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Western Blotting

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We use this protocol and it's working

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Abstract

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

Attachments

Protein Lysis Buffer... 44KB

Protein Isolation.do... 56KB

Western Blot Protoco... 19KB

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.

Materials

STEP MATERIALS

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

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.

4 Collect Cells and either stored as a pellet for lysis later, or lyse directly now,
100X Protease/Phosphatase Inhibitor **Cell Signaling Technology Catalog #**5872

STEP CASE

Direct lysis, adherent only 28 steps

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 18000 x g

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

Protocol

NAME

BCA Assay for protein quantification

CREATED BY

Karina L Conkrite

Preview

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 Standard (ug)	Volume 0.1% BSA Volume 0.1% BSA (uL)	Volume buffer 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.

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

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

9.1.6 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.

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

9.1.8 Cool samples to room temp. 1m

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

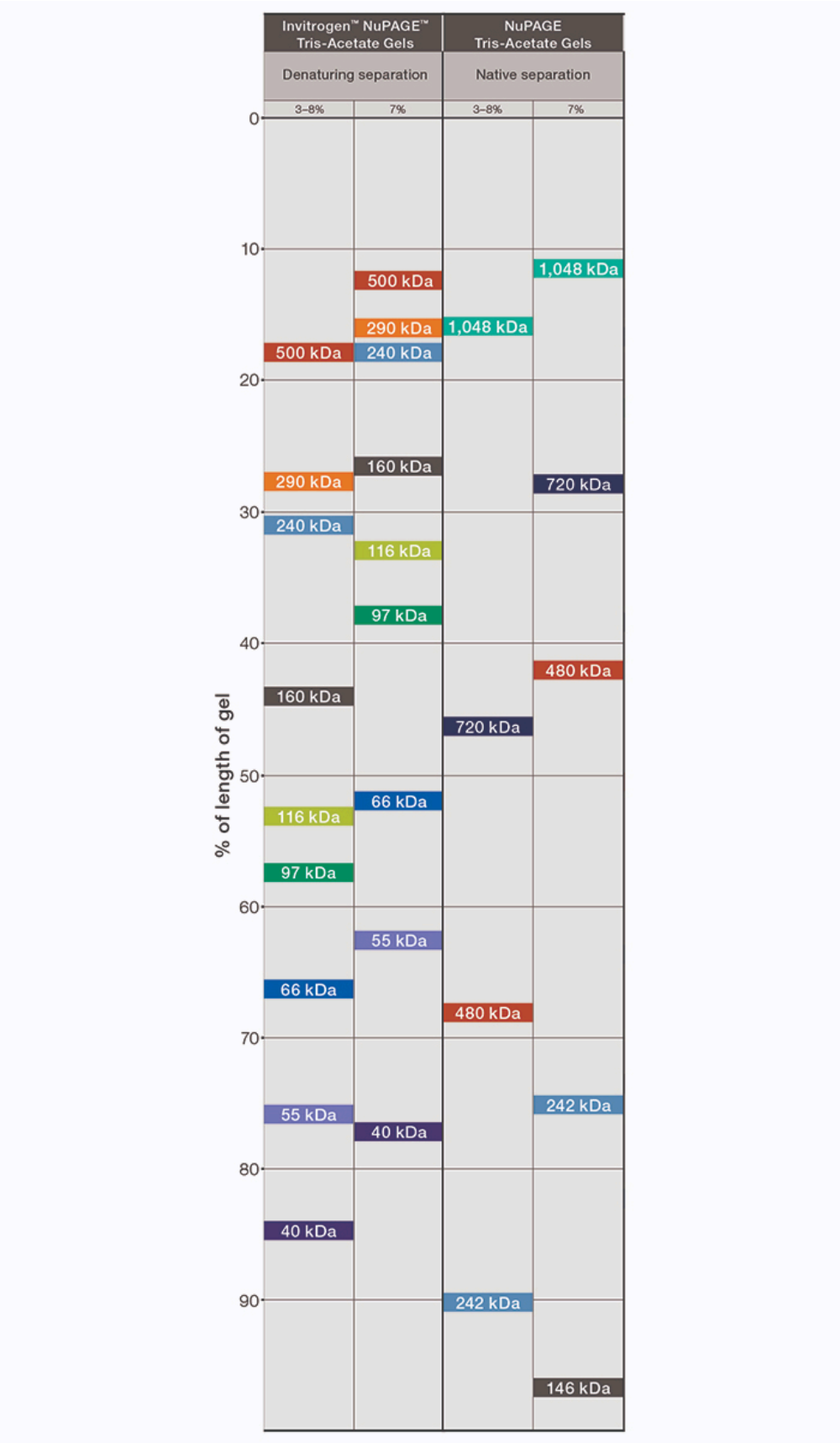
9.1.10 Read on BCA program on GloMax plate reader at λ = 562 nm

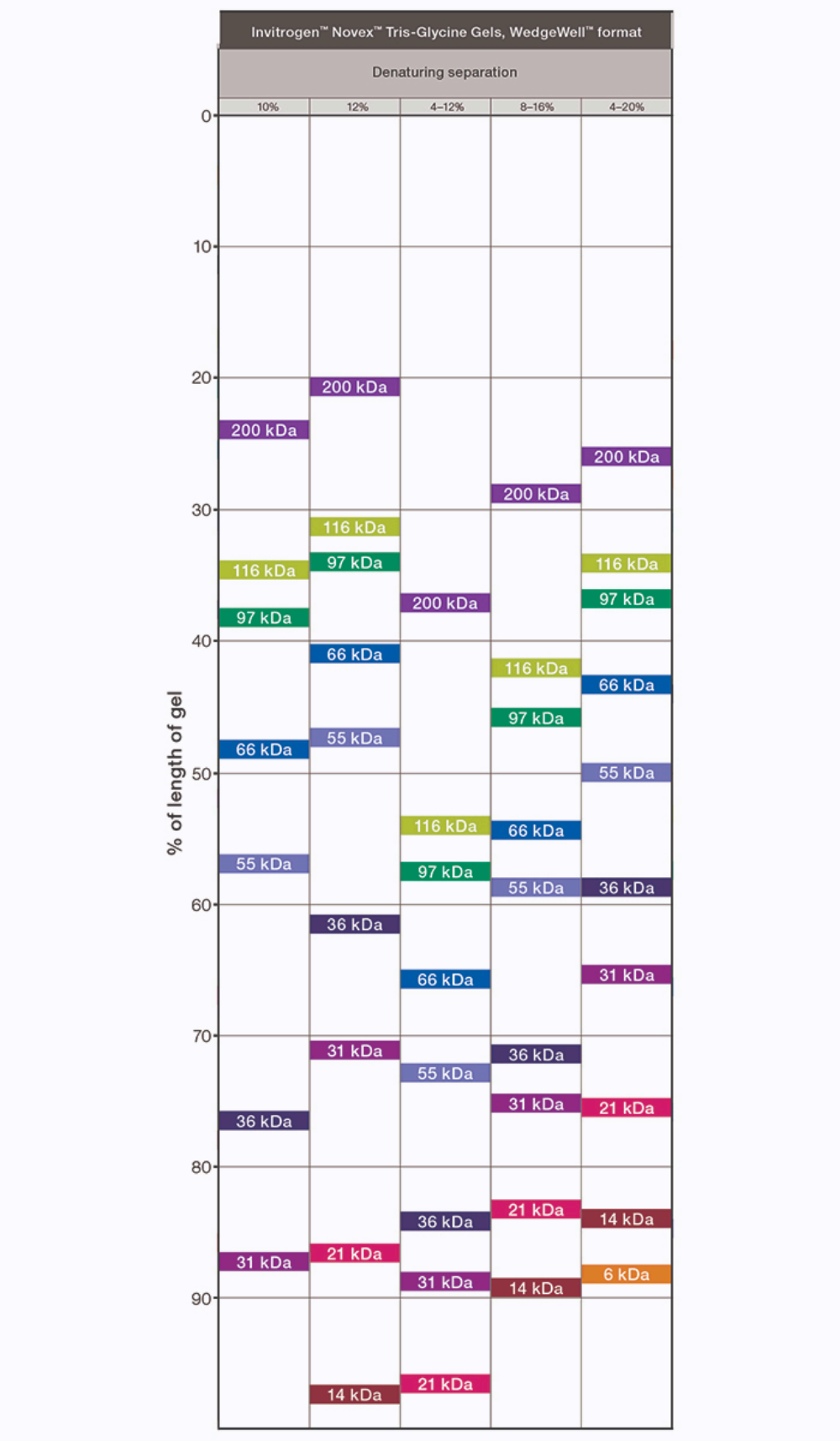
9.1.11 Use values from BSA standards to create standard curve. Can be done in Excel, Statmost, or GraphPrizm. Seeking R2 value as close to 1 as possible. Ensure all your samples are within the standard curve range. If not, need to be redone.

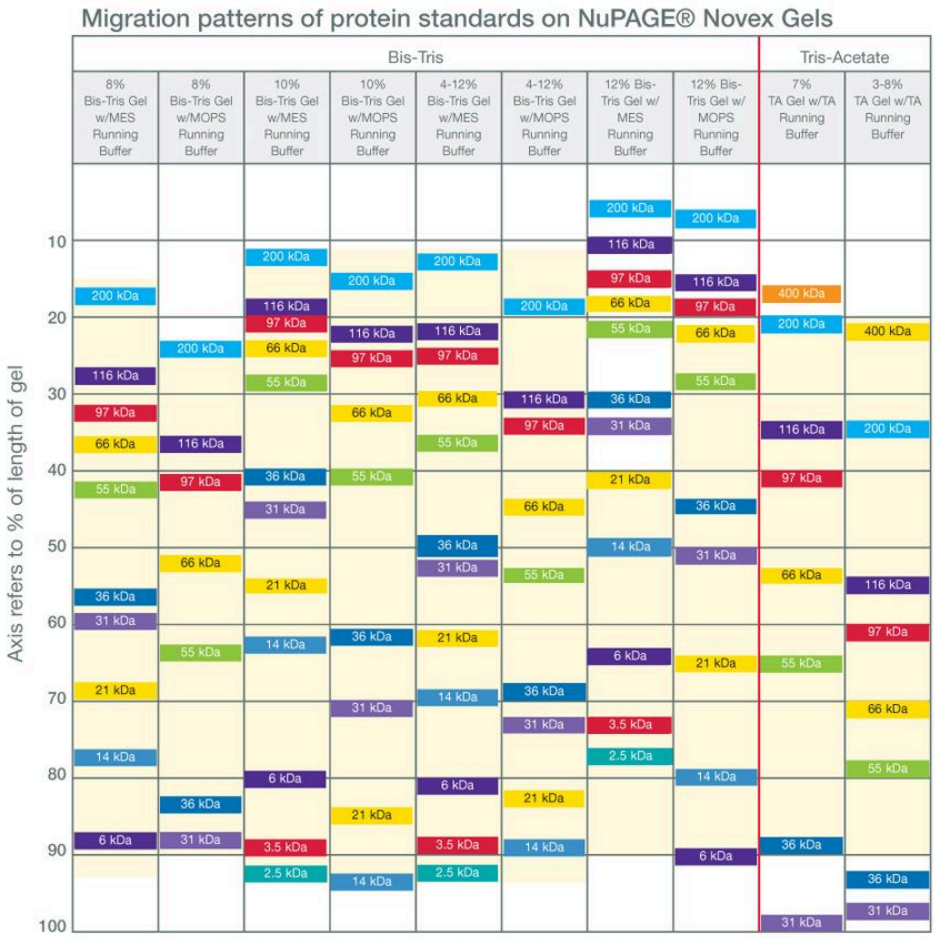
9.1.12 Use the resultant y=mx+b equation to quantify the amount of protein in each sample. Dividing by 3 will give you your protein concentration per uL.

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
Denaturing separation
3–8%
7%
NuPAGE Tris-Acetate Gels
Native separation
3–8%
7%
% of length of gel
0
10
20
30
40
50
60
70
80
90
500 kDa
290 kDa
240 kDa
160 kDa
116 kDa
97 kDa
66 kDa
55 kDa
40 kDa
1,048 kDa
720 kDa
480 kDa
242 kDa
146 kDa


Invitrogen™ Novex™ Tris-Glycine Gels, WedgeWell™ format
Denaturing separation
10%
12%
4–12%
8–16%
4–20%
% of length of gel
0
10
20
30
40
50
60
70
80
90
200 kDa
116 kDa
97 kDa
66 kDa
55 kDa
36 kDa
31 kDa
21 kDa
14 kDa
6 kDa



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 **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 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**IPVH00010

Whatman Grade 3MM Chr Cellulose Western Blotting Membranes, GE Healthcare, Grade 3MM Chr Blotting Paper, roll, 10 cm × 100 m, **VWR International (Avantor) 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

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 13 steps

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

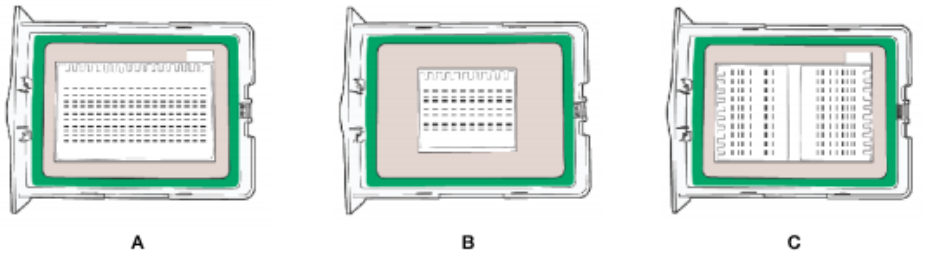
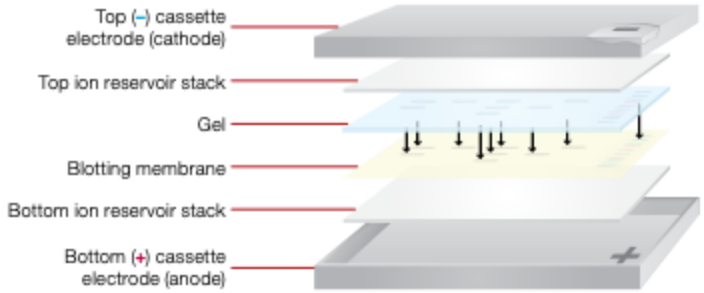


Fig. 12. Suggested placement of assembled transfer stacks in a cassette. A, midi stack and gel placement; **B,** mini stack and gel placement; **C,** two mini gels on a midi stack.

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 × 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.



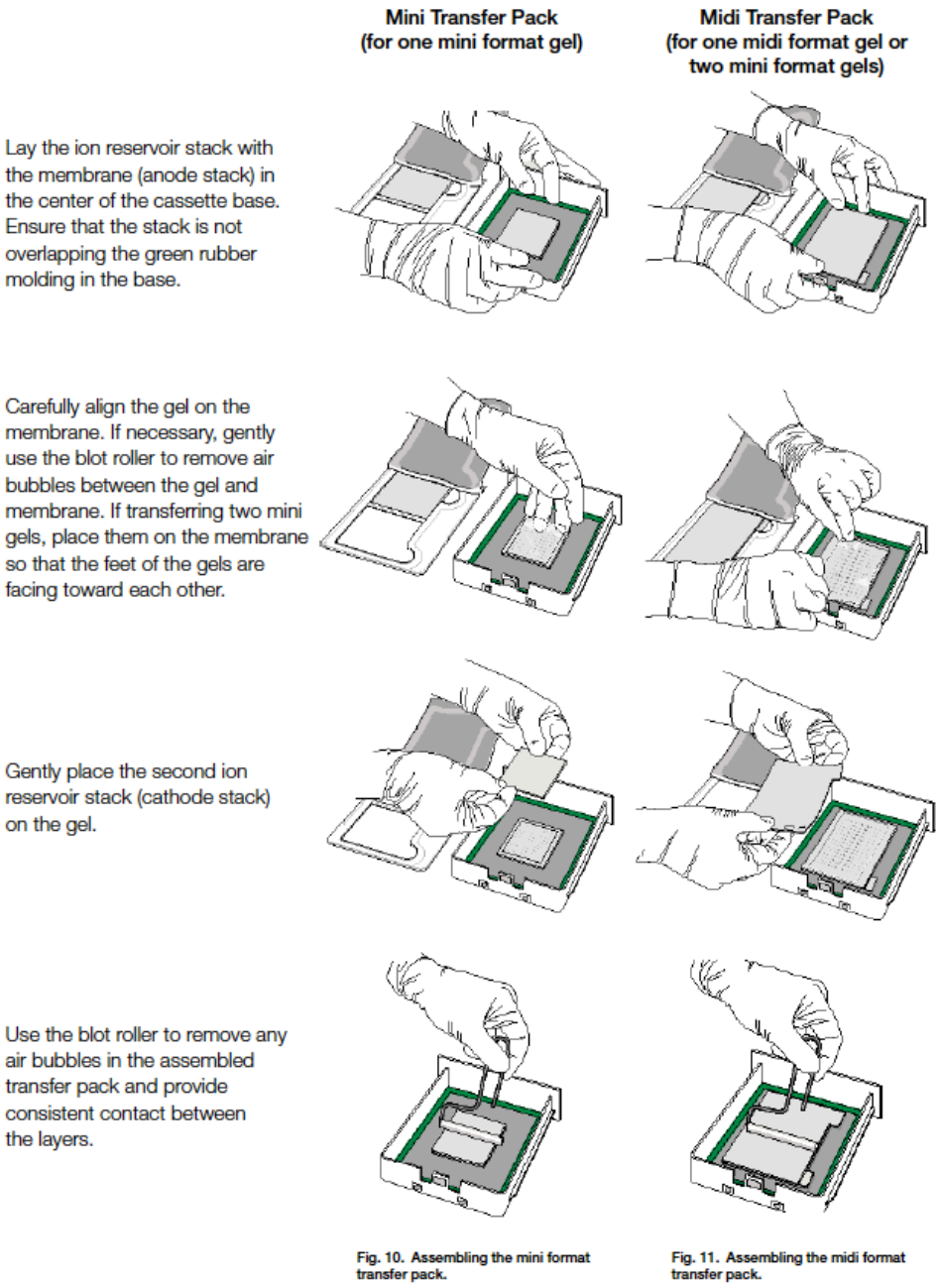


Fig. 10. Assembling the mini format transfer pack.

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)) **Jackson ImmunoResearch Laboratories, Inc. 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 **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 × 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.