Western Blot protocol
As taught to Jessie by Jen Goeckeler in the Brodsky lab 3-11-08 to 3-12-08

This protocol includes protein separation by SDS-PAGE (poly-acrylamide gel electrophoresis) and the immunoblotting of Hsp101.

Western Blot protocol:

1. Pour the Gel
2. Run the gel
3. Transfer to nitrocellulose
4. Ponceau S stain (optional)
5. Block
6. Incubate with primary antibodies
7. Wash off primary antibody
8. Incubate with secondary antibody(s)
9. Wash off secondary antibody
10. Develop with enhanced chemiluminescent reagent (ECL)
11. Imaging

Important Notes:
Do not touch nitrocellulose except with forceps on the corners of the membrane.
Do not allow nitrocellulose to dry out at any step after wetting. This means don't drain buffer or antibody until you are prepared for the next step.

Safety notes:

- Unpolymerized acrylamide is a neurotoxin and carcinogen. Although in its polymerized state (in liquid), it is not toxic, there can be traces of unpolymerized acrylamide in the gels so always use gloves when handling the gel and the gel-running equipment. The Brodsky lab does dispose of these gels in the regular trash.
- All buffers except transfer buffer can be disposed of in the drain. Transfer buffer is reused until it's yellow and then collected and disposed of through chemical waste, due to the amount of methanol (20%).

1. Pouring the Gel - 10% acrylamide with 5% stacking gel
    - I did not learn this yet
    - Find out spacer thickness
2. Run the Gel (1.5 hours if gel is already made)
    - remove a gel with plates from the stack – insert a razor blade between the back white plate of your gel and the front glass plate of the next one and use that to pry them apart
    - wipe off/scrape excess acrylamide from the surface of the plates, including the "fringe" at the top above the loading wells and the thin layer on the back plate
    - mark the wells on the glass plate using a marker (plate must be dry)

- remove the comb gently, pulling straight out from the middle so that you don't disturb the wells
- wipe off excess acrylamide "fringe" again moving the kimwipe in only the direction going parallel to the direction that the plate will run. The part of the wells you will load into is under the glass plate. (if you wipe across the wells in a perpendicular direction, you might bend them)
- if running more than one gel, label the glass plates with a marker
- clip the gels to the rig, with the widest part of the red clamp facing outward
- fill the center and trough on the rig with 1x running buffer (SDS buffer – get recipe) The center part should be filled to the top of the gel(s) and the trough should be about half full
- load ladder in first well– we used 3-4 uL of Fermentas Pageruler Prestained Protein Ladder Plus (cat # SM1811) – use extra long (gel loading) tips
- load samples using marks made with marker to visualize placement of pipette tip
- dispense sample starting at the bottom of the well and slowly moving the tip toward the top of the well as you go so that if you expel a bubble at the end it doesn't blow too much of your sample out of the well
- run at 20mAmps PER GEL (or 40 mAmps per 2 gels etc.) on CONSTANT CURRENT setting for about an hour, or when the front of the bromophenol blue has migrated to the bottom of the gel, or when you have achieved "good enough" separation based on the positions of the marker bands; voltage will increase as the samples move from the stacking to separating gel

3. Transfer to nitrocellulose (a few hours or overnight; for more concentrated protein samples, longer, slower transfers are better. The general times are as follows: overnight at 60 mAmps, 3 hours at 180 mAmps, 1.5 hours at 360 mAmps)
    - cut out 2 pieces of filter paper larger than the gel
    - cut out nitrocellulose squares about the size of the gel, being careful to not touch the nitrocellulose with fingers, only on the edges with forceps when necessary (it is highly pressure sensitive and finger indentations will affect the protein transfer). Nitrocellulose: Biotrace NT Roll 30cm x 30cm prod # 66485
    - soak filter paper and nitrocellulose in transfer buffer (see recipe). After this point the nitrocellulose should stay moist at all times
    - fill the transfer tank with transfer buffer
    - lay out "gel cassette" with the gray side away from you (gray, away) and black side toward you (black to your back)
    - Make sandwich on black surface with layers in this order

        filter paper

        gel – gently pull out one spacer and use as a wedge to lift the glass plate from the surface of the gel. Lift the gel with a razor blade and carefully transfer to the filter paper. Invert the gel so that it is backward on the filter paper. The marker lane should now be on

the RIGHT side. This way, when the proteins are transferred to the nitrocellulose, they will be present in the same order that you loaded the gel initially. Use a Pasteur pipet wet with transfer buffer to flatten the gel if there are bubbles

nitrocellulose- gently lay it over the gel, try to match up 2 sides so that you only have to trim the other 2. Trim the membrane to the size of the gel with a razor blade; cut a shape into a corner to distinguish between blots if you have more than one. Use the wet pipet to gently roll out any bubbles between the gel and nitrocellulose.

filter paper – lay gently over the sandwich, don't worry about bubbles for this layer.

- Close "gel holder" and load into the transfer tank with the gray side facing towards the positive terminal (red).
- Double check that you have loaded the tank with the nitrocellulose on the positive side of the gel.
- Transfer at 60mAmps overnight on constant current. Volts will be low (under 20) and get lower…
- After removing the membrane to a Tupperware dish with water, throw away the gel and filter paper and pour the transfer buffer back into the carboy. It can be reused until it is yellow.

4. Optional – Ponceau S staining. (15min)
   This step is just to check that you have protein on the gel. Can also be useful to check for any bubbles in your lanes or to verify even loading.
   - rinse the gel in water to remove the transfer buffer (a minute is sufficient)
   - put in a small container and add enough Ponceau S stain (2% Ponceau S in 5% acetic acid) to cover
   - rotate/shake for five minutes
   - rinse ponceau stain off with water to remove the pink background (you should see protein bands)
   - can cut blots into strips at this point if desired – keep it wet!
5. Blocking (15-30 min).
   This step coats (blocks) all areas of the membrane that are not already covered by your proteins so that the antibody doesn't stick to the membrane
   - put blot back into container and add enough blotto or blocko or both to cover gel (see recipes)
   - rotate/shake for at least 15 minutes (this step can be done overnight or longer)
6. Incubate with primary antibody (2 hours to overnight)
   - make up antibodies if not made, 15mL per blot in blotto. Blotto helps to block non-specific antibody binding.

     for Hsp101 (labeled AZ204): dilute 1:1000 in blotto, so 15 ul in 15mL of blotto

     for alpha-Tubulin: recommended to dilute 1: between 4600 and 9200. We diluted 1:5000 so 3ul in 15mL

(since that wasn't quite enough this time, I'd try it at 1:2000 next time and make sure you incubate overnight at 4°C)

- until we know that Hsp101 antibody and loading control antibodies do not cross-react, do each antibody separately
- cut blot between where 2 proteins should be – for Hsp101 and alpha-tubulin, cut at red 72 kD band of the Pageruler ladder.
- Cover blot in antibody solution. If you are incubating more than one blot, make sure that they are not stuck together.
- Incubate in primary antibody for 1 – 4 hours at room temp or over night at 4°C. (Vierling protocol says 1 hour, we did 2.)
- SAVE AND RE-USE ANTIBODY SOLUTIONS??

7. Wash 3 times (20 min)
   - cover gels with TBST and rotate/shake for 5 min.
   - drain and cover with fresh TBST and again rotate/shake for 5 min
   - repeat one more time then drain
8. Incubate with secondary antibody (30 min to 1 hour)
   - make up secondary antibodies in TBST (HRP-linked sheep anti-mouse and HRP-linked donkey anti-rabbit from the Brodsky Lab; GE Healthcare cat #s NA-931 and NA-934 respectively)

     ** do not use blotto for secondary antibodies. The sodium azide in the blotto will inhibit the horseradish peroxidase in ECl.
   - Cover blot in antibody. If you are incubating more than one blot, make sure that they are not stuck together.
   - Incubate for 30 min to 1 hour at room temp (We did 1 hour).
9. Wash 3 time (20 min)
   - cover gels with TBST and rotate/shake for 5 min.
   - drain and cover with fresh TBST and again rotate/shake for 5 min.
   - repeat one more time then drain
10. Developing with enhanced chemiluminscent (ECL) reagent (Pierce cat# 34080) (10 min)
    - make sure TBST is complete drained from container
    - set up a piece of saran wrap in which to wrap the blots when developing
    - add 1 mL of each chemiluminescent agent (one contains horseradish peroxidase substrate and one contains an enhancer/stabilizer) per whole blot (or 2 halves)
    - manually shake blot(s) around in the mixture for about a minute and make sure blot(s) are completely covered in the reagent. Add more of each reagent if you have to.
    - Working quickly, lay each blot protein side down as flat as possible on saran wrap. Repeat with additional blots. Wrap up the blot(s) keeping only one layer of unwrinkled saran wrap on the protein side. Don't let the blot(s) dry out!
11. Imaging (10 min; highly dependent on antibody)
    - use Kodak imaging program on Brodsky/Arndt lab image station. Do not touch any part of the computer or camera with gloves. The wrapped blots

are not toxic at this point so you can use your hands, but be careful to not smudge the saran wrap on the protein side.

- Adjust only magnification on the camera (the middle moveable ring)
- The ring on the right should be set to 1.2 and the one on the left should be aligned with the little white dot. The UV light should NOT be on and the filter should be set at "0".
- To begin, place your blots on the imager surface and in the program click preview and expose. This will allow you to see the position of your blot and make adjustments to the zoom if desired. When your blots are the size and position you want, click stop and then unclick preview. Set your exposure times and capture numbers.
- Exposure – start at 2 minutes, 5 captures. Expose more if necessary. For weak signal, fewer but longer exposures are better, for very strong signals, shorter fewer exposures are ok.

RECIPES:
All recipes are based on the Laemmli method of Western Blotting.

SDS running buffer,
10X Stock: 0.25M Tris, 1.92M glycine, 1% SDS.
Currently the Brodsky Lab purchases this from the stockroom, National Diagnostics brand, CH-094.
Dilute to 1X for use.

1X Transfer Buffer:
This can be reused until yellow.
Add, in order:

	1L	2L	3L	4L
10X SDS Running Buffer	100mL	200mL	300mL	400mL
10% SDS	1mL	2mL	3mL	4mL
Volume ddwater	fill to 800mL	fill to 1600mL	fill to 2400ml	fill to 3200mL
Methanol*	200mL	400mL	600mL	800mL

*The methanol must be added after the water to keep the SDS from precipitating out.

Blotto- for 1 liter:
begin with 500 mL DDW,
add:

- 100 mL of 10X TBS pH 7.4,
- 20g non-fat dry milk,
- 1 mL Tween-20,
- 10 mL of 0.5M Sodium Azide (NaN3),

stir for a while to be sure that the milk is dissolved and the tween is incorporated,

volume to 1L with DDW
store at 4°C.

Blocko:
same as above except 20 g of BSA instead of milk

Chemiluminscent Agents:
The Brodsky Lab purchases these. They use Thermo Scientific(Pierce) "Supersignal West Pico Chemiluminscent" Product # 34080.

See Also:
http://www.abcam.com/index.html?pageconfig=resource&rid=11375&sc_ql=1702&intGoUser=5026238