Large Gel Shift (EMSA) Protocol

1. Set up gel plates:
   - Clean glass with water followed by ethanol. Setup the glass plate apparatus as described in the gel apparatus manual:
     a. Put the blue rubber gasket around the glass with the rounded edges so that the thick side of the gasket is facing up. Align the notches in the gasket with the rounded corners of the plate.
     b. Place the gray spacers just inside the gasket on the bottom plate. Rest the larger glass plate with the notched end on top of the spacers and put the plastic clamps on the glass plate sandwich. Put 3 clamps along the bottom and stand the plates up in a vertical position.

2. Gel percentage will depend on size of complex you wish to resolve. E.g. 5% was used to resolve 20-mer RNA (Krystle, full length UAP56) or 8% for an 11-mer (Bill, TNFα RNA for TIA-RRM123).

Notes:

- Mix the reagents (except TEMED) in an Erlenmeyer side-arm flask and de-gas the solution for 15 min while stirring

Stock	Final concentration	Volume for 28 cm gel
5x TBE (recipe below)	0.5x	10 mL
40% acrylamide/bis (37.5:1)	8%	20 mL
Water	-	69.2 mL
10% APS (100 mg/ml) Degas mixture under vacuum with stirring for 15 mins or more.	0.75 mg/mL	690 µL
TEMED Swirl to mix then pour as polymerization occurs quickly.		90 µL

- Final volume 100 mL for **one** gel

- Add TEMED and swirl to mix. Using a 25mL pipette, put the solution between the plates (while vertical) being careful not to create any bubbles. Be sure that the well comb is clean and readily available.
- Fill the plates to the top with acrylamide solution and insert the comb between the glass without trapping any air beneath the comb.
- Label each well with a sharpie marker to help with loading.

3. Let the gel set and pre-run the gel in cold box for 1 hour at 200 V in 0.5x TBE.

4. Meanwhile, prepare gel shift reaction.
   - No-BSA was added; since we are working with purified proteins (BSA is generally used with nuclear extracts)

- Superase-In is in the -20 degrees freezer (where the antibiotics are kept)
- Gel Plates are kept separately from the plates used for SDS-PAGE (in the same bench)

Stock	Final concentration	Volume
100 mM Hepes pH 7.4	10 mM	1 µL
80 mM MgCl2	8 mM	1 µL
0.5M KCl	50 mM	1 µL
50% glycerol	5%	1 µL
20 U/µL Superase*	1 U	0.5 µL
25 µM Fl-RNA*	2.5 µM	1 µL
Water		3.5 µL
Protein* 12.5, 25, 50, 100, 200 µM	 1.25, 2.5, 5.0, 10.0, 20.0 µM	1 µL
Final volume		10 µL per reaction

(Can also make a stock solution with 100mm Hepes pH7.4, 80mM $MgCl_2$, 0.5M KCl, 50% glycerol and water in the ratios used above and add 7.5uL to save time)

*Superase•In from Ambion, Inc/Applied Biosystems. Cat. # 2694 2500 U = ~$100.
*50µM Fl-RNA stock for brighter bands. This will require more protein.
*[Protein] ranges from 0.1-10x [Fl-RNA] in 2-fold dilutions. Ankit used: 2.5, 5, 10, 20 µM final concentrations of protein with 5 µM final [Fl-RNA]. U2AF-RRM123 saturates AdML at 4:1Protein:RNA molar ratio.

5. Incubate gel shift reaction at 30°C for 20 minutes. The reactions then can be kept at room temperature. Incubation times and temperatures will vary between proteins. Proteins with higher affinities may be incubated for shorter times or at lower temp.

6. Load gel the pre-run gel in 0.5x TBE. Include one lane of agarose gel loading dye as a running marker and one lane with just protein if you plan on staining with Coomassie as well. Load quickly to limit diffusion of the sample which will result in fuzzy bands.

7. Run in cold box until orange dye reaches bottom of gel / xylene cyanol dye (light blue) reaches half-way down gel. This may take over 5 hours. The degree of separation required will be determined by the sizes of the macromolecules and the number of bands.Ankit found a constant current of 25 mA, ~250 V worked (maybe ~20mA, 200 V better?). Voltage and time will need to be optimized on a case-by-case basis.

8. Use Storm phosphorimager to look at fluorescein fluorescence in gel. Located in room across from Joe's lab. You can scan the gel without removing it from the glass plates. For imaging fluorescein, use blue fluorescence (Excitation wavelength = 490 nm).

A. Login as: kielkopf Password: cklab
B. Click on MDSCANSI.exe
C. Select scanner as STORM860-Blue Fluorescence/Chemifluorescence

D. In Scanner control, select the area where the gel is placed by looking at the position on the glass edges in the imager. The top-middle seems to have the fewest scratches on the glass surface.
E. Click on setup to change the PMT voltage (>700 V is generally good, can take scans up to 1000 V)
F. Click on scan to write the file name for the scan. The data can be saved in My documents/ck_data folder and later transferred to a USB drive.
G. Adjust the intensity and background in ImageQuant using the slide-bar saturation tool.
H. Remove the gel and clean the glass with water using a kimwipe.
I. Log off the computer.

7. Stain/destain gel as usual if you want to visualize the protein. The gel can be folded up into a 1mL pipette tip box to save stain or spread out in a larger plastic container.

8. Wash glass plates and the plastic comb. Rub with ethanol (95% for plastic plate) to finish.

Recipe for 5x TBE (Maniatis):

What:	**How much:**
Tris Base (MW 121.1)	54 g
Boric acid (MW 61.8)	27.5 g
0.5 M EDTA pH 8.0	20 mL
Water	To 1 L final volume.