The following steps may differ depending on the type of antibody used and the amount of block:

1) The presence of SDS in the transfer buffer (using 0.1% SDS in transfer buffer facilitates the transfer of high molecular weight proteins from the gel to the membrane).
2) The type of membrane used—for smaller molecular weight proteins, the lab uses 0.2 um nitrocellulose or PDVF, for high molecular weight proteins (used by JEM), 0.45 um nitrocellulose works.
3) The amount of milk used to block the membrane after transfer-ranges from 3%-5% (5% used by JEM)
4) The concentration of primary and secondary antibodies used depends upon the amount of protein loaded on the gel and the strength of the antibody.
5) The amount of milk or the presence of milk in the antibody incubation can also be varied depending on background and antibody signal.

- 0.45 um PVDF membrane works well with the Bio-Rad scanner. You must pre-wet the PVDF first in methanol prior to setting up the transfer. Blots are blocked in 5% milk with TBS/tween.
- Typical secondary anti-rabbit antibody concentration is 1:2000 in 2.5% milk/tween/TBS and for anti-mouse, 1:5000.
- For loading controls, when the GAPDH lot is new, then 1:100,000 concentration likely suffices if you are loading 25 ug or higher of total protein but as the stock ages, you need to increase the concentration up to 1:1000.

Note- Protein extraction done in Ripa buffer
Ripa buffer contents: from Kelsey Martin lab (Kim’s recipe)
1% NP-40 substitute (USB cat#19628)
0.5% Deoxycholate
0.1% SDS
Bring to volume with 1x PBS (w/o Ca or Mg)
+ Sigma protease inhibitor cocktail- 10ul/100ul for cell culture extract but 10ul per 1ml of brain tissue.
Homogenize.

1) Prepare glass slides for gels. Clean with windex or alcohol and dry. You can put parafilm over the casting stand grey strips which allows for a tighter seal between the glass and the casting stand.
2) Clamp glass plates into green clamps-right side up is with the green little buttons showing. Make sure it’s even with countertop. Press down firmly on glass onto rubber strips.
3) Prepare 10% SEPARATING gel in 15ml tube. Do not let sit.
Separating gel:
3.3 mls Acrylamide mix (29:1 acrylamide:bis-acrylamide) found at 4°C
2.5ml 1.5M Tris pH 8.8
4.1ml dH2O
100ul 10% SDS
50ul 10% ammonium persulfate found at 4°C
5ul TEMED mix whole solution by inversion
4) Using a transfer pipet, transfer separating gel into space between glass slides. Do not allow bubbles to remain. Fill to about ¾ of the way up so you have enough room to put the stacking gel and the comb-ie so there is room in-between the separating gel and the stacking gel.
5) Overlay top of separating gel with isopropanol to prevent evaporation (ethanol will mix with the gel contents so this alcohol is better).
6) 30 minute period for solidifying. Suck remaining separating gel into transfer pipet to determine solidification later. Thaw out protein standard.
Protein samples should be prepared as early as possible using the formula for 2x Loading sample buffer:
- 25ul of Laemmli 2x dye with betamercaptoethanol
- 25ul of your protein sample of desired concentration (if you load less than 25 ul, makeup the difference in dPBS or lysis buffer) = 50ul total
- which is the maximum each well can hold for a 10% polyacrylamide gel (gradient gels can only hold 30ul total volume). Use a boiling water bath to boil protein samples for 2-3 min then cool on ice for 5 min and do a quick centrifuge to spin down sample because buffer “soapy.”

**Sample buffer is 950ul Laemmli dye + 50ul beta-mercaptoethanol. 2x sample buffer made to match 1:1 the volume of the protein and dilution amount.**

Pour out overlaying isopropanol. Carefully dry space with filter paper. DO not touch gel.

**Prepare STACK gel in 15ml tube.**

**Stack gel:**
- 0.67ml Acrylamide mix (29:1 acrylamide:bis-acrylamide) at 4°C
- 0.5ml 1M Tris pH 6.8
- 2.4ml ddH2O
- 40ul 10% SDS
- 30ul 10% ammonium persulfate at 4C
- 3ul TEMED

This solidifies quickly so you need to work fast!

Transfer stack gel to glass slides using transfer pipette. Fill to glass edge. No bubbles. Need careful and consistent flow.

Immediately insert 10 well comb of 1.5mm all the way. 60ul per well maximum.

15 minutes for solidifying. Suck remaining liquid into pipette.

Prepare running buffer. 10% of xml/10x ml of total solution.

Use 10x TGS buffer. 80ml of 10XTGS in 800ml total dH20 (for Terasaki setup)

After 15 min for stack gel, remove glass sandwich from green clamp. Lie it down so you can slowly remove comb then insert it into gasket with wells facing inward. Push clamps shut to lock.

If you are running one gel, you need to insert buffer dam in back.

Fill area in-between wells and clear plastic window with running buffer (step 12) to top. Watch for leakage.

Insert yellow well guide above gel wells. Load wells.

Apply green cap with positive and negative electrodes (red&black).

Turn on switch on right side back, keep “pause”

Set 100V for gel. Run for 1 hour 15 min. Do not let protein run off gel.

Prepare buffer for transferring gel to the membrane.

100ml buffer 10x TG (found at RT)

200ml methanol

10 ml of 10% SDS to make up 0.1% SDS of total solution (aids in transferring protein off gel, do not use for small MW proteins)

Bring to 1300 mls to completely fill the container.

Pour some buffer into flat container. Under buffer remove gel by using green tool to pry open the two glass plates or using filter paper to stick gel to it. Mind orientation of gel-cut corner so you know orientation. Usually cut lower left corner.

22) Trim gel where protein has not run-use razor or spatula.
23) Under buffer–assemble transfer sandwich in the following order. Debubble by rolling with a plastic pipet tip as each layer is added. Use gloves, use forceps for blot. 
Order: from the bottom up:
   a. black plastic 
   b. foam white sheet/pad which was presoaked in transfer buffer 
   c. filter paper 
   d. gel protein side up 
   e. nitrocellulose membrane-first wet in buffer (if you use PVDF-must first be pre-wet in methanol for a few minutes) 
   f. filter paper 
   g. white foam sheet/pad-missing so use thick filter paper 
   h. clear plastic top
24) insert sandwich into black and red gasket with black side with holes facing black wall of gasket and if you have a second cassette make sure it faces the black side of the gasket. 
25) insert into clear plastic box 
26) insert ice box (lab freezer-fill with dH20 if needed) 
27) pour in transfer buffer, put in stir bar 
29) while stirring, apply cap and run at 400mA for 2 hours (note-Schweizer lab uses a shorter transfer time-1-1.5 hours for smaller molecular weight proteins) 
Prepare non-binding fluid-milk 
mixed in erlemeyer flask 
For 5% blocking: 3.0 ml of tween (10% tween 20) room temp (0.1% of total solution) 
60ml of 5x TBS (tris-buffered saline) room temp (10% of total solution) 
15g of nonfat dry milk (5% of total solution) 
----ddh20 
~300 mls total 
For 2.5% fluid for primary and secondary antibody incubation-take 100mls of tween/TBS mixture and add 2.5g of milk
30) following transfer, disassemble sandwich under transfer buffer, before removing nitrocellulose membrane use a razor to cut around the shape of the gel incl cutting the needed corners, and using forceps to prevent hand contamination. 
31) Take blot and stain for 1 minute or so with Ponceau solution (PVDF-WET MEMBRANE IN METHANOL FIRST, THEN RINSE WITH DH20 PRIOR TO ADDING PONCEAU; DO NOT LET PVDF DRY OUT!)-which will stain protein bands and indicate if transfer was successful, then rinse off with dH2O; take gel and stain with Coomassie blue-this will tell you whether protein is left on the gel. Nitrocellulose membrane can be directly stained for Ponceau then rinsed with ddH2O. 
32) Put membrane protein side up in container and block for 1-1.5 hours in 5% milk 
33) Apply primary antibody in milk/tween/TBS solution- about 4mls (if blot is smaller, you can use less) and incubate overnight in a heat-sealed bag at 4ºC while rocking. 
Note-to seal it in-cut open one side, place blot in, then seal that side, open up the top of the bag, put solution in.
The following day: prepare tween/.TBS washes 
(3ml tween (10% tween 20) (0.1% solution) 
60ml 5x TBS (10% of solution) (JEM makes stock 5x TBS) 
300 ml dH2O
------300ml total 
34) Remove membrane from plastic with tweezers, wash in tween/TBS a few quick rinses then wash membrane 3x 10 minute washes in tween/TBS on rocker at room temp.
35) For secondary antibody, make dilution in 2.5% milk/tween/TBS (leave out milk if called for) and incubate membrane for 2 hours at room temp.
36) Wash membrane 5x 10 min each on rocker with the wash solution:
- 3ml tween (10% tween 20) (0.1% solution)
- 60ml 5x TBS (10% of solution)
- 300 ml dH2O

------~300ml total
Then develop using GE Healthcare Amersham ECL detection plus kit
1. Add appropriate amounts of A+B and put on blot sitting in blot box in the dark for 5 min.
2. Image

5x TBS buffer for Western blot washing:
(100mM Tris-HCl, 2.5M NaCl)

1) Put ~1800mls of ddH20 in a beaker
Add 292.9g NaCl (slowly otherwise won’t dissolve)
31.52g Tris-HCl (not Tris base)
Resulting pH is ~5 so you need to add 1M NaOH to bring final pH up to 7.5
Bring up to 2L total volume with ddH20

To make TBT/tween wash buffer for Western-

Take 60ml of 5x TBS (=1x TBS)
3 mls of Tween 20 (0.1% of total solution)
Bring to 300mls total volume with ddH20