I. SDS-PAGE:

1. Assemble glass sandwich
   a. Clean the short and 1.5 mm spacer plates with 70% alcohol before starting (one short and one spacer plate per gel).
      - *If there is residual polyacrylamide dried on either of the glass plates, it needs to be scraped off carefully with a razor blade; otherwise, the gel will leak during casting.*
   b. On a smooth level surface (i.e., the lab bench), place together the short and 1.5 mm spacer plates so that both plates are flush at the bottom.
   c. Slide the 2 plates into the casting frame with the smaller plate-facing front. Lock the pressure cams to secure the glass plates.
      - *Do not drop the glass plates into the casting frame, or else it will chip the base of the glass plates and cause leaks*
      - *Be sure not to tilt the casting frame and glass plates when locking the pressure cams. This will cause the gel to leak.*
   d. Place a gray rubber gasket on the bottom of the gel assembly apparatus.
   e. Insert the glass sandwich into the gel-casting apparatus: Place the glass sandwich on top of the gray casting stand gasket and engage the top of the spacer plate with the spring-loaded lever. The pressure of the lever will hold the spacer plate down so both plates are perpendicular against the gray rubber gasket.
      - *Do not push the glass plates down on the gray rubber gasket with more force than is provided by the spring-loaded lever. This will damage the gasket and cause the gel to leak.*
2. Prepare the separating gel solution.
   a. Consult the Sambrook table for the recipes using different volumes of separating gel (10 ml volume per gel) and percentages of acrylamide. Make sure to prepare the APS fresh and add it last to the separating gel solution.
   b. Swirl the separating gel gently after APS addition and use a 10-ml pipette to add the gel between the glass plates up to ~1 mm of the bottom of the green bar on the casting frame.
      - *Do not add the separating gel beyond the bottom of the green bar. This will cause the gel comb to push into the separating gel during Step #3.*
   c. Immediately after adding the separating gel, use a P-200 micropipet to add water-saturated butanol as gently as possible to the top of the gel to smooth out the gel interface.
      - *Pull the butanol from the top layer of the butanol-water solution.*
   d. Allow the separating gel to polymerize. Test the progress of the polymerization by squeezing the top of the used disposable pipette: if the solution has solidified, the polymerization has occurred.
      - *Work reasonably quickly, because the polymerization typically occurs within 5–10 min*
      - *Older bottles of acrylamide will take longer to polymerize because of monomer oxidation.*
   e. In the meantime, prepare the stacking gel. Consult the Sambrook table for the recipes using different volumes of stacking gel (~2.5 ml volume per gel, 5 ml minimum). Do not add the APS until Step #3.
   f. When separating gel has polymerized, pour off the butanol in the sink and rinse gently with tap water. Remove the water by capillary action with a paper towel.
      - *Ensuring a flat interface is critical for applications that involving looking at electrophoretic mobility shifts*
3. Stacking gel
   a. Add the APS and use a disposable pipette to lay the stacking gel on top of the polymerized separating gel. Fill with separating gel up to the shorter gel plate. Insert a 1.5mm comb between the glass plates while making sure no air bubbles are trapped between the teeth of the comb.
      - *The best way to do this is touch the bottom of the combs to the separating gel first; then, bring the comb parallel with the spacer plate and insert the comb between the glass plates.*
      - *When inserting the comb, keep your face away from the gel, because stacking gel commonly squirts out at this step.*

- *If a bubble gets stuck below the comb, remove the comb, refill with stacking gel to the top, and try again*

b. In the meantime, prepare the running buffer by diluting the 5× Tris-glycine stock in H_2O (1 L running buffer per tank).

c. When the stacking gel has polymerized, carefully remove the comb. Rinse and irrigate the wells with 1× running buffer thoroughly to remove unpolymerized acrylamide.

- *Remove the comb gently to avoid creating a vacuum suction that deforms the acrylamide lane separators of the stacking gel.*

4. Remove the gel plates from the casting frames and place the plates in the electrophoretic apparatus with the gels firmly seated at the base of the electrophoretic apparatus and the short plates facing inward.
   - *Putting the plates in backwards will not allow current to flow through the gel, and it is very difficult to fix the plate orientation after the samples have been loaded.*
   - *If only running one gel, use the plastic buffer dam on the other side of the electrophoretic apparatus, although some have noted that single gels tend to leak more*
   - *Be sure that the apparatus has two metal leads pointing upwards, that red-black on the leads matches red-black on the tank, and that only two gels are run per tank (Biorad says four can be run in the same tank, but this setup is troublesome).*
5. Fill the inner chamber full with running buffer. Fill the outer chamber up to the level specified on the tank.
   - *The liquid level in the inner chamber must stay above the short plates for current to flow through the gel. If the liquid level in the inner chamber is dropping, then there is a leak and the gel plates have not been properly installed.*
   - *If the leak remains after fixing the plates, fill the outer chamber up to the level of the inner chamber so that there is no fluid head between the inner and outer chambers.*
6. Load the samples:
   a. 5 μl Precision Plus dual color markers (Bio-Rad #161-0374)
      - *To save reagents, one can typically dilute the newest Precision Plus markers five-fold in 1× sample buffer and still have enough color to indicate the markers after transfer*
   b. 40 μl total volume for samples when using a 10-well comb (including 10 μl of 4× sample buffer). 20 μl total volume for samples when using a 15-well comb (including 5 μl of 4× sample buffer).
      - *Up to 40 μl can be loaded in the 15-well comb, and up to 60 μl can be loaded in the 10-well comb, but care must be taken to avoid having the sample overflow into the adjacent well*
      - *Do not load more than 20–25 μg total cellular extract, because this will typically saturate the band intensities*
   c. Fill each well slowly—the goal is to get all of the sample solution into each well. Try to avoid bubbles in wells that may cause overflow of the solution into other wells
   d. When working with two gels, make sure to label on the outside of the container which gel is which.
7. Match the + and – electrode ends of the lid to the power supply, and set the apparatus to run ~1.25 hr at 130V constant voltage.
   - *Lower voltages and longer times can improve the separation and resolution of some proteins*
8. Check to make sure that the blue dye front has been run all the way to the bottom of the gel plate.
   - *For colorimetric staining of total proteins instead of Western blotting, stain for 1 hr in Coomassie Blue staining solution, destain periodically with destaining solution, and destain overnight with longer destaining solution*
9. Set up transfer apparatus
   a. Prepare 1L of transfer buffer (100 ml 10× transfer buffer stock, 800 ml H_2O, 100 ml methanol for most proteins).
      - *More methanol (20%) may be required for small proteins (<20 kDa) and less methanol (5%) may be required for very large proteins (>150 kDa)*

b. Heat 1L of deionized water in the microwave for 4 mins and dip the fiber transfer pads in several times to dissolve any leftover buffer salts.
   - *This step avoids any problems with transfer "hot spots" that could be caused by residual buffer salts leftover from the last transfer.*

c. Set up the transfer cassette on a cafeteria tray
   i. Start with the clear half of the transfer cassette on the bottom
   ii. Fiber pad: -soak and squeeze out air bubbles in transfer buffer
   iii. Filter paper: -pre-wet in transfer buffer
   iv. PVDF membrane: -briefly soak in methanol (0.45 μm pore size Immobilon-FL for most proteins, 0.2 μm pore size can be tried for proteins less than ~20 kDa)
   v. Gel: -remove gel from glass plates by cracking plates open with a wet razor, gently cutting the stacking gel off, and laying the gel on top of the PVDF membrane using a wet razor
   vi. Filter paper: -pre-wet in transfer buffer
   vii. Fiber pad: -soak and squeeze out air bubbles in transfer buffer

d. Close together cassette and insert into transfer housing so that the black side of the cassette faces the black side of the housing. Insert the cassette gently to avoid generating air bubbles within the transfer sandwich.

10. Put in the ice block, fill the tank with transfer buffer, and surround the transfer tank in ice.
    - *When filling the tank with transfer buffer, do not pour the buffer directly on the transfer cassettes, as this will generate air bubbles within the transfer sandwich.*
11. Set up the wet transfer to run for 1 hr at 100V constant voltage.
12. While the transfer is running, clean up the electrophoresis setup by rinsing the tank, chamber, and glass plates with tap water.
    - *Take extra care when cleaning the glass plates to remove all residual polyacrylamide from the plates; this can be achieved by rubbing the surfaces of the glass plates with your gloves.*
    - *The razor blade used to lay down the gel will rust unless it is sprayed down with ethanol or water after use.*
13. Wipe down the bench with a wet paper towel to remove buffer salts that may have spilled.

II. Western Blotting

1. Remove the PVDF membrane from the transfer apparatus. Mark which side the gel proteins transferred onto by writing your initials and the date. Mark the MW markers with a **pencil** and indicate the pink "landmark" bands. The blue markers will fluoresce (in the 700 channel), but the pink will not.
   - *Pencil will fluoresce but the signal will not leach away from the marks on the membrane.*
   - *Some pencils use lead that is not autofluorescent, so it is best to identify one that works well and stick with it (do not oversharpen or it will tear the membrane)*
   - *Black or blue ink will fluoresce and cause background problems across the entire membrane.*
2. Place the membrane in 0.5× Odyssey blocking solution for 1 hr @ RT on the nutator.
   - *Dilute Odyssey blocking solution 1:1 in PBS if using Licor #927-40000 or in TBS if using Licor #927-50000; use 1× blocking solution if there are too many nonspecific bands.*
   - *The TBS-based Licor blocking solution is generally better for phospho-specific antibodies, but the PBS-based Licor blocking solution is otherwise preferred.*
   - *Conserve Odyssey blocking solution by using 5 ml per blot in a sealed bag*
   - *Handle membrane carefully with flat forceps or clean gloves (fingerprints will show up on the Odyssey)*
   - *Work quickly so that the membrane does not dry out during handling; this can cause problems during blocking*
3. Clean up the transfer setup by rinsing the tank, chamber, assembly tray, transfer cassette, and transfer housing in tap water and the fiber pads in deionized water.
   - *It is important to submerge the fiber pads completely in deionized water to remove residual buffer salts that can cause transfer "hot spots" if they are left to dry on the fiber pad.*

- *Make sure that the metal wires within the transfer housing are thorougly rinsed to avoid salt-related transfer artifacts.*

4. Place the membrane in 5 ml of primary antibody solution (dilute in 0.5× Odyssey blocking buffer + 0.1% Tween) in a sealed bag overnight @ 4ºC on the nutator. Two-color detection requires primary antibodies raised in different host species.
   - *Use the same Tris or phosphate buffer as in the original blocking solution.*
   - *5% nonfat milk in TBS can also be used if the Odyssey blocking buffer is determined to be too stringent for weak antibodies.*
   - *As a convenient loading control that can be combined with mouse or rabbit primary antibodies, we use a chicken anti-tubulin antibody (Abcam #ab89984, 1:20,000 dilution; there is a 1:20 dilution of the stock antibody in the 4ºC antibody box)*
   - *Convenient mouse loading controls that can be combined with anti-tubulin are anti-vinculin (Millipore #05-386, 1:10,000 dilution) and anti-GAPDH (Ambion #AM4300, 1:20,000 dilution)*
   - *Convenient rabbit loading control that can be combined with anti-tubulin are anti-Hsp90 (SCBT #sc-7947, 1:2000 dilution) and anti-p38 (SCBT #sc-535, 1:5000 dilution)*
5. Discard the primary antibody solution and wash the membrane for 4 × 5 min in PBS + 0.1% Tween or TBS + 0.1% Tween.
   - *Use the same Tris or phosphate buffer as in the original blocking solution.*
   - *The scissors used to cut open the antibody bags will rust unless they are sprayed down with ethanol after use.*
   - *Use separate trays for blots or blot pieces that have been stained with different primary antibodies (the residual primary antibody can move from one membrane to another during the early washes)*
6. In the meantime, prepare secondary antibody solution (1:20,000 dilution in 0.5× Odyssey blocking buffer + 0.01% SDS + 0.1% Tween).
   - *Be careful not to introduce contamination into the antibody vial*
   - *Use the same Tris or phosphate buffer as in the original blocking solution.*
   - *Addition of SDS to the secondary solution is important to reduce background staining*
   - *IrDye 800 is brighter with lower background and should always be used to image the more-important primary antibody*
   - *Use IrDye 680LT to image anti-tag antibodies or loading-control antibodies*
7. Place the membrane in secondary solution @ RT for 1 hr on the nutator.
   - *Licor says to cover membrane with foil during incubation, but the IrDyes are so photostable that this is not necessary*
   - *Incubation times longer than 1 hr may increase background*
8. Discard the secondary antibody solution and wash the membrane for 4 × 5 min in PBS + 0.1% Tween or TBS + 0.1% Tween.
   - *Use the same Tris or phosphate buffer as in the original blocking solution.*
   - *The scissors used to cut open the antibody bags will rust unless they are sprayed down with ethanol after use.*
   - *Use separate trays for blots or blot pieces that have been stained with different secondary antibodies (the residual secondary antibody will move from one membrane to another during the early washes)*
9. Rinse the membrane with PBS or TBS to remove residual Tween.
10. The membrane is now ready to scan.
    - *Wet membranes may be stored for up to 4 days, protected from light*
11. If needed, store the blot wet with PBS or TBS at 4ºC for stripping and reprobing.
    - *Once a membrane is dry, stripping is ineffective*

III. Stripping & Reprobing

1. Place the blot in 10–15 ml of high-stringency stripping buffer in a sealed bag for 30 min @ 50ºC. Agitate the bag 2–3 times during the 30-min incubation.

- *After stripping, the PVDF membrane is very susceptible to handling artifacts (creasing, smudging, etc.) that give high fluorescence background upon reprobing; handle the membrane very gently*

2. Wash the membrane for 3 × 5 min in PBS or TBS.
   - *Do not use Tween-containing solutions during this wash step, as it will cause severe background in the 700 channel*
   - *The high-stringency stripping buffer contains β-mercaptoethanol, which must be disposed of as hazardous waste*
3. Go to Step #2 of Western blotting.

- *Alternatively, strip the blot in 10 ml of low-stringency stripping buffer for 10 min @ RT, wash once with PBS, and go to Step #2 of Western blotting*
- *The low-stringency strip can partially preserve phospho-epitopes and will better retain low-abundance proteins but will not remove tight-binding primary antibodies*

Buffer recipes

- **4X sample buffer** Store @ -70 °C in 1 ml aliquots
  0.5 ml 2.5 M Tris, pH 6.8 (250 mM)
  2 ml 20% SDS (8%)
  2 ml glycerol (40%)
  0.2 ml 1% bromphenol blue in 10% EtOH (0.04%)
  (Add 400 mM DTT = 0.3084 g if reducing conditions are desired)
  Fill to 5 ml with H_2O (~ 0.3 ml)

- **5X Tris-glycine running buffer** Store @ RT
  15.1 g Tris base (125 mM)
  94 g glycine (1.25 M)
  5 g SDS (0.5%)
  Volume to 1 L (don't pH)

- **10X transfer buffer** Store @ RT
  29 g Tris base (240 mM)
  144 g glycine (1.9 M)
  3.75 g SDS (0.375%)
  Volume to 1 L (don't pH)

- **Coomassie blue staining solution**
  0.1% w/v Coomassie blue R250
  40% methanol
  10% glacial acetic acid

- **Destaining solution**
  30% methanol
  10% glacial acetic acid

- **Longer destaining solution**
  5% methanol
  7.5% glacial acetic acid
  5% glycerol

- **TBS-T**
  100 ml 10× Tris-buffered saline (0.2 M Tris-HCl [pH 7.5], 1.37 M NaCl)
  1 ml Tween-20 (0.1%)
  Volume to 1 L (don't pH)

- **PBS-T**
  100 ml 10× phosphate-buffered saline (pH 7.4) (0.1 M Na_2HPO_4,
  18 mM KH_2PO_4, 27 mM KCl, 1.37 M NaCl)
  1 ml Tween-20 (0.1%)
  Volume to 1 L (don't pH)

- **High-stringency stripping buffer**
  6 ml 10% SDS (2%)
  0.75 ml 2.5 M Tris, pH 6.8 (62.5 mM)
  0.21 ml β-mercaptoethanol, 14.3 M stock liquid (100 mM)
  23.04 ml H_2O

- **Low-stringency stripping buffer**
  6 M guanidine-HCl in H_2O

TABLE A8-9 Solutions for Preparing Resolving Gels for Tris-glycine SDS-Polyacrylamide Gel Electrophoresis

⇓ Components / Gel Volume ⇒	Volume (ml) of Components Required to Cast Gels of Indicated Volumes and Concentrations							
	5 ml	10 ml	15 ml	20 ml	25 ml	30 ml	40 ml	50 ml
6% gel								
H_2O	2.6	5.3	7.9	10.6	13.2	15.9	21.2	26.5
30% acrylamide mix <!>	1.0	2.0	3.0	4.0	5.0	6.0	8.0	10.0
1.5 M Tris (pH 8.8)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
10% SDS	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
10% ammonium persulfate <!>	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED <!>	0.004	0.008	0.012	0.016	0.02	0.024	0.032	0.04
8% gel								
H_2O	2.3	4.6	6.9	9.3	11.5	13.9	18.5	23.2
30% acrylamide mix <!>	1.3	2.7	4.0	5.3	6.7	8.0	10.7	13.3
1.5 M Tris (pH 8.8)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
10% SDS	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
10% ammonium persulfate <!>	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED <!>	0.003	0.006	0.009	0.012	0.015	0.018	0.024	0.03
10% gel								
H_2O	1.9	4.0	5.9	7.9	9.9	11.9	15.9	19.8
30% acrylamide mix <!>	1.7	3.3	5.0	6.7	8.3	10.0	13.3	16.7
1.5 M Tris (pH 8.8)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
10% SDS	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
10% ammonium persulfate <!>	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED <!>	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02
12% gel								
H_2O	1.6	3.3	4.9	6.6	8.2	9.9	13.2	16.5
30% acrylamide mix <!>	2.0	4.0	6.0	8.0	10.0	12.0	16.0	20.0
1.5 M Tris (pH 8.8)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
10% SDS	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
10% ammonium persulfate <!>	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED <!>	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02
15% gel								
H_2O	1.1	2.3	3.4	4.6	5.7	6.9	9.2	11.5
30% acrylamide mix <!>	2.5	5.0	7.5	10.0	12.5	15.0	20.0	25.0
1.5 M Tris (pH 8.8)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
10% SDS	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
10% ammonium persulfate <!>	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED <!>	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02

Modified from Harlow and Lane (1988).

TABLE A8-10 Solutions for Preparing 5% Stacking Gels for Tris-glycine SDS-polyacrylamide Gel Electrophoresis

⇓ Components / Gel Volume ⇒	Volume (ml) of Components Required to Cast Gels of Indicated Volumes							
	1 ml	2 ml	3 ml	4 ml	5 ml	6 ml	8 ml	10 ml
H_2O	0.68	1.4	2.1	2.7	3.4	4.1	5.5	6.8
30% acrylamide mix <!>	0.17	0.33	0.5	0.67	0.83	1.0	1.3	1.7
1.0 M Tris (pH 6.8)	0.13	0.25	0.38	0.5	0.63	0.75	1.0	1.25
10% SDS	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1
10% ammonium persulfate <!>	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1
TEMED <!>	0.001	0.002	0.003	0.004	0.005	0.006	0.008	0.01

Modified from Harlow and Lane (1988).