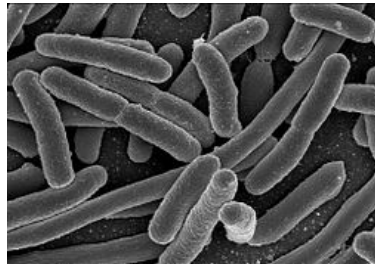


# TX-TL preparation overview

TX-TL workshop

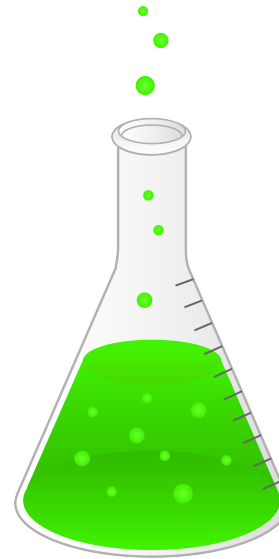
August 29, 2013

# What makes up TX-TL?



E. coli extract

+



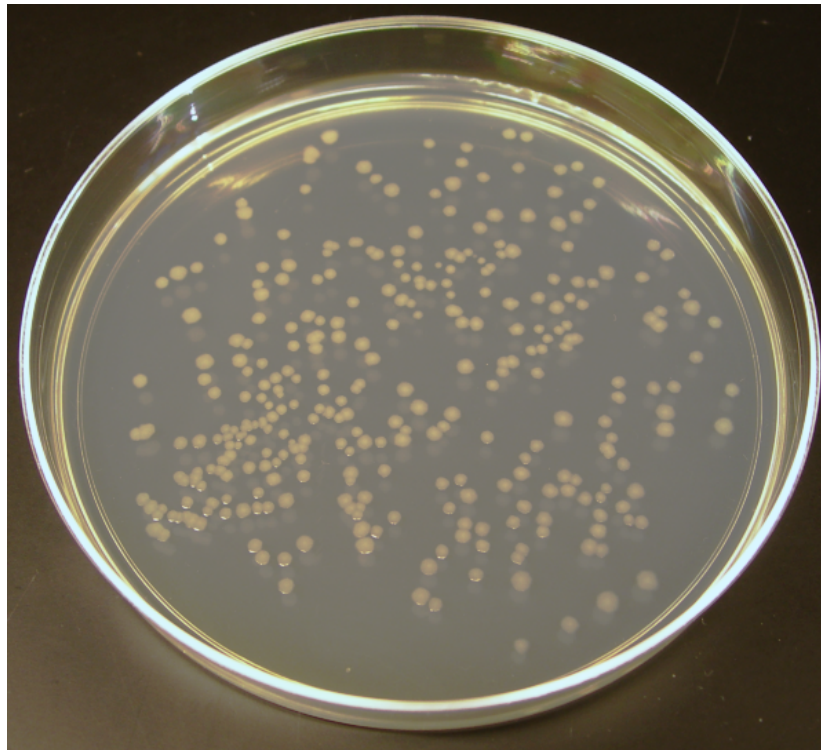
Buffer  
(Energy solution + Amino Acids)

# Extract Preparation Summary

- Streak out colonies
- Grow up cultures
- Pellet and wash cells
- Lyse cells through bead-beating
- Filter to remove beads
- Spin to remove cell debris
- Incubate at 37C to remove endogenous DNA and RNA
- Spin down to remove debris
- Dialyze to exchange buffer

# Day 1: Streak out cells

- Streak out or spread from frozen stock *E. coli* cells, strain BL21 Rosetta, on a 2xYT + chloramphenicol plate.



## Day 2: Mini-cultures

- 2 pm: Add 4 mL of 2xYT + phosphates + chloramphenicol into a culture tube. Inoculate with a few colonies (1-3). Incubate shaking at 220 rpm and 37C for 8 hours.
- 10 pm: Add 100 uL of the first mini-culture to a flask with 50 mL of 2xYT + phosphates + chloramphenicol. Incubate shaking at 220 rpm and 37C for 8 hours.

## Day 3

- 6 am: Pour 660 mL of 2xYT + phosphates into 6 x 4L flasks. Add 6.6 mL of mini-culture 2 into each flask. Incubate shaking for 3-3.5 hours, until an OD of 1.5 – 2.
- Transfer cultures into centrifuge bottles, and spin for 12 minutes at 5000g and 4C.
- Pour out supernatant and wash the pellet by adding 200 mL of S30 buffer A (Mg and K glutamate, pH 7.7). Shake until the cells are completely resuspended.
- Spin down again. Repeat twice.
- Weight 4 x 50 mL Falcon tubes. Transfer pellets into tubes, and weigh to find the pellet mass.

# Bead-beating calculations

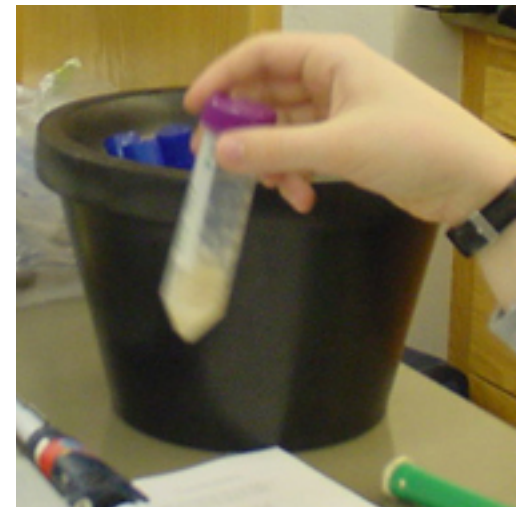
	falcon			
	1	2	3	4
Empty 50mL falcon (g)				
50mL falcon with pellet (g)				
Pellet mass ( <b>50mL falcon with pellet – empty 50mL falcon</b> ) (g)				
S30A buffer volume to add ( <b>pellet mass* 0.9</b> ) (mL)				
Total mass of beads to add ( <b>pellet mass * 5.0</b> ) (g)				



<http://wordpress.com>



<http://ricfacility.byu.edu>

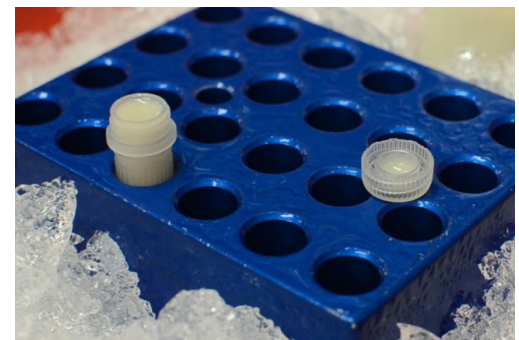


<http://web.mit.edu>

- Add 0.9\*pellet mass of S30A buffer (mL) to each Falcon tube and vortex.
- Add 5\*pellet mass of beads to each Falcon tube, in 3 aliquots. Vortex.
- Using a pipette with the tip cut off, add the mixture into bead-beating tubes. Fill the tubes up completely and avoid air bubbles.
- Beat each tube for 30 seconds at 4600 rpm. Place them upside down on ice and wait 30 seconds before beating again.



<http://static.coleparmer.com>





# Construct Filter Contraption

- Remove the cap of the beat tube and replace it with a filter.
- Place a clean tube onto the end of the filter.
- Put the cap of the clean tube into the bottom of a 15 mL Falcon tube.
- Place the tube + filter upside down in the Falcon tube.
- Centrifuge for 6000g at 4C for 5 minutes.

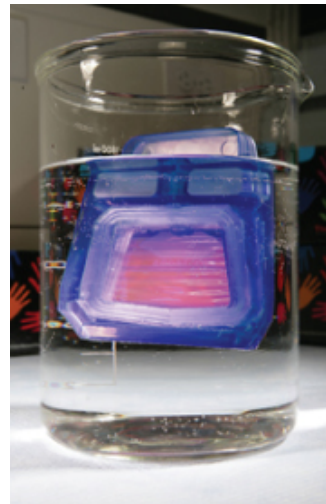
# Spins and Incubation

- Remove the filter and transfer the supernatant inside the tubes into clean tubes. Discard pellet.
- Centrifuge the supernatant at 12000g for 10 minutes at 4C.
- Put supernatant into clean tubes, 500 uL each. Discard pellet.
- Place tubes without caps into 15 mL culture tubes. Incubate for 80 minutes at 37C and 220 rpm.



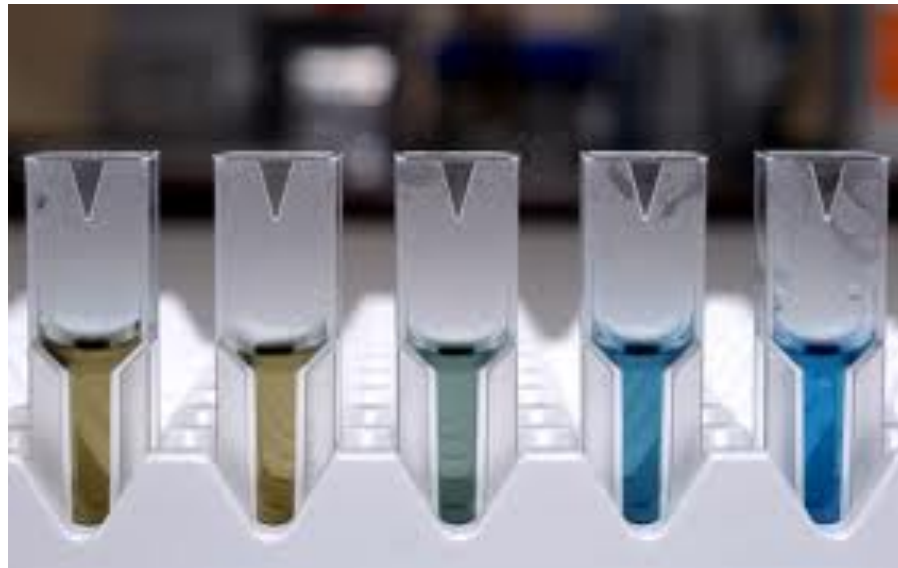
# Dialysis

- Remove tubes from incubator and consolidate extract into 1.5 mL micro-centrifuge tubes. Spin at 12000g for 10 minutes at 4C.
- Transfer supernatant into a 15 mL Falcon tube and invert to mix.
- Using a syringe transfer supernatant into 10K MWCO dialysis cassettes.
- Place cassettes into beakers with 1L S30B buffer (pH 8.2). Dialyze for 3 hours at 4C, stirring.



# Bradford Assay

- Before dialyzing save a small sample of extract (10 uL).
- Determine protein concentration with a Bradford Assay.
- The protein concentration will determine the volume of the final extract aliquots.



# Spin and Aliquot

- After dialysis remove extract from the cassettes and aliquot into micro-centrifuge tubes.
- Spin at 12000g for 10 minutes at 4 C.
- Transfer supernatant into a 15 mL Falcon tube and invert to mix.
- Aliquot extract into small tubes and flash freeze with liquid nitrogen.

# Amino Acids

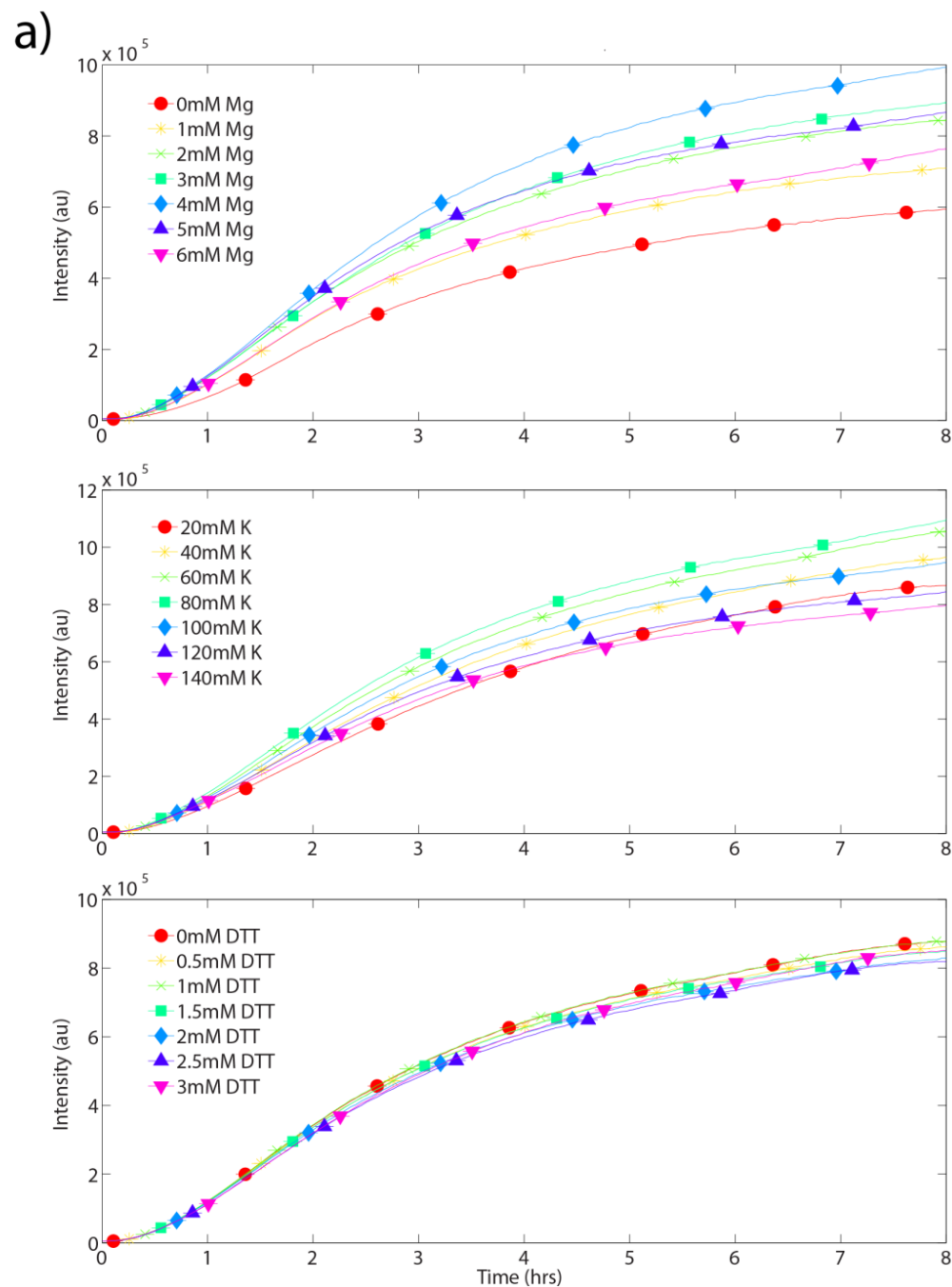
- Use the 5 Prime RTS Amino Acid Sampler.
- Mix the Amino Acids together and flash freeze in small aliquots.



<http://www.5prime.com>

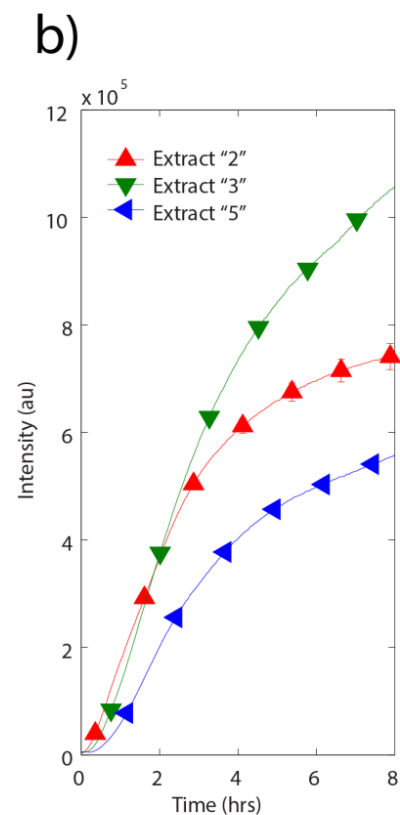
# Energy Solution

Name	Concentration	Amount	Sterilization	Notes
HEPES	2M, pH 8	4mL	None	To reach pH 8, titrate with KOH.
Nucleotide Mix	156mM ATP and GTP, 94mM CTP and UTP, pH 7.5	1.5mL	None	To reach pH 7.5, titrate with KOH.
tRNA	50 mg/ml	600uL	None	
CoA	65mM	600uL	None	
NAD	175mM, pH 7.5-8	300uL	None	To reach pH 7.5-8, titrate with Tris at 2M.
cAMP	650mM, pH 8	200uL	None	To reach pH 8, titrate with Tris at 2M.
Folinic Acid	33.9mM	300uL	None	Although only 300uL is needed, recipe in supplemental is for 1.15mL.
Spermidine	1M	150uL	None	Store at 4C.
3-PGA	1.4M, pH 7.5	3.2mL	None	To reach pH 7.5, titrate with Tris at 2M.

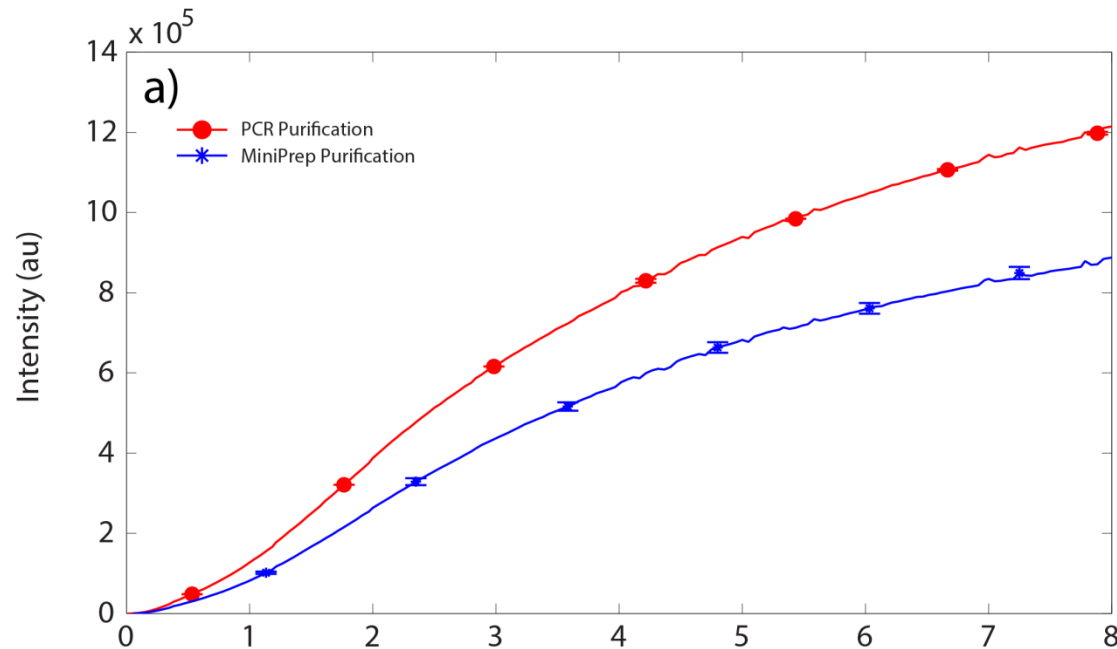


a) Mg, K and DTT calibration curves. Final buffer composition is adjusted to the concentrations that give max expression.

b) Variability between 3 different extract batches.

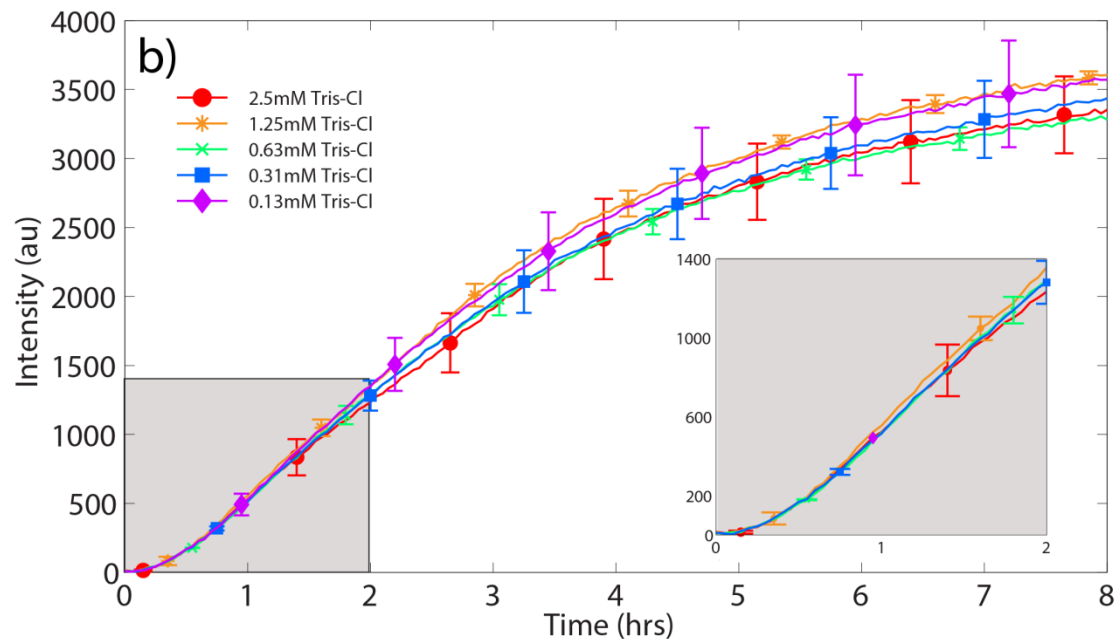






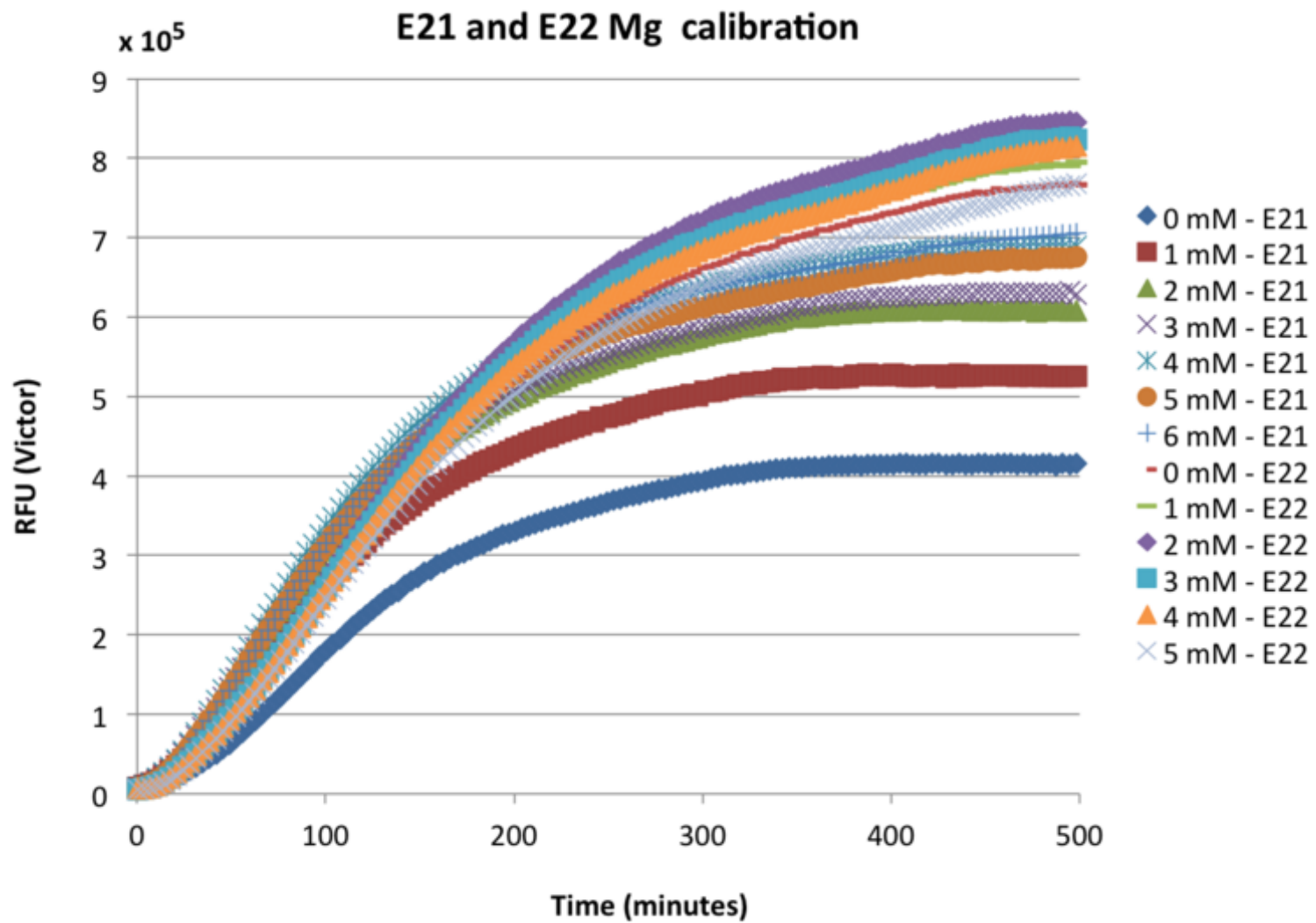
Why we always PCR purify our miniprep products:

a) The blue curve shows expression in TX-TL of miniprepplasmids, while the red curve shows expression of plasmids that have been both miniprepplasmids and PCR purified.



Does eluting in EB vs. water affect expression?

b) An assay of TX-TL expression with varying EB (Tris-Cl) concentrations. EB concentration has little to no effect on expression.

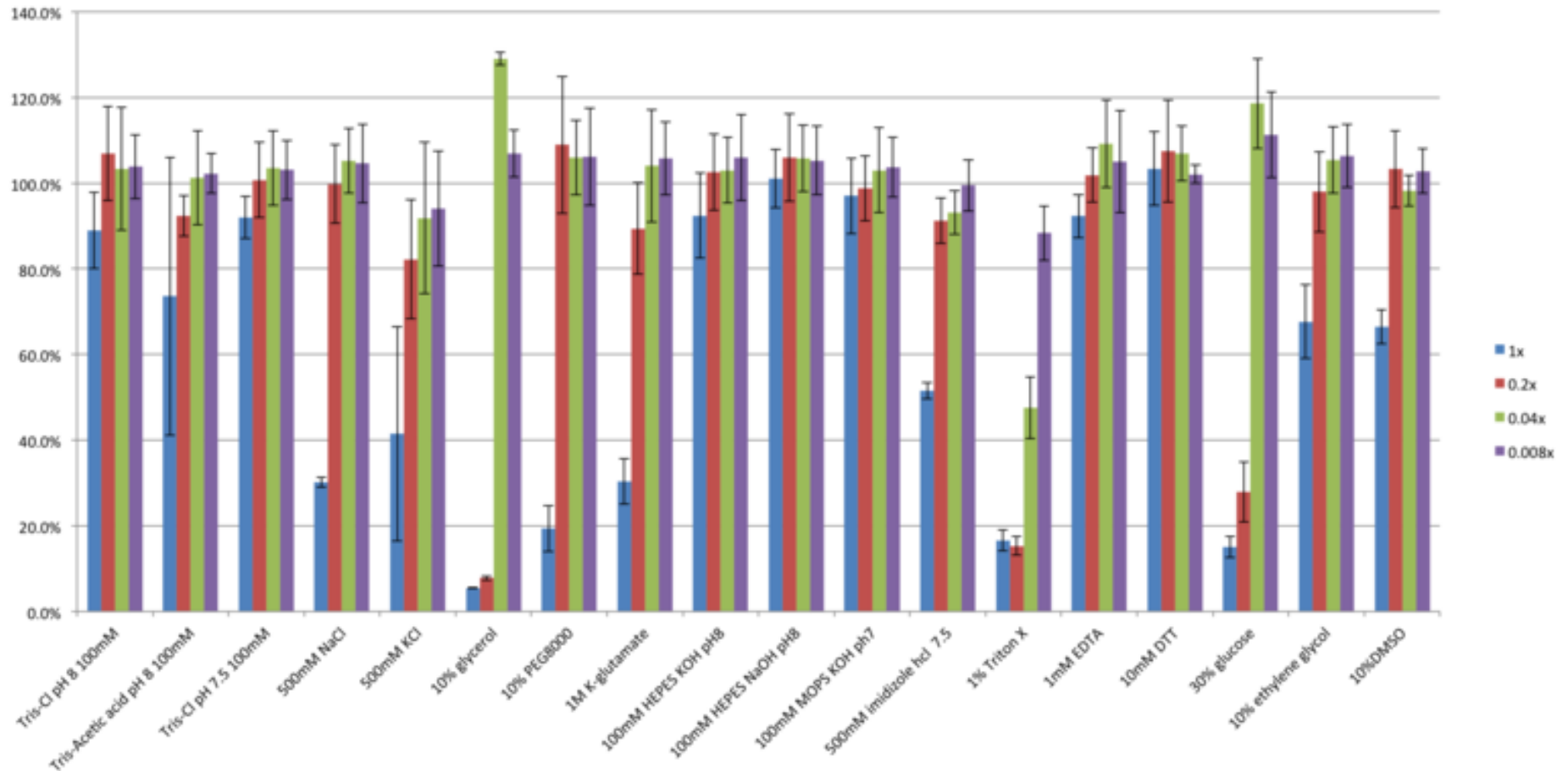


Extract variability:

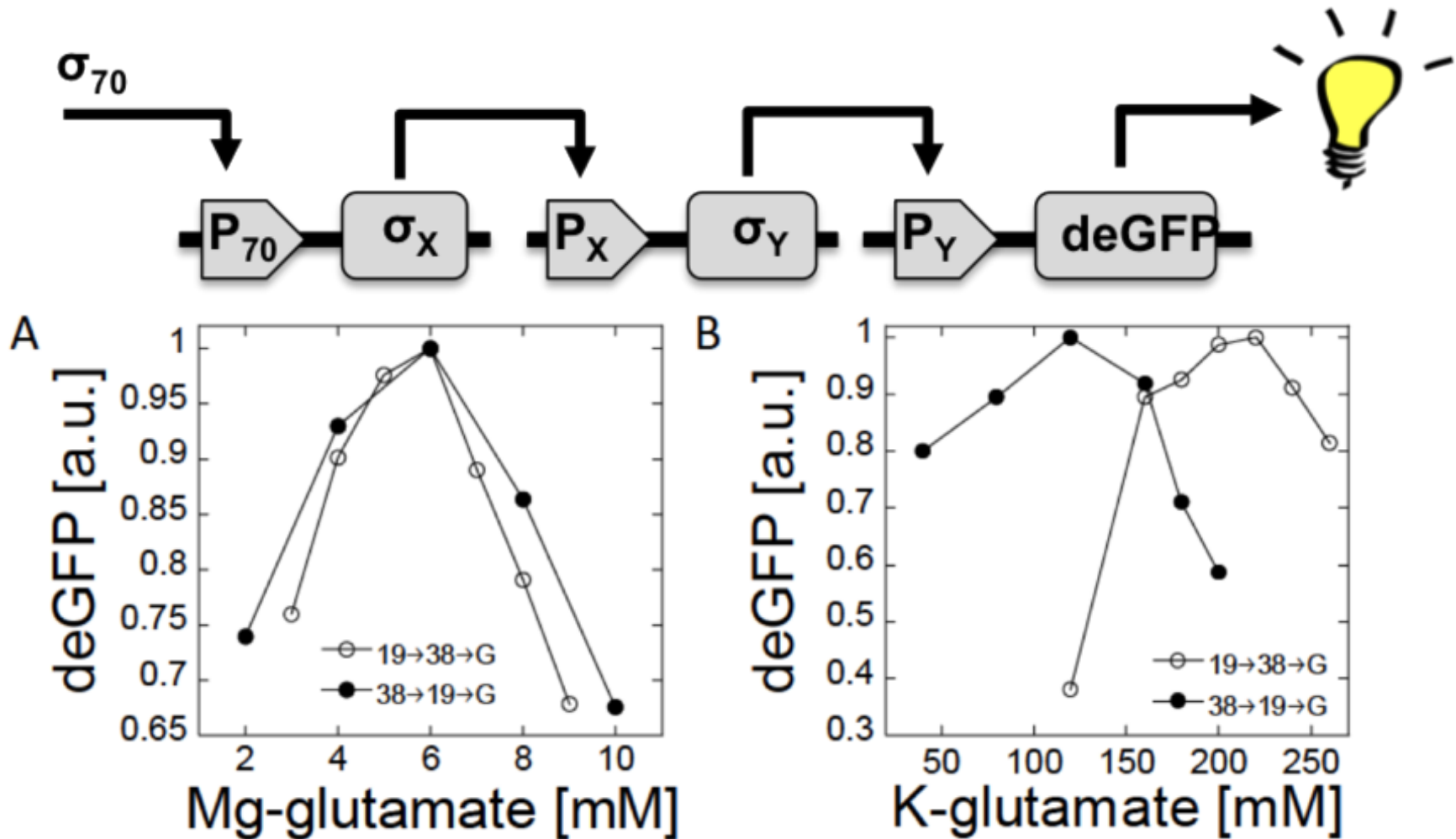
E21 – lower final signal, faster initial rate

E22 – higher final signal, slower initial rate

# Toxicity Assay



Effects of different additives on TX-TL efficiency. A variety of different additives commonly used in protein buffers was tested for toxicity. 25% of the reactions containing the above additive was added to positive control and endpoint fluorescence after 8 hours was determined – percentages are against a control with no additive internal to the experiment. Error bars represent one standard deviation from three independent experiments.



Response of two two-stage transcriptional activation cascades as a function of magnesium and potassium. Top: schematic of the circuit where x and y are either  $\sigma_{19}$  or  $\sigma_{38}$ . Bottom: response of the two circuits as a function of magnesium (A) and potassium (B).

# Supplemental Recipes

- 2xYT+P+Cm agar plate: Prepare 1.24g 2xYT, 1.6mL potassium phosphate diabolic solution @1M, 0.88mL potassium phosphate monobasic solution @1M, 0.6g agar, and water to 40mL. Autoclave. Let cool to 50C and add 40uL Cm. Aliquot 25mL into a 100x15mm petri dish, and let cool for an hour.
- 2xYT+P media: Prepare 124g 2xYT, 160mL potassium phosphate diabolic solution @1M, 88mL potassium phosphate monobasic solution @1M, and water to 4L. Aliquot out into 2x1.88L and 0.24L. Autoclave.
- Tris base, 2M: Prepare 60.57g Tris base and water to 250mL. Sterilize, store at RT for later use.
- DTT, 1M: Prepare 2.31g DTT and water to 15mL. Filter sterilize (0.22uM), aliquot to 1mL tubes, store at -20C for later use.
- S30A buffer: Prepare 10.88g Mg-glutamate and 24.39g K-glutamate, 50mL Tris at 2M, acetic acid (to pH 7.7), and water to 2L. Autoclave, store at 4C, add 4mL 1M DTT before use.
- S30B buffer: Prepare 10.88g Mg-glutamate and 24.39g K-glutamate, Tris at 2M (to pH 8.2), and water to 2L. Autoclave, store at 4C, add 2mL 1M DTT before use.