Then place the wet fiber pad on top of the second filter paper.
j. Lower the clamp-side of the cassette, and lock in the closed position.

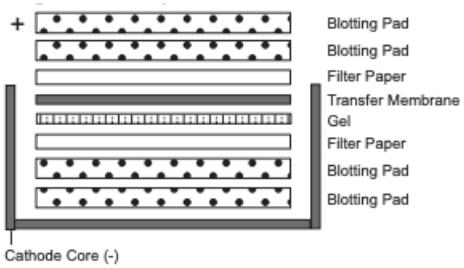
18 Once you are set up with the appropriate sandwich/buffer/apparatus combo....
Check your current! And transfer...

step case

Novex Sure-Lock blotting module

Remove the gel from the cassette for transfer after completion of electrophoresis as described below. If you are not ready to perform transfer immediately, you may continue electrophoresis of your gel at a low voltage of 5 V. The gel can be left in the unit for a few hours until you are ready to transfer the gel.
1. After electrophoresis, separate each of the 3 bonded sides of the gel cassette by inserting the gel knife into the gap between the cassette's 2 plates. The notched ("well") side of the cassette should face up.
2. Push up and down on the knife handle to separate the plates. Repeat on each side of the cassette until the plates are completely separated. Caution: Use caution while inserting the gel knife between the 2 plates to avoid excessive pressure towards the gel.
3. The gel may adhere to either side of plates upon opening the cassette. Carefully remove and discard the plate without the gel The gel remains on the other plate.
4. Remove wells on the gel with the gel knife.
5. Place a piece of pre-soaked filter paper (as prepared on page 10) on top of the gel, and lay just above the "foot" at the bottom of the gel (leaving the "foot" of the gel uncovered). Keep the filter paper saturated with transfer buffer and remove all trapped air bubbles by gently rolling over the surface using a glass pipette.
6. Turn the plate over so the gel and filter paper are facing downwards over a gloved hand or clean flat surface covered with a piece of Parafilm®.
7. Remove gel from the plate using the following methods: • If the gel rests on the longer (slotted) plate, use the gel knife to push the foot out of the slot in the plate and the gel will fall off easily. • If the gel rests on the shorter (notched) plate, use the gel knife to carefully loosen the bottom of the gel and allow the gel to peel away from the plate.
8. When the gel is on a flat surface, cut the "foot" off the gel with the gel knife. Proceed to Transferring One Gel, below.

Transferring the gel:
1. Wet the surface of the gel (step 8, above) with the transfer buffer and place pre-soaked transfer membrane on the gel. Remove air bubbles by rolling a glass pipette over the membrane surface.
2. Place the pre-soaked filter paper on top of the transfer membrane. Remove any trapped air bubbles.
3. Place 2 soaked blotting pads into the cathode (–) core of the blot module. The cathode core is the deeper of the 2 cores and the corresponding electrode plate is a darker shade of gray. Carefully pick up the gel membrane assembly with your gloved hand and place on the pad in the same sequence, such that the gel is closest to the cathode plate (see figure below).



4. Add enough pre-soaked blotting pads to rise 0.5 cm over the rim of the cathode core. Place the anode (+) core on top of the pads. The gel/membrane sandwich should be held securely between the two halves of the blot module ensuring complete contact of all components. Note: To ensure a snug fit, use an additional pad since pads lose their resiliency after many uses. Replace pads when they begin to lose resiliency and are discolored.
5. Position the gel membrane sandwich and blotting pads in the cathode core of the XCell II™ Blot Module to fit horizontally across the bottom of the unit. There should be a gap of ~ 1 cm at the top of the electrodes when the pads and assembly are in place (see figure below).
6. Hold the blot module together firmly and slide it into the guide rails on the lower buffer chamber. The blot module fits into the unit in only one way, such that the (+) sign is seen in the upper left hand corner of the blot module. The inverted gold post on the right hand side of the blot module fits into the hole next to the upright gold post on the right side of the lower buffer chamber.
7. Depending on the mini-cell that you are using, follow the appropriate instructions for positioning the wedge: • For XCell SureLock™ Mini-Cell, place the gel tension wedge such that the vertical face of the wedge is against the blot module. Push the lever forward to lock it into place.
8. Fill the blot module with transfer buffer until the gel/membrane sandwich is covered in transfer buffer. Do not fill all the way to the top as this will generate extra conductivity and heat.
9. Fill the outer buffer chamber with ~ 650 mL deionized water by pouring in the gap between the front of the blot module and front of the lower buffer chamber. The water level should reach approximately 2 cm from the top of the lower buffer chamber. This serves to dissipate heat produced during the run. Note: If you accidentally fill the outer buffer chamber with the transfer buffer, it will not adversely affect the transfer. The liquid in the outer buffer chamber serves as a coolant. We recommend adding deionized water to the outer buffer chamber to avoid any exposure of the mini-cell to methanol as the mini-cell is susceptible to methanol.
10. Place the lid on top of the unit. CAREFULLY. DO NOT FORCE ANYTHING.
11. With the power turned off, plug the red and black leads into the power supply.

18 Once you are set up with the appropriate sandwich/buffer/apparatus combo....
Check your current! And transfer...

Check the transfer

step case

Novex transfer

Type of the Gel	Transfer Buffer (1X)	Membrane	Transfer Conditions	Expected Current
Novex® Tris–Glycine Novex® Tricine	Novex® Tris Glycine Transfer Buffer with 20% methanol. 1X Transfer Buffer should be pH 8.3 before addition of SDS or methanol. Do not adjust the pH.	Nitrocellulose or PVDF	25 V constant for 1–2 hours	Start: 100 mA
NuPAGE® Novex® Bis–Tris	NuPAGE® Transfer Buffer with 10% methanol for transfer of one gel. NuPAGE® Antioxidant for reduced samples	Nitrocellulose or PVDF	30 V constant for 1 hour	Start: 170 mA End: 110 mA
NuPAGE® Novex® Tris–Acetate	NuPAGE® Transfer Buffer with 10% methanol for transfer of one gel. NuPAGE® Antioxidant for reduced samples	Nitrocellulose or PVDF	30 V constant for 1 hour	Start: 220 mA End: 180 mA
Novex® TBE, TBE–Urea, and DNA Retardation	45 mM Tris 45 mM boric acid 1 mM EDTA	Nylon	30 V constant for 1–2 hours	Start: 360 mA End: 270 mA
Novex® IEF*	0.7% acetic acid, pH 3.0, see page 27	Nitrocellulose or PVDF	10 V constant for 1 hour	Start: 65–85 mA

For overnight blotting, perform transfer in the cold room with low power to prevent overheating. Transfer at constant voltage of 10–15 V overnight. Depending on the transfer efficiency, adjust the transfer conditions accordingly. This must be done in the ventillated cold room!

19 Transfer efficiency can be checked by staining and destaining the membrane with Ponceau stain.

0.1% Ponceau S (page 32) in 7% trichloroacetic acid (TCA) for 5 minutes. Rinse the membrane in deionized water to obtain transient staining or 10% acetic acid to obtain permanent staining, and air dry.

If planning to blot immediately, do not allow membrane to dry. Immediately apply blocking solution, or place in TBST until ready to continue.

Blotting

20 After confirming successful transfer, block membrane in 5% milk made in TBST for 1 hour prior to overnight incubation with primary antibody. This should be down with rocking.
For 1 mini gel (9X10cm) 8 mL milk is plenty to block in the Perfect Western boxes.
This will help cut down on non-specific binding of antibodies.

Per 100mL: add 5g powdered milk to 50 mL TBST in bottle with stir-bar. Shake. Add remaining 50 mL TBST and set on stir plate to mix until uniform.

21 If splitting membrane into sections, cut now.
This is most easily done with a clean scalpel, while the membrane is enclosed in a sheet protector. Recommended to line up the sections with ladder marks on both sides of the membrane and use a ruler for straight edges.
Splitting the membrane is only recommended when you are certain of the antibody's reactivity and patterns. This does allow for staining of multiple targets simultaneously, and the use of less antibody, but can disrupt patterns.

Check the transfer

step case

Criterion

Criterion Blotting:
Table 3.1 SDS-PAGE Gels These conditions were determined empirically using 12.5% Tris-HCl Criterion gels and total proteins from E.coli lysates. Buffer:

1X Tris/Glycine (see section 3.3 Buffer formulation)

	20% Methanol	10% Methanol	15% Ethanol
Criterion Blotter with plate electrodes	100 V 30 minutes	100 V 30 minutes	Not recommended*
Criterion Blotter with wire electrodes	100 V 60 minutes	100 V 30 minutes	Not recommended*

* Our tests show only 60% transfer of E.Coli proteins in 1 hour at 100V. The ethanol buffer might work if longer transfers are acceptable or if your target protein transfers under this condition.

For overnight blotting, perform transfer in the cold room with low power to prevent overheating. Transfer at constant voltage of 10–15 V overnight. Depending on the transfer efficiency, adjust the transfer conditions accordingly. This must be done in the ventillated cold room!

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If planning to blot immediately, do not allow membrane to dry. Immediately apply blocking solution, or place in TBST until ready to continue.

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Per 100mL: add 5g powdered milk to 50 mL TBST in bottle with stir-bar. Shake. Add remaining 50 mL TBST and set on stir plate to mix until uniform.

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This is most easily done with a clean scalpel, while the membrane is enclosed in a sheet protector. Recommended to line up the sections with ladder marks on both sides of the membrane and use a ruler for straight edges.
Splitting the membrane is only recommended when you are certain of the antibody's reactivity and patterns. This does allow for staining of multiple targets simultaneously, and the use of less antibody, but can disrupt patterns.

step case

TransBlot Turbo

Recommended is the preprogrammed "mixed MW" setting, which will run at 1.3A, 25V for 7 minutes. Insert cassettes and hit run!

3.4.3 Preprogrammed Protocols

Pressing the Bio-Rad preprogrammed protocols button accesses the protocols described in Table 3.

- **STANDARD SD** provides typical semi-dry transfer conditions for use with conventional semi-dry western blotting consumables (see section 3.7)
- **1.5 mm GEL** uses a longer transfer time (10 min) for more efficient transfer when using 1.5 mm thick gels
- **HIGH MW** is optimized for more efficient transfer of large proteins (>150 kD)
- **LOW MW** is optimized for more efficient transfer of small proteins (<30 kD)
- **MIXED MW** is for efficient transfer of proteins over a broad range of molecular weights (5–150 kD). This protocol is also accessed via the Turbo navigation button
- **1 Mini-TGX** is an ultrafast protocol that will transfer a single Mini-PROTEAN TGX Gel with mixed MW proteins (5–150 kD) in 3 min with excellent efficiency

Table 3. Bio-Rad preprogrammed protocols.

Protocol Name	MW, kD	Time, min	2 Mini Format Gels or 1 Midi Format Gel (per cassette)	1 Mini Format Gel (per cassette)
Standard SD	Any	30	Up to 1.0 A; 25 V	
1.5 mm GEL	Any	10	2.5 A, up to 25 V	1.3 A, up to 25 V
High MW	>150	10		
Low MW	<30	5		
Mixed MW (Turbo)	5–150	7		
1 Mini-TGX	5–150	3	–	2.5 A, up to 25 V

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0.1% Ponceau S (page 32) in 7% trichloroacetic acid (TCA) for 5 minutes. Rinse the membrane in deionized water to obtain transient staining or 10% acetic acid to obtain permanent staining, and air dry.

If planning to blot immediately, do not allow membrane to dry. Immediately apply blocking solution, or place in TBST until ready to continue.

Blotting

20 After confirming successful transfer, block membrane in 5% milk made in TBST for 1 hour prior to overnight incubation with primary antibody. This should be down with rocking.
For 1 mini gel (9X10cm) 8 mL milk is plenty to block in the Perfect Western boxes.
This will help cut down on non-specific binding of antibodies.

Per 100mL: add 5g powdered milk to 50 mL TBST in bottle with stir-bar. Shake. Add remaining 50 mL TBST and set on stir plate to mix until uniform.

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This is most easily done with a clean scalpel, while the membrane is enclosed in a sheet protector. Recommended to line up the sections with ladder marks on both sides of the membrane and use a ruler for straight edges.
Splitting the membrane is only recommended when you are certain of the antibody's reactivity and patterns. This does allow for staining of multiple targets simultaneously, and the use of less antibody, but can disrupt patterns.

Check the transfer efficiency

step case

Criterion Wet transfer

Criterion

Criterion Blotting:
Table 3.1 SDS-PAGE Gels These conditions were determined empirically using 12.5% Tris-HCl Criterion gels and total proteins from E.coli lysates. Buffer:

1X Tris/Glycine (see section 3.3 Buffer formulation)

	20% Methanol	10% Methanol	15% Ethanol
Criterion Blotter with plate electrodes	100 V 30 minutes	100 V 30 minutes	Not recommended*
Criterion Blotter with wire electrodes	100 V 60 minutes	100 V 30 minutes	Not recommended*

* Our tests show only 60% transfer of E.Coli proteins in 1 hour at 100V. The ethanol buffer might work if longer transfers are acceptable or if your target protein transfers under this condition.

For overnight blotting, perform transfer in the cold room with low power to prevent overheating. Transfer at constant voltage of 10–15 V overnight. Depending on the transfer efficiency, adjust the transfer conditions accordingly. This must be done in the ventillated cold room!

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0.1% Ponceau S (page 32) in 7% trichloroacetic acid (TCA) for 5 minutes. Rinse the membrane in deionized water to obtain transient staining or 10% acetic acid to obtain permanent staining, and air dry.

If planning to blot immediately, do not allow membrane to dry. Immediately apply blocking solution, or place in TBST until ready to continue.

Blotting

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For 1 mini gel (9X10cm) 8 mL milk is plenty to block in the Perfect Western boxes.
This will help cut down on non-specific binding of antibodies.

Per 100mL: add 5g powdered milk to 50 mL TBST in bottle with stir-bar. Shake. Add remaining 50 mL TBST and set on stir plate to mix until uniform.

Check transfer efficiency

step case

Trans-Blot system

Using TransBlot system

transfer occurs from top-down!!
https://www.bio-rad.com/webroot/web/pdf/lsr/literature/10016505E.pdf

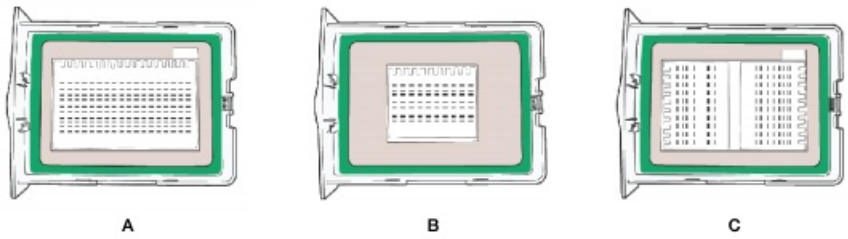
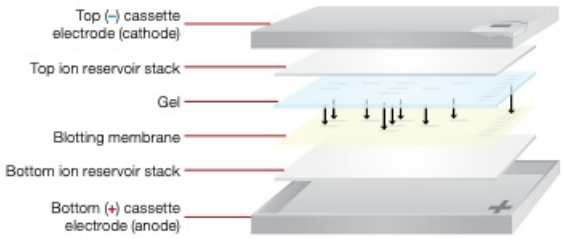


Fig. 12. Suggested placement of assembled transfer stacks in a cassette. A, midi stack and gel placement; **B,** mini stack and gel placement; **C,** two mini gels on a midi stack.

Stack arrangement in the Trans-Blot Turbo
Using RTA Transfer Kits [1704273 Ready-to-assemble transfer kit includes 40 midi-sized PVDF membranes (8.5 x 13.5 cm), 80 transfer stacks, 2 L 5x transfer buffer, and 2 gel trays for wetting and equilibrating membranes and transfer stacks]:
1. Prepare Trans-Blot Turbo Transfer Buffer: 1 Part 5X trans-blot turbo buffer, 1 part 100% ethanol, 3 parts water. For 1 mini stack, make 100mL. For 2 minis or 1 midi, use 200 mL
2. Wet and equilibrate the membrane and transfer stacks:
PVDF membrane: immerse in 100% methanol or ethanol until the membrane is translucent. Transfer to a soaking tray with 30mL of 1X transfer buffer, submerge the membrane, equilibrate 2-3 minutes
Tranfer stacks: Midi stacks- immerse 2 stacks separated by blue sheet in two soaking trays, each containing 50-70 mL transfer buffer for 2-3 minutes
3. Assemble the sandwich according to figures below.



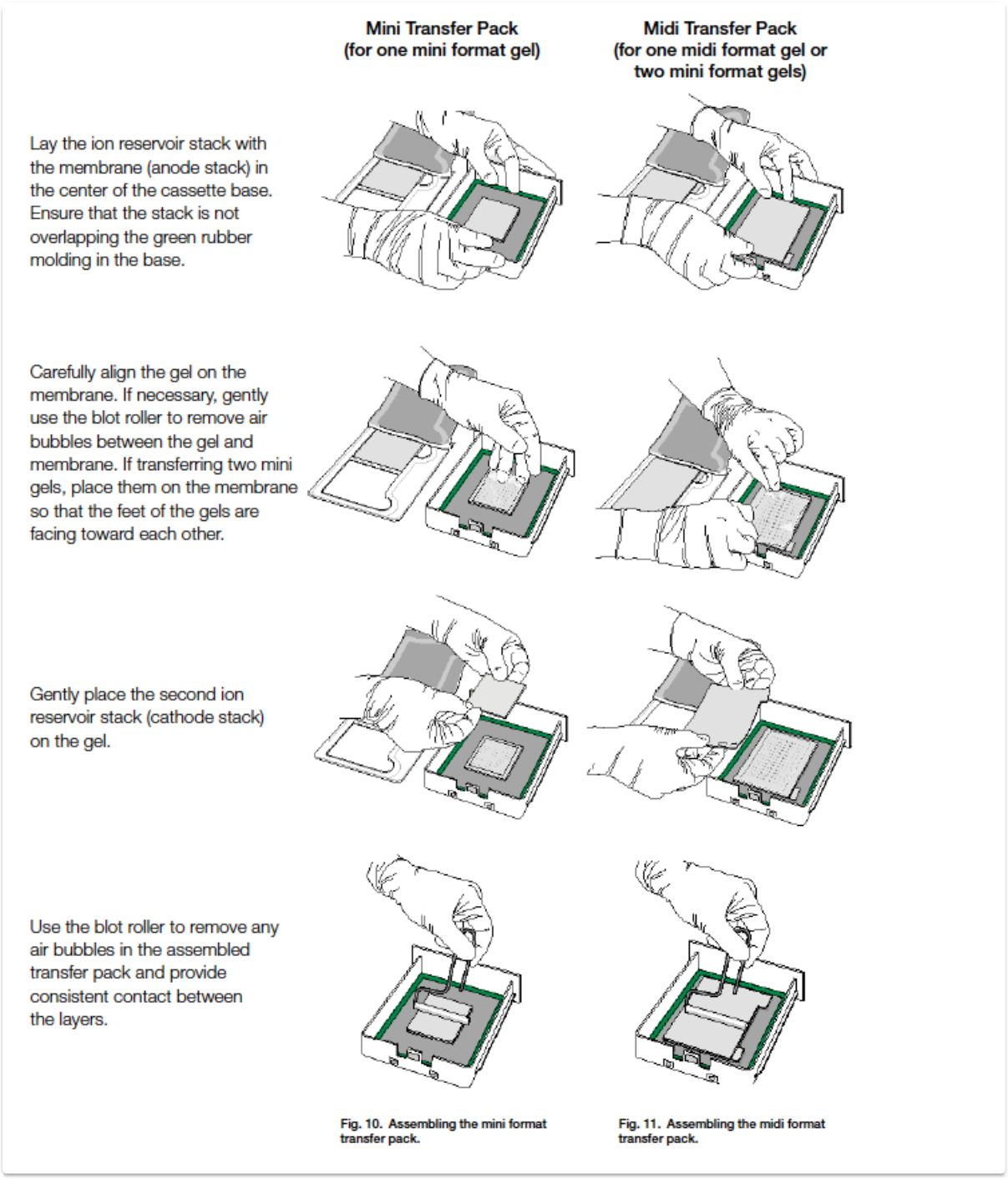


Fig. 10. Assembling the mini format transfer pack.

Fig. 11. Assembling the midi format transfer pack.

4. Once assembled, remove excess transfer buffer by inverting the cassette base with the assembled stack carefully held in place. Place the casette lid on and lock into place. Proceed with transfer step.

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0.1% Ponceau S (page 32) in 7% trichloroacetic acid (TCA) for 5 minutes. Rinse the membrane in deionized water to obtain transient staining or 10% acetic acid to obtain permanent staining, and air dry.

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