Supplementary Information for:

Deconstructing cell-free extract preparation for *in vitro* activation of transcriptional genetic circuitry

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SUPPLEMENTARY FIGURES

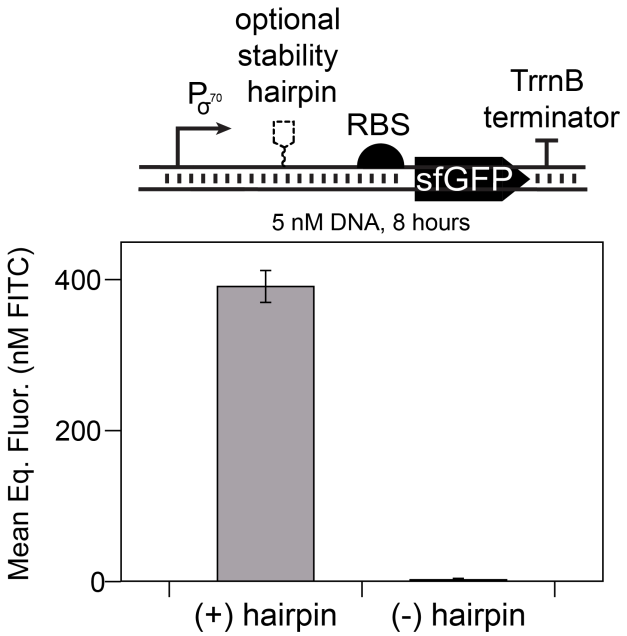


Figure S1. Including a 5' stability hairpin is necessary for efficient cell-free gene expression from a bacterial promoter. Comparison of eight-hour endpoint fluorescence from a reporter template plasmid with a bacterial promoter shows no sfGFP production unless the PHP14 stability hairpin[67] is included in the 5' untranslated region. Error bars represent standard deviation of the mean from three independent reactions drawn from a single batch of unprocessed extract.

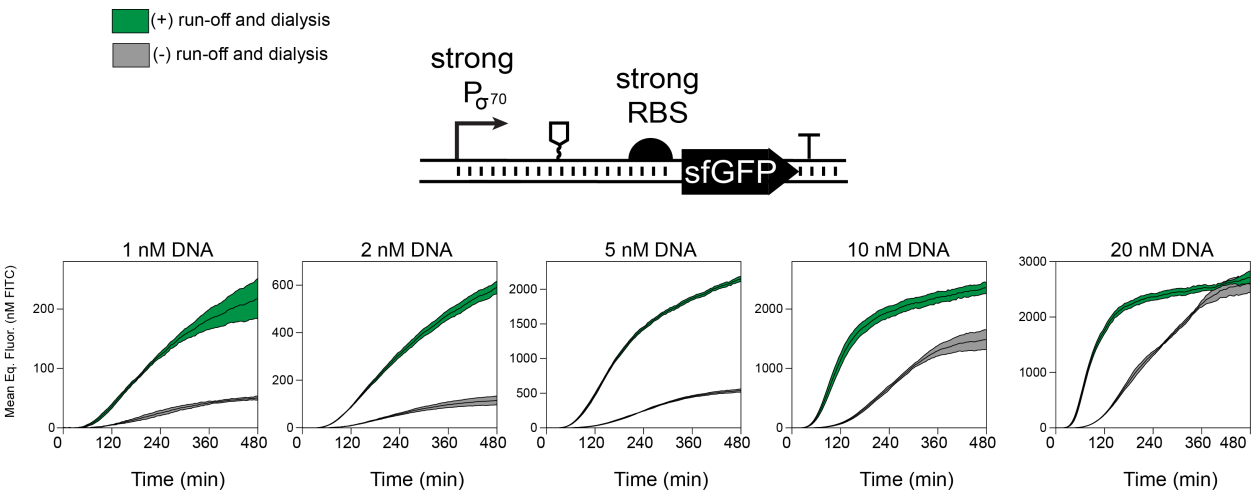


Figure S2. Kinetics of cell-free gene expression as a function of DNA concentration from Figure 2c. Shaded area represents plus-minus one standard deviation of the mean from three technical replicates drawn from a single batch of extract. All y-axes are individually scaled to their final values.

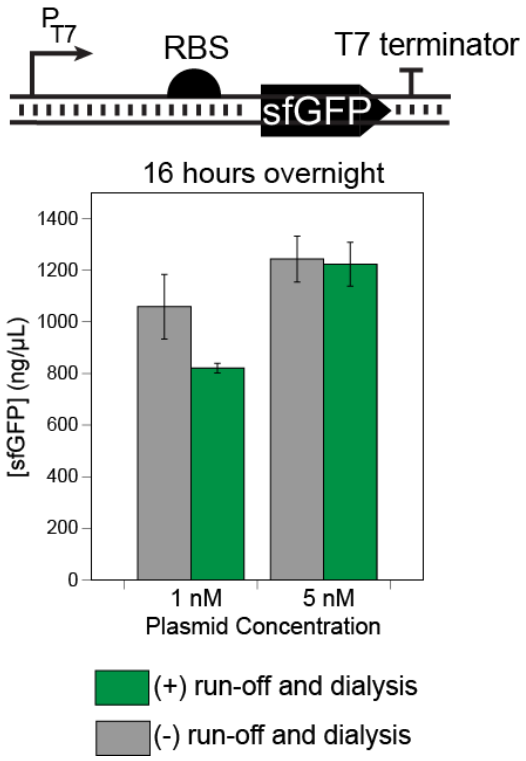


Figure S3. Additional reporter DNA supplemented to a cell-free gene expression reaction weakly improves sfGFP production when expressed from a T7 promoter. When sfGFP production is driven from a T7 promoter, increasing plasmid concentration from 1 nM to 5 nM increases final protein titer by 17% in the non-processed lysate and 49% in the processed lysate. Experiments were performed overnight at 30ºC and sfGFP yield was correlated to a known fluorescent calibration standard. Green represents postlysis processed extracts and gray represents non-processed extracts. Error bars represent the standard deviation of the mean across three technical replicates drawn from a single batch of extract.

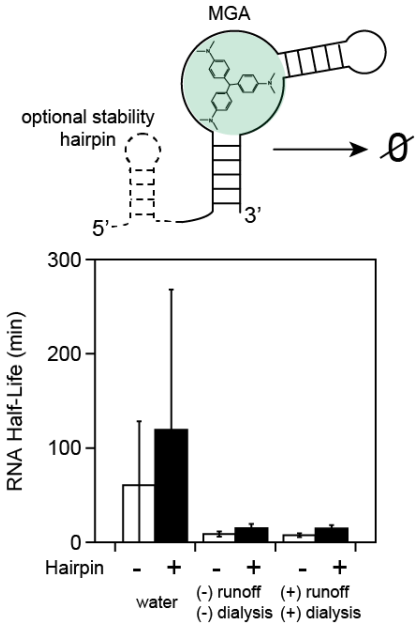
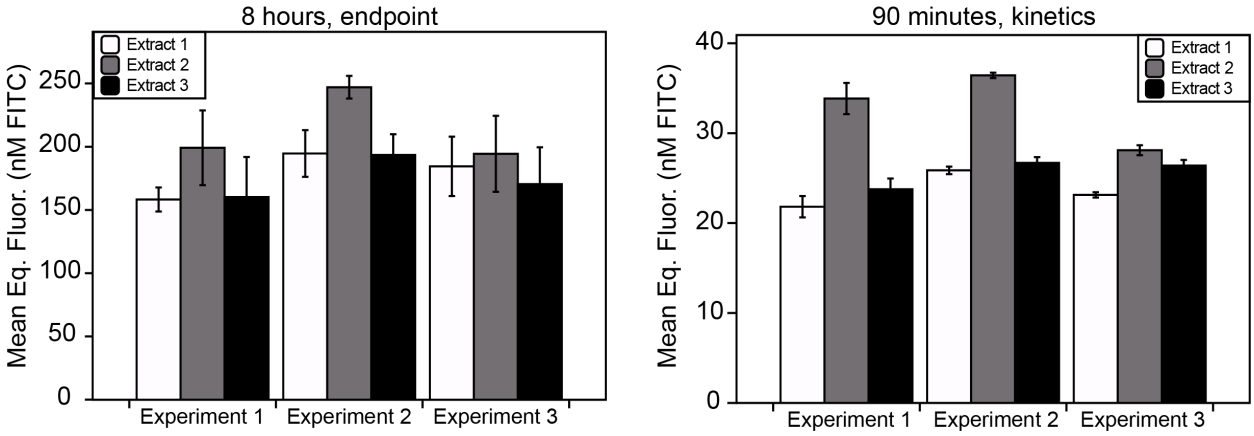


Figure S4. The 5' stability hairpin improves half-life of RNAs in extract. The malachite green RNA aptamer was purified with and without the pHP14 5' stability hairpin, mixed with the malachite green dye and 10% extract by volume and the decay in fluorescence over time was measured in a plate reader to indicate RNA degradation (see Figure 3C). The RNA half-life was estimated by fitting an exponential decay function to the fluorescence kinetics over the first 30 minutes. Errors in half lives are propagated from three independent measurements and reported as standard deviation.

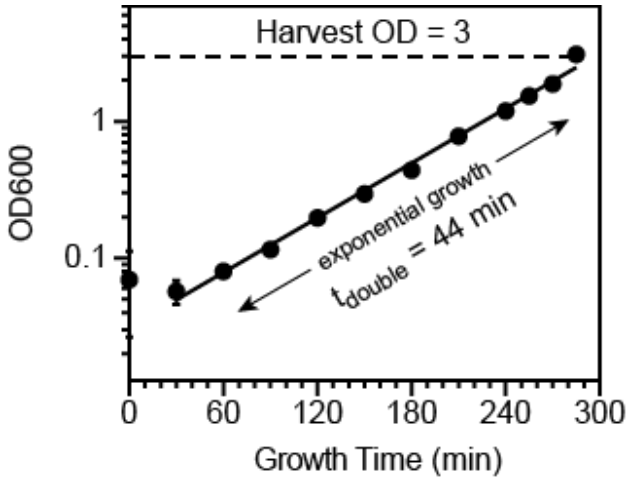
A



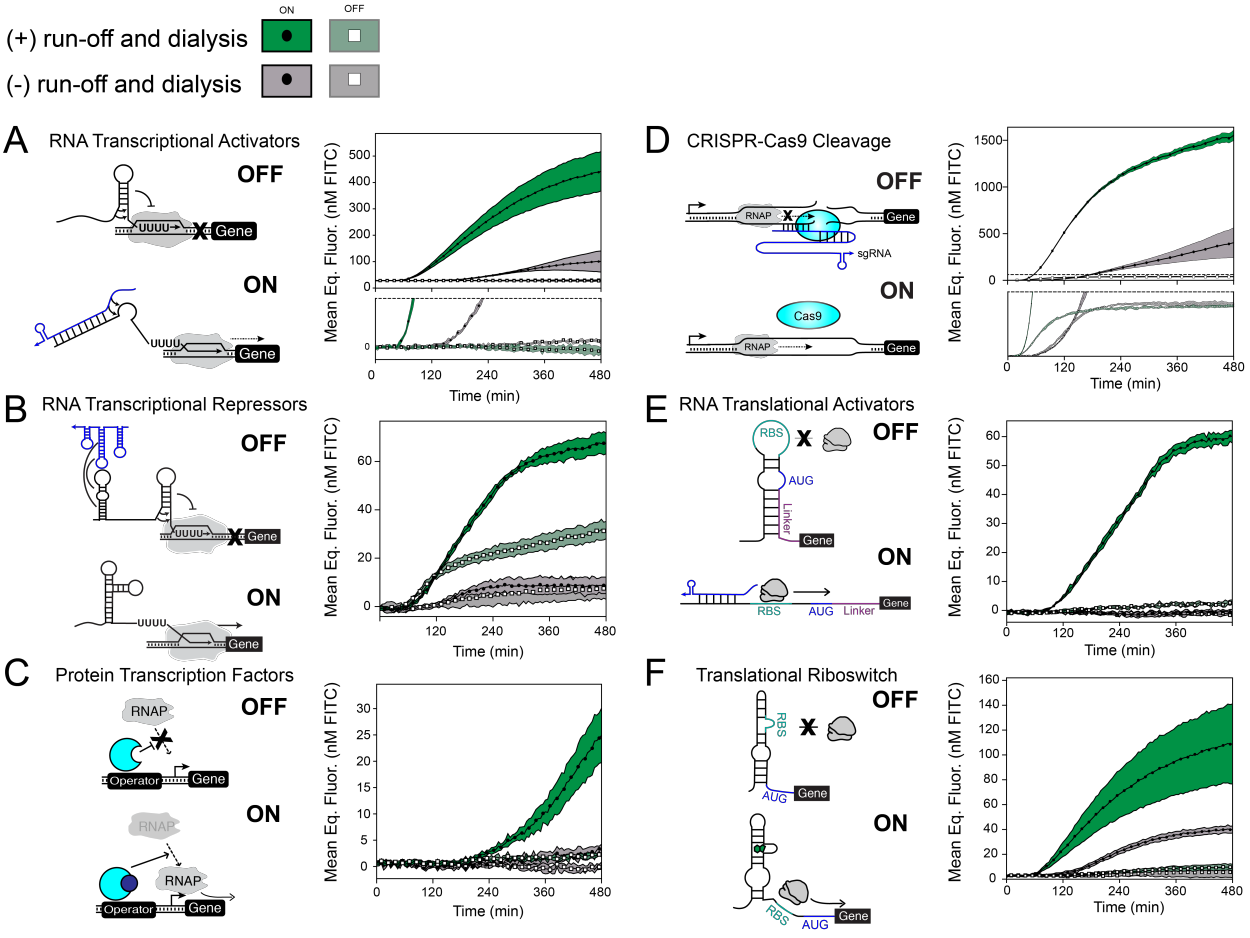
B

p value				Experiment, Endpoint								
				1			2			3		
				Extract			Extract			Extract		
				1	2	3	1	2	3	1	2	3
Experiment	1	Extract	1		0.090	0.193	0.149	0.001	0.177	0.479	0.077	0.362
			2	0.090		0.376	0.452	0.044	0.497	0.206	0.482	0.264
			3	0.193	0.376		0.359	0.051	0.404	0.287	0.385	0.363
	2	Extract	1	0.149	0.452	0.359		0.121	0.457	0.210	0.438	0.263
			2	0.001	0.044	0.051	0.121		0.108	0.051	0.031	0.054
			3	0.177	0.497	0.404	0.457	0.108		0.239	0.491	0.299
	3	Extract	1	0.479	0.206	0.287	0.210	0.051	0.239		0.210	0.412
			2	0.077	0.482	0.385	0.438	0.031	0.491	0.210		0.269
			3	0.362	0.264	0.363	0.263	0.054	0.299	0.412	0.269	

Supplementary Figure S5. Batch and experimental effects contribute roughly equally to variation in CFE yields in transcriptionally-limiting conditions. (A) Three independent extracts (1-3) were prepared identically on consecutive days and tested under transcriptional limitations (1 nM template DNA with a strong bacterial σ^{70} promoter and RBS. 10 µL sfGFP synthesis experiments were conducted for 8 hours in a plate reader at 30ºC. Fluorescence at endpoint (left) and 90 minutes (right) are reported. Two-factor ANOVA analysis on measured endpoint fluorescence values shows p = 0.030 for extract variability (comparing extracts across experiments) and 0.026 for experimental variation (comparing experiments across extracts). (B) Pairwise two-tailed t tests between all combinations of experiment and extracts showing difference between mean endpoint fluorescence values. All statistically significant ($p<0.05$; red text) differences involve Experiment 2, Extract 2, which had the highest measured value.



Supplementary Figure S6. Rosetta2 (DE3) pLysS in 2X YTP media grows at exponential phase at the harvest optical density. The observed slower doubling time for Rosetta2 compared to the source strain BL21 is likely a consequence of the plasmid burden. Bacterial growth was monitored by measuring the optical density at 600 nm on a spectrophotometer every thirty minutes and every fifteen minutes after four hours across two biologically independent growth cultures. Error bars represent one standard deviation of the mean.



Supplementary Figure S7. Kinetics of genetic circuitry in processed and unprocessed lysates. Expanded kinetic results from Figure 5. Circuit conditions are presented in Supplementary Table 1. Shaded area represents plus-minus one standard deviation of the mean from three technical replicates drawn from a single batch of extract.

SUPPLEMENTARY TABLE

Supplementary Table 1. Plasmid identities and reaction concentrations used in this study. Refer to the Supplementary Plasmids used in this study for sequence information.

Figure	Plasmid 1	Conc. (nM)	Plasmid 2	Conc. (nM)	Plasmid 3	Conc. (nM)	Reaction Notes
1B-C	pJBL7023	5	pJBL7010	5			T7 RNAP supplemented for pJBL7023; run at 12 mM Mg for T7 expression
2A	pJBL7010	1	pJBL7011	1	pJBL7016	1	
2B	pJBL7010	1	pJBL7017	1	pJBL7020	1	
2C-D	pJBL7010	0, 1, 2, 5, 10, 20					
3A	pJBL7007	20					100 µM malachite green dye
3B	pJBL7004	20					100 µM malachite green dye
3C	N/A (purified from pJBL7004 and pJBL7005)	2200 (purified RNA)					100 µM malachite green dye
4A-C	pJBL7010	1					
5A	pJBL5816	1	pJBL5817	8	pJBL002	8	
5B	pJBL7022	2	pJBL004	8	pJBL002	8	
5C	pJBL2812	5	pJBL2814	20			AHL 100 nM
5D	pJBL7010	5	pJBL632	5	pJBL623	5	purified SpyCas9 at 50 nM
5E	pJBL3859	1	pJBL3860	4	pJBL002	4	Reaction run at 12 mM Mg
5F	pJBL7008	5					theophylline 2 mM
S1	pJBL7010	5	pJBL7022	5			
S2	pJBL7010	0, 1, 2, 5, 10, 20					
S3	pJBL7022	1, 5					
S4	N/A (purified from pJBL7004 and pJBL7005)	2200 (purified RNA)					100 µM malachite green dye
S7	pJBL7010	1					
S7A	pJBL5816	1	pJBL5817	8	pJBL002	8	

S7B	pJBL7022	2	pJBL004	8	pJBL002	8	
S7C	pJBL2812	5	pJBL2814	20			AHL 100 nM
S7D	pJBL7010	5	pJBL632	5	pJBL623	5	purified SpyCas9 at 50 nM
S7E	pJBL3859	1	pJBL3860	4	pJBL002	4	Reaction run at 12 mM Mg
S7F	pJBL7008	5					theophylline 2 mM

Supplemental Note: Supplemental Extract Preparation Protocol

Day 1: (5 minutes)
1. Streak a fresh plate of the host bacterial strain onto a fresh plate (with antibiotic if appropriate). We typically use Rosetta2 (DE3) pLysS with chloramphenicol at 25 µg/mL, but have observed that generally any derivative of a protein production strain (*e.g.*, BL21) will work.

Day 2: (1 hour)
2. Prepare and autoclave one liter of 2X YT + P media (16 g tryptone, 10 g yeast extract, 5 g sodium chloride, 7 g potassium phosphate dibasic, 3 g potassium phosphate monobasic, and water to one liter).

3. Prepare and autoclave 200 mL of wash buffer (50 mM Tris base, 14 mM magnesium glutamate, 60 mM potassium glutamate) and pH to 7.7 using acetic acid.

4. Prepare and autoclave 600 mL of dialysis buffer (5 mM Tris base, 14 mM magnesium glutamate, 60 mM potassium glutamate) and pH to 8.2 using Tris.

5. Separately autoclave two 1L Nalgene centrifuge bottles (ThermoFisher 3120-1000) and lids, a stir bar, a spatula, and a one-liter beaker.

6. Sixteen hours before the beginning of the culture prep on Day 3, inoculate a 30 mL starter culture from the plate with antibiotic as appropriate into a 250 mL flask and grow overnight.

Day 3: (12 hours, or 6 + 6 hours split across two days)
7. Thirty minutes before inoculation, warm up the 2X YT+P to 37°C in an incubator.

8. After the 16-hour growth culture is complete, remove 20 mL of the 2X YT+P media to serve as the blank for measuring optical density. Then, inoculate the 1L growth culture with 20 mL of the saturated starter culture with antibiotic as appropriate.

9. During the growth phase, chill all centrifuge rotors (ThermoScientific F14-14x50cy and ThermoScientific F10-4x1000 LEX), buffers, centrifuge bottles, and glassware to 4°C, and thaw 1.1 mL of 1M DTT. Grow to optical density (OD600) 3.0 ± 0.2 at 37°C and shaking at 200 rpm, which should require around 3.5-5 hours depending on the strain.

10. Upon reaching the harvest optical density, immediately divide the cell culture into two centrifuge bottles on ice, balance, and centrifuge (ThermoScientific Sorvall Lynx 4000 Centrifuge) for 15 minutes at 5000XG at 4°C. From this point forward, keep the cells on ice at all times.

11. During the first spin, add 400 µL of 1M DTT to the wash buffer so it is at a final concentration of 2 mM. Weigh and record the mass of two clean 50 mL Falcon tubes. Keep on ice.

12. At the end of the spin, carefully decant the supernatant, taking care not to disturb the pellet. Using the spatula, transfer each pellet to a 50 mL Falcon tube, wiping the wet pellet on the side of the Falcon tube to maximize exposed surface area.

13. Resuspend each cell pellet in 25 mL of wash buffer, vortexing each tube for 20 seconds at a time until the suspension is homogeneous and no solid is visible.

14. Balance the Falcon tubes and centrifuge for 10 minutes at 5000XG at 4°C.

15. At the end of the second spin, carefully decant the supernatant and resuspend each pellet in 25 mL of wash buffer, again vortexing in 20 second intervals. Once homogeneous, balance the two Falcon tubes and centrifuge for 10 minutes at 5000XG at 4°C.

16. At the end of the third spin, carefully decant the supernatant and resuspend each pellet in 25 mL of wash buffer, again vortexing in 20 second intervals. Once homogeneous, balance the two Falcon tubes and centrifuge for 10 minutes at 7000XG at 4°C.

17. At the end of the fourth spin, carefully decant the supernatant. Wipe all exposed surfaces of the tube and dry any residual liquid inside the tube. Weigh each pellet. If harvested around OD 3.0, each pellet should weigh around 1.5-3 g.

18. *Optional:* At this point, the cells can be flash-frozen in liquid nitrogen and stored at -80°C. The subsequent steps take around 5 hours and should be done in the same day. After flash freezing, allow the cells to thaw for 1 hour on ice before attempting the next step.

19. Resuspend each pellet in 1 mL wash buffer per gram of cell pellet by vortexing.

20. Let the suspensions rest for 5-10 minutes. Chill a tabletop centrifuge to 4°C prior to the next step.

21. Aliquot out 1.4 mL of the suspension into four total 1.7 mL Eppendorf tubes (total of 5-6 mL suspension from the two pellets).

22. Sonicate the suspensions on ice. The optimal sonication energy may be strain-specific and require some tuning; for Rosetta2 (DE3) in this manuscript, around 350J was found to be optimal. The lysed suspensions should turn brown and become much less viscous.

23. Add 4 µL of 1 M DTT to each 1.7 mL tube after lysis.

24. After lysing all four tubes, centrifuge for 10 minutes at 12,000XG and 4°C.

25. If the lysis has gone well, there will be a separation of the clarified lysate into three bands--a clear top band with milkier opaque cell debris at the bottom and an intermediate band between. Pipette off the top supernatants (approximately 800 µL per tube from 1.4 mL lysate) and combine into two fresh Eppendorf tubes.

26. Cover the tubes with aluminum foil and incubate shaking at 37°C and 200 rpm for 80 minutes for the runoff reaction.

27. After the runoff reaction is complete, centrifuge the tubes at 12,000XG for 10 minutes at 4°C. The tubes will go opaque again during the runoff reaction, and this centrifugation step will remove any additional protein translated during the incubation.

28. Add 600 µL of 1M DTT to the dialysis buffer to a final concentration of 1 mM and transfer to the beaker with a stir bar. Soak a 10K MWCO dialysis cassette in the buffer for at least five minutes. Dry the cassette without blotting the membrane.

29. After the centrifugation is complete, pipette off the supernatant, taking care not to remove any pellet. Load the dialysis cassette with this clarified extract. If no extract has yet been lost, this should be around 2-3 mL and will be faintly yellow but transparent.

30. Dialyze with no buffer exchanges for three hours at 4°C.

31. After the dialysis is complete, remove the extract from the cassette into two fresh 1.7 mL tubes and do a final spin at 12,000XG for 10 minutes at 4°C. There should be a very small pellet. Transfer the supernatant from this spin into a fresh tube--this is the final extract. Aliquot into smaller tubes at 35 µL each and flash-freeze on liquid nitrogen. From a one-liter culture prep, the yield should be around 2.5 mL total, or about 70 tubes containing 35 µL. Store at -80°C until use.

Reagent Preparation

The cell-free reaction is composed of three major constituents: the extract (approximately 30% by volume; refer to Supplemental; Extract Preparation Protocol for detailed preparation notes); the Reaction Buffer (approximately 30% by volume); and the mixture of DNA and inducers supplied to each individual reaction (the balance, approximately 40% and made up to the extra volume with water). The reaction buffer itself has five major constituents which are maintained in separate stocks and mixed fresh for every reaction:

Salt Solution Stock (23.2% of Reaction Buffer by volume):

15X* mM magnesium glutamate (Sigma 49605; stock solution prepared in water to 500 mM)

150 mM ammonium glutamate (MP Biomedicals 02180595; stock solution prepared in water to 2.8M)

1950 mM potassium glutamate (Sigma G1501; stock solution prepared in water to 3.5M)

*The magnesium concentration of the salt solution stock should be prepared to 15X the desired final reaction concentration. For example, if the final concentration of Mg in the reaction is set to be 16 mM Mg, then the salt solution stock should be at 15X16 = 240 mM. We recommend preparing a range of salt solutions and optimizing reaction conditions for each new extract or cell-free construct, as magnesium has an especially strong impact on the reaction yield.

NTP Master Mix Stock (23.2% of Reaction Buffer by volume):
Prepare to a final stock concentration:

18 mM ATP (Sigma A-2383; prepared from an approximately 0.5M stock solution in water and pHed with 10N KOH)*

12.75 mM GTP (Sigma G-8877; prepared from an approximately 0.5M stock solution in water and pHed with 10N KOH)*

12.75 mM UTP (Sigma U-6625; prepared from an approximately 0.5M stock solution in water and pHed with 10N KOH)*

12.75 mM CTP (Sigma C-1506; prepared from an approximately 0.5M stock solution in water and pHed with 10N KOH)*

0.51 mg/mL folinic acid (Sigma 47612; prepared from a 9 mg/mL stock solution in water)

2.559 mg/mL *E. coli* Mre 600 tRNA (Roche 10109541001; prepared from a 50 mg/mL stock solution in water)

*Each nucleotide stock concentration is validated using a NanoDrop and the following molar extinction coefficients:

ATP: 15.4 mM^{-1} cm^{-1} at 259 nm
GTP: 13.7 mM^{-1} cm^{-1} at 253 nm

UTP: 10.0 mM^{-1} cm^{-1} at 262 nm
CTP: 9.0 mM^{-1} cm^{-1} at 271 nm

Reagent Mix Stock (27.8% of Reaction Buffer by volume)*:*

372 mM HEPES (Sigma H3375, from a 1M, pH 7.4 stock solution prepared in water, brought to pH with 10N KOH, and filter-sterilized)

9.8 mM spermidine (Sigma S2626, from a 250 mM stock solution prepared in water)

6.6 mM putrescine (Sigma P5780, from a 250 mM stock solution prepared in water)

26 mM oxalic acid (Sigma P0963, from a 1M stock solution prepared in water)

1.76 mM CoA (Sigma C3144, from a 50 mM stock solution prepared in water)

2.61 mM NAD (Sigma N8535, from a 100 mM stock solution prepared in water) from a 100 mM stock solution prepared in water)

The desired concentrations are obtained by mixing 860 µL of 1M HEPES, 90 µL of 250 mM spermidine, 60 µL of 250 mM putrescine, 60 µL of 1M oxalic acid, 80 µL of 50 mM CoA, and 60 µL of 100 mM NAD.

Amino Acid Stock (13.9% of Reaction Buffer by volume)*:*
Amino acids are prepared from separate powders to a total final concentration in the stock of 50 mM of each amino acid. The following instructions are for preparing 40 mL of this stock solution:

1. Add 0.234 g L-valine (Sigma V0500), 0.408 g L-tryptophan (Sigma T0254), 0.33 g L-phenylalanine (Sigma P2126), and 0.262 g L-isoleucine (Sigma I2752) to 25 mL sterile water. Shake 10 minutes at 37ºC or until soluble.
2. Add 0.262 g L-leucine (Sigma L8000) and 0.242 g L-cysteine (Sigma C7352). Shake 10 minutes at 37ºC or until soluble.
3. Add 0.298 g L-methionine (Sigma M9625), 0.178 g L-alanine (Sigma A7627), 0.348 g L-arginine (Sigma A8094), 0.264 g L-asparagine (Sigma A0884), 0.266 g L-aspartic acid (Sigma A8949), 0.406 g L-glutamic acid, potassium salt (Sigma G1501), 0.150 g L-glycine (Sigma G8898), and 0.292 g L-glutamine (Sigma G3126). Shake 10 minutes at 37ºC or until soluble.
4. Add 0.308 g L-histidine (Sigma H8000), 0.365 L-lysine (Sigma L5501), 0.230 g L-proline (Sigma P0380), 0.210 g L-serine (Sigma S4500), 0.238 g L-threonine (Sigma T8625), and 0.362 g L-tyrosine (Sigma T3754).
5. Add water to a final volume of 40 mL. Amino acids will not go into solutions after the addition of tyrosine, so vortex frequently to measure.
6. Shake for 15 minutes in a 37ºC incubator.
7. Flash freeze the amino acid solution.

Energy Mix Stock (11.6% of Reaction Buffer by volume)*:*

Prepare 1M PEP (phosphoenolpyruvate monopotassium salt; Roche 10108294; prepared to 1M at pH 7 using 10N KOH).

The final buffer mix is then prepared at a ratio of 1 : 1 : 1.2 : 0.6 : 0.5 of salt solution : NTP master mix : reagent mix : amino acids : energy mix (see the Supplemental Reaction Spreadsheet for experimental setup).