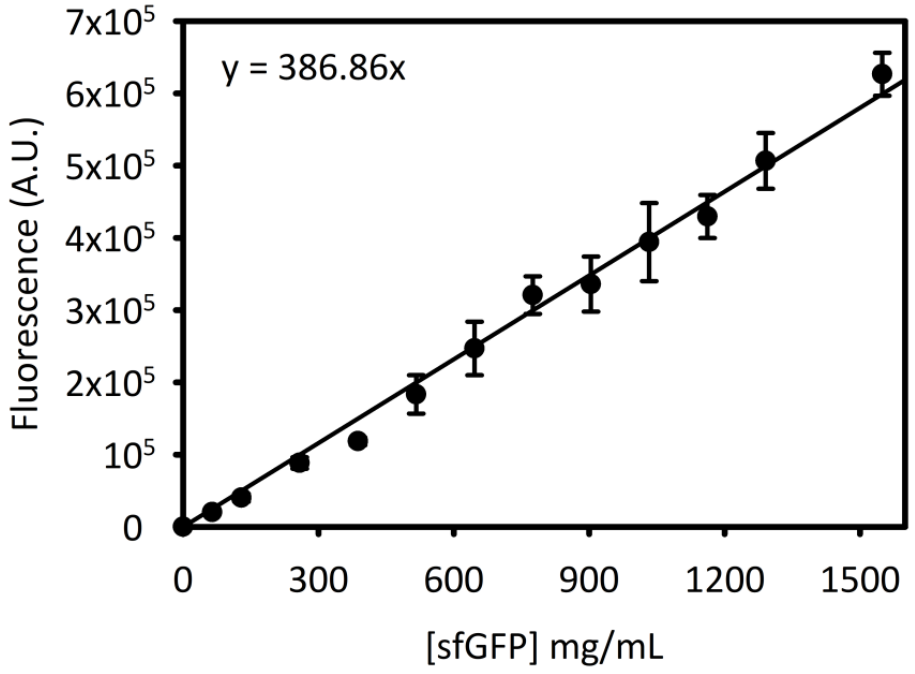
Preparation of a sfGFP Standard Curve

To prepare a standard curve for quantification of sfGFP, a single colony of BL21* PY71 sfGFP was inoculated into 5 mL of LB with Kanamycin in a round bottom test tube and grown overnight. The next day, this culture was centrifuged at 10,000 x g for 5 minutes in a 15 mL falcon tube, with the appropriate balance. The supernatant was discarded and the pellet was flash frozen in liquid nitrogen and stored at -80°C. Strep Tag purification was then carried out as follows using a Strep Tag Purification column (Strep-TactinXT, IBA, Göttingen, Germany). The pellet was retrieved from the -80°C and 500uL of diluted Buffer W (provided with column) was added to the falcon tube, and the mixture was placed on ice for 20-30 minutes to thaw. The pellet was then completely resuspended through gentle vortexing, with resting periods, to minimize bubble formation. The resuspended mixture was transferred to an Eppendorf tube and sonicated for 10 seconds on then 10 seconds off, until the total Joules delivered was about 200J. The sample then centrifuged at 10,000 xg for 5 minutes, and the supernatant was collected and saved in a separate Eppendorf tube. Next, the column was prepared by allowing the storage buffer to drip through and equilibrating the column with 2 column volumes of Buffer W (400 uL). 500 uL of the supernatant was then applied to the column, and the flowthrough was collected. The column was washed with 5 column volumes of Buffer W (1000 uL). Elution was performed by adding 7 separate 0.5 column volumes of Buffer BXT (100 uL each), and collecting the elution in 7 separate Eppendorf tubes. Lastly, the column was washed with 3 column volumes of 10 mM NaOH (600uL) followed by 10 column volumes of buffer W (2000 uL). The column was then capped and buffer W was added for storage at 4°C. SDS-PAGE was performed on all collected samples to determine purity, and those sample with pure sfGFP were combined. The combined stock was then used to create dilutions in HEPES buffer (0.05 M, pH 7.0), and the absorbance at 280 nm was obtained to determine the concentration of the stock sfGFP solution, given that the extinction coefficient for His tagged sfGFP is 18910 1/M*cm. Serial dilutions of the stock sfGFP were then prepared, ranging from 0 to 800 ug/mL of protein. Each dilution was then quantified via a multi well plate fluorometer (Cytation5, BioTek, Winwooski, VT) as follows: 48ul 0.05 M HEPES pH 7.0 buffer and 2 uL of the respective sfGFP dilution were added to each well of a flat bottom 96-well half area black plate (Corning Incorporated, Kennebunk, ME), and each dilution was quantified in triplicate. Excitation and emission wavelengths for sfGFP fluorescence quantification were 485 and 510, respectively. A standard curve was created from the data of known concentrations in order to convert from fluorescence readings to concentration of sfGFP in ug/mL.



Supplemental Figure 1. **Standard curve for sfGFP on Cytation 5.** This curve was determined using the methods outlined above.

T7 RNAP Preparation

T7 RNA Polymerase was purified by affinity tag chromatography as previously described.[40]

Materials Preparation

- Tris(OAc): Prepare 6.057 g Tris Base and bring volume up to 50 mL and pH with Glacial Acetic Acid to pH 8.2.
- $Mg(OAc)_2$: Prepare 15.01 g $Mg(OAc)_2$ and bring final volume to 50 mL using nanopure water.
- K(OAc): Prepare 29.442 g K(OAc) and bring final volume to 50 mL using nanopure water.
- DTT: Prepare 1.54 g of DTT and bring final volume to 10 mL. Aliquot 1 mL of solution per tube and store at -80°C.
- S30 components: Prepare 1 mL Tris(OAc), 1 mL $Mg(OAc)_2$, 1 mL K(OAc), and 0.200 mL DTT and bring volume to 100 mL using nanopure water. Do not add DTT until day of use and store at 4°C.
- 2x YTP Media: Prepare 5 g NaCl, 16 g Tryptone, 10 g Yeast Extract, 7 g Potassium Phosphate Dibasic, and 3 g Potassium Phosphate Monobasic and bring volume to 375 mL using nanopure water. Adjust pH to 7.200 using 5 M KOH. Dilute solution to 750 mL. Autoclave in 2.5 L Tunair Baffled Flask at liquid 30 setting. Store at 37°C until use.

- D-Glucose Solution: Prepare 18 g D-Glucose and bring volume to 250 mL using nanopure water. Autoclave solution in glass bottle at liquid 30 setting. Store at 37°C until use. Combine with 2x YTP media prior to inoculation of 2x YTPG media with overnight BL21*DE3 culture.
- IPTG: Prepare 2.38 g IPTG and bring final volume to 10 mL using nanopure water. Store 1 mL aliquots at -80°C.

For the following stocks, we recommend keeping log sheets for each batch. Over time, this will help identify batch-to-batch variation in reaction performance.

- NAD: Prepare 0.050 g and bring volume to 0.750 mL molecular grade water. Store at -80°C
- PEP: Prepare 0.206 g and bring volume to 0.500 mL using molecular grade water. pH solution to 7.0 by adding 10 M KOH. Bring final volume to 1 mL using molecular grade water. Store at -80°C.
- CoA: Prepare 0.010 g and bring volume to 0.260 mL using molecular grade water. Store at -80°C.
- Putrescine: Prepare 0.011 g and bring final volume to 0.500 mL using molecular grade water. Store at -80°C.
- Spermidine: Prepare 0.018 g and bring final volume to 0.500 mL molecular grade water. Store at -80°C.
- HEPES: Prepare 2.38 g HEPES and bring volume to 10 mL using molecular grade water. Store at -80°C.
- Folinic Acid: Prepare 0.015g folinic acid and bring volume to 1.5 mL using molecular grade water. Store at -80°C.
- tRNA: Prepare 0.050g tRNA and bring volume to 1 mL using molecular grade water. Store at -80°C.
- 15X MasterMix: 180 uL ATP, 127.5 uL GTP, 127.5 uL CTP, 127.5 uL UTP (NTPs were purchased at a stock concentration of 100 mM), 47.22 uL folinic acid, and 51.18 uL tRNA. Store at -80°C.
- 15X Salt Solution: Prepare 0.290 g of Magnesium Glutamate, 0.120 g of Ammonium Glutamate, and 1.98 g of Potassium Glutamate and bring volume to 5 mL using molecular grade water. Store at -80°C.
- Oxalic Acid: Prepare 0.92 g and bring volume to 5 mL using molecular grade water. Store at -80°C.
- 20 Amino Acids: Prepare 0.234 g L-Valine, 0.408 g L-Tryptophan, 0.330 g L-Phenylalanine, 0.262 g L-Isoleucine, 0.262 g L-Leucine, 0.242 g L-Cysteine, 0.298 g L-Methionine, 0.178 g L-Alanine, 0.348 g L-Arginine, 0.264 g L-Asparagine, 0.266 g L-Aspartic Acid, 0.406 g L-Glutamic Acid Potassium Salt Monohydrate, 0.150 g Glycine, 0.292 g L-Glutamine, 0.308 g L-Histidine, 0.365 g L-Lysine, 0.230 g L-Proline, 0.210 g L-Serine, 0.238 g L-Threonine, 0.362 g L-Tyrosine and add molecular grade water to a

final volume to 40 mL. Shake 15 min in 37°C incubator. pH of solution should be ~6.7. Store at -80°C.

Solutions A and B are generated upon mixing the aforementioned stock solutions as described below:

Solution A	
Reagent	**Amount**
Master mix	1000 µL
NAD	60 µL
CoA	80 µL
Oxalic Acid	60 µL
Putrescine	60 µL
Spermidine	90 µL
HEPES	855 µL
Total Volume	2205 µL
*Makes enough for one thousand 15 µL reactions * Add 2.2 µL to each 15 µL reaction	

Solution B	
Reagent	**Amount**
15x SS	1000 µL
20 Amino Acids	600 µL
PEP	495 µL
Total Volume	2095 µL
*Makes enough for one thousand 15 µL reactions * Add 2.1 µL to each 15 µL reaction	

CFPS Reaction Setup Guide (excel sheet provided)

Name:
Date:
Purpose:
Reaction Size (uL): 15

Reagent Information

Cell Extract				
Cell Type	Growth Date	Extract Preparation Date	Volume per Reaction (µL)	Note
BL21* DE3	7/26/2018	7/27/2018	5.00	

Solution A		Solution B		
Lot #	Volume per Reaction (µL)	Lot #	Volume per Reaction (µL)	Note
1	2.20	1	2.10	

DNA Template				
Template Name	Stock concentration (ng/µL)	Final concentration in reaction (ng/µL)	Volume per Reaction (µL)	Note
pJL1-sfGFP	240	16	1.00	Must be less than volume of water in negative control

Reaction Set Up
(perform each in triplicate)

	Molecular Grade Water (µL)	Solution A (µL)	Solution B (µL)	Cell Extract (µL)	DNA Template (µL)
Negative	5.70	2.20	2.10	5.00	0.00
Positive	4.70	2.20	2.10	5.00	1.00
Experimental	4.70	2.20	2.10	5.00	1.00