EMSAs

Probe Preparation:

1. Make 250uM dNTP mix (store extra at -20°C): 10ul 1mM dATP + 10ul 1mM dCTP + 10ul 1mM dGTP + 10ul 1mM dUTP(Cy5.5 labeled, from Lumiprobe)
2. For 25ul reaction: 150ng DNA fragment + 6ul dNTPs + 2.5ul 10x NEB buffer 2 + 1ul Klenow exo- (NEB, 5U/ul). Add water to 25ul
3. Incubate at 37°C for 45 minutes
4. Add 5ul sheared E.coli DNA and mix. Save 1ul of this for gel analysis.
5. Prepare P-30 spin column: https://www.bio-rad.com/sites/default/files/webroot/web/pdf/lsr/literature/4006051.pdf
6. Flow through P-30 spin column

In vitro transcription/translation with RRLs:

- Thaw components on ice (quick thaw the RRL in your hand and put on ice)
- Per reaction: 20ul RRL + 0.5ul Methionine +1ul PCR enhancer + 1ul plasmid + 2.5ul water
    - Adding the enhancer: final concentration of $Mg(OAc)_2$ will be 0.5mM, KCl will be 20mM
    - If doing a labeled reaction to quantify expression, add 1ul Transcend tRNA and only add 1.5ul water
- Make a master mix of RRL+Met+enhancer and add 21.5ul to each reaction tube
- Add water, tRNA, and plasmid (include a no DNA condition for RRL background)
- Incubate at 30°C for 60-90 minutes (no observable difference in expression within this range)
- Flash freeze the samples if you're not immediately using them for an experiment

Quantification of labeled translation products:

- Add 2ul labeled reaction to 15ul 1x laemmli buffer and heat for 15 minutes at 70°C
- Run a Bis-Tris gel (load entire sample)
- Transfer onto nitrocellulose or PVDF, 90V for 60 minutes at 4°C
- Block for 45-60 minutes in TBST
- Add 6ul Strep to 15mL TBST, add to the membrane and incubate for 45-60 minutes
- Wash two times for 1 minute in TBST, then two times for 1 minute in water
- Incubate the membrane in Western Blue Stabilized Substrate for Alkaline Phosphatase in the dark, until bands appear. Quench the reaction with water. Take an image and protect from light if you want to keep the membrane.

EMSA

- Thaw components at room temperature and transfer to ice
    - 80% glycerol: allows sample to sink in the gel
    - 1ug/ul sheared E.coli DNA: nonspecific competitor that will absorb DNA binding proteins in the crude lysate
    - 150mM Tris-HCl pH 7.5: optimal pH
    - 100ng/ul b-casein: blocks non-specific protein binding
    - Labeled probe (~25nM)
    - 20mM EDTA: quenches enzymes in RRL, optimizes protein-DNA binding
    - 2M NaCl

- Make master mix

	[Final]	Vol (uL) per rxn	MM 20X
80% Glycerol	4%	0.5	10
1µg/µl sheared coli DNA	25ng/uL	0.5	10
150 mM Tris-HCl pH 7.5	15mM	1	20
100 ng/µl b-casein	2.5ng/uL	0.25	5
labeled probe (~25nM)	0.5nM	0.25	5
20mM EDTA	1mM	0.5	10
2M NaCl	100mM	0.5	10
TRF2/no DNA in vitro translation (last)		0.5, 1, 2.5	
dH2O (for No DNA)		6.5	
dH2O (for 0.5uL Protein)		6	
dH2O (for 1uL Protein)		5.5	
dH2O (for 2.5uL Protein)		4	

- Make 0.8% agarose gel in 0.1X TBE using a 20-well comb
- Add appropriate volume of water to each reaction tube
- Add 3.5ul master mix to each tube
- Add RRL protein samples last, and gently tap to mix
- Move samples to room temperature and incubate for 20 minutes
- Return samples to ice and load all 10ul in gel. Add 2ul orange G to the probe alone condition to track where the free probe is running.
- Run the gel at 200V for 40 minutes
- Image using fluorescent R-short on Typhoon scanner