Supplementary Information

Establishing a cell-free *Vibrio natriegens* expression system

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Materials and Methods

Materials: All cell-free protein synthesis (CFPS) reaction components were purchased from Sigma-Aldrich (St. Louis, MO) with the exception of the *E. coli* MRE 600 tRNA mixture which was purchased from Roche Applied Science (Indianapolis, IN). ATP, GTP, CTP, UTP, T7 RNA Polymerase, and Murine RNase Inhibitor were purchased from New England Biolabs (Ipswich, MA). The 20 amino acids were purchased from BioTechRabbit, GmbH (Hennigsdorf, Germany) as the RTS Amino Acid Sampler. A Q125 Sonicator and CL-18 probe with a ⅛-inch tip diameter (Qsonica - Newtown, CT) was used for all cell-free crude extract preparations.

Bacteria Strains and Plasmids: *Vibrio natriegens* #14048 (ATCC - Manassas, VA) and *Escherichia coli* strain A19 (Coli Genetic Stock Center #5997- New Haven, CT) were used for this study. *V. natriegens* was grown in LB-V2 media (LB Miller supplemented with 200 mM NaCl, 23.1 mM $MgCl_2$ and 4.2 mM KCl) unless indicated otherwise. *E. coli* A19 was grown in 2xYTP media (10g/L yeast extract, 6 g/L tryptone, 5 g/L NaCl) [1]. Plasmid DNA was used as template for all CFPS expression reactions unless otherwise indicated. Plasmid pJL1 (Addgene #69496) was used for expression of super-folder GFP (sfGFP) under control of a T7 promoter. Plasmids were purified using Qiagen Maxi-Prep Kit (Qiagen - Hilden, Germany) as per manufacturer's instructions and eluted in MilliQ water. Plasmid concentration was determined with a Nanodrop 2000 UV-Vis spectrophotometer (Thermo Scientific - Waltham, MA). Additional growth media were formulated according to manufacturer instructions and supplemented with 1.5% (w/v) final Ocean Salts (Aquarium System, Inc.) to make high salt versions of Brain Heart Infusion (BHI-O), Nutrient Broth (NB-O), and Lysogeny Broth (LB-O). No additional salts were added to Marine Broth (MB). High salt lysogeny broth (LB3) media is prepared by supplementing LB Miller Broth (Fisher BP9723-500) with 3% (w/v) final NaCl.

Cell Growth & Preparation of Cells Pellets: Initial liquid cultures were inoculated from glycerol stocks and incubated overnight shaking at 225 rpm in the appropriate medium. Overnight cultures were then harvested, washed once using the same growth media, and 1mL was used to inoculate 1 L of fresh growth media in a 4 L baffled flasks. For smaller scale cultures, 250 mL of fresh growth media was inoculated in 1 L baffled flasks. Culture optical density at 600 nm (OD_{600}) was monitored by a spectrophotometer and was used to determine overall growth

kinetics. At the desired OD_{600}, cells were harvested by centrifugation spinning at 3500 x G for 20 minutes at 4 C. The resulting pellet was then immediately stored at -80 C until preparation of the crude cell extract.

Preparation of Crude Cell Extracts: Thawed cell pellets were suspended in 10 mL of S30A buffer (50 mM Tris, 14 mM Mg-Glutamate, 60 mM K-Glutamate, 2 mM Dithiothreitol (DTT), adjusted with Glacial Acetic Acid to pH 7.7) in a 15 mL Falcon tube and centrifuged at 3500 x G for 20 minutes at 4°C. The supernatant was aspirated and this process was repeated once more. Pellets were then resuspended in 200-500 µL cold S30A buffer and transferred to 2 mL Eppendorf tubes with a wide bore pipette for sonication. The total transfer volume of the S30A buffer and cell suspension was minimized to keep crude extract total protein as concentrated as possible. Cell suspensions were lysed with the Q125 Sonicator by pulse sonication at frequency of 20 kHz and 50% amplitude for 10 seconds on and 60 seconds off three times. Six sonication pulses were used to lyse larger volume cell suspensions (> 500 µL). Sonication was performed in an ice-water bath to minimize heat damage. The lysates were then centrifuged at 16,000 x G for 30 minutes at 4 C and the resultant supernatants were transferred to new 2 mL Eppendorf tubes without disturbing the debris pellet. The total protein concentration was quantified by Qubit Fluorometer Protein Kit (Invitrogen) according to manufacturer's protocol. Cell extracts were then aliquoted in cryotubes, flash frozen in liquid nitrogen, and stored at -80°C until use. On average, a well lysed cell pellet yielded 20-30 mg/mL of total protein in the crude extract; extracts that were > 20 mg/mL were not used for CFPS.

Standard Cell Free Protein Synthesis Reaction: Standard CFPS reactions were performed in 10 uL volumes in black, flat-bottom 384-well assay plates (Corning - Corning, NY) or clear, 384-well PCR plates (Eppendorf - Hamburg, Germany). A standard CFPS reaction consisted of 35% crude cell extract, 1x Energy Solution Master Mix, 2 mM of each of the 20 amino acids (1x Amino Acid Master Mix), 3.5 mM Mg-glutamate, 80 mM K-glutamate, 2% PEG-5000, 50 Units of T7 RNA Polymerase, 4 Units of Murine RNase Inhibitor, and 500 ng of plasmid. CFPS reactions using *E. coli* A19 crude extract were performed at 37°C and reactions using *V. natriegens* crude extract were performed at 30°C, unless otherwise specified. The use of Mg- and K-glutamate mirrored the ion sources in calibrating *E. coli* based cell-free expression systems [2, 3].

Energy Solution Master Mix: A 10x Energy Solution Master Mix was prepared by combining 500 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 8.0), 15 mM ATP, 15 mM GTP, 9 mM CTP, 9 mM UTP, 2 mg/mL *E. coli* MRE 600 tRNA mixture, 2.6 mM Coenzyme A (CoA), 3.3 mM β-Nicotinamide adenine dinucleotide (NAD), 7.5 mM Adenosine 3′,5′-cyclic monophosphate (cAMP), 0.70 mM L-5-formyl-5, 6, 7, 8-tetrahydrofolic acid (Folinic Acid), 10 mM Spermidine, and 300 mM 3-Phosphoglyceric Acid (3-PGA). Energy Solution Master Mix was aliquoted into 2 mL Eppendorf tubes, flash frozen in liquid nitrogen, and stored until needed at -80°C. We choose 3-PGA as our cell-free reaction energy source as it was

previously shown to be an improvement over other energy sources such as phosphoenolpyruvate (PEP) and creatine pyruvate (CP) [4].

Amino Acid Master Mix: A 4X Amino Acid Master Mix was prepared by diluting each amino acid to a final concentration of 8 mM in MilliQ water in the following order: (ALA, ARG, ASN, ASP, GLN, GLU, GLY, HIS, ILE, LYS, MET, PHE, PRO, SER, THR, VAL, TRP, TYR, LEU, CYS). Amino Acid Master Mix was aliquoted into 2 mL Eppendorf tubes, flash frozen in liquid nitrogen, and stored until needed at -80°C.

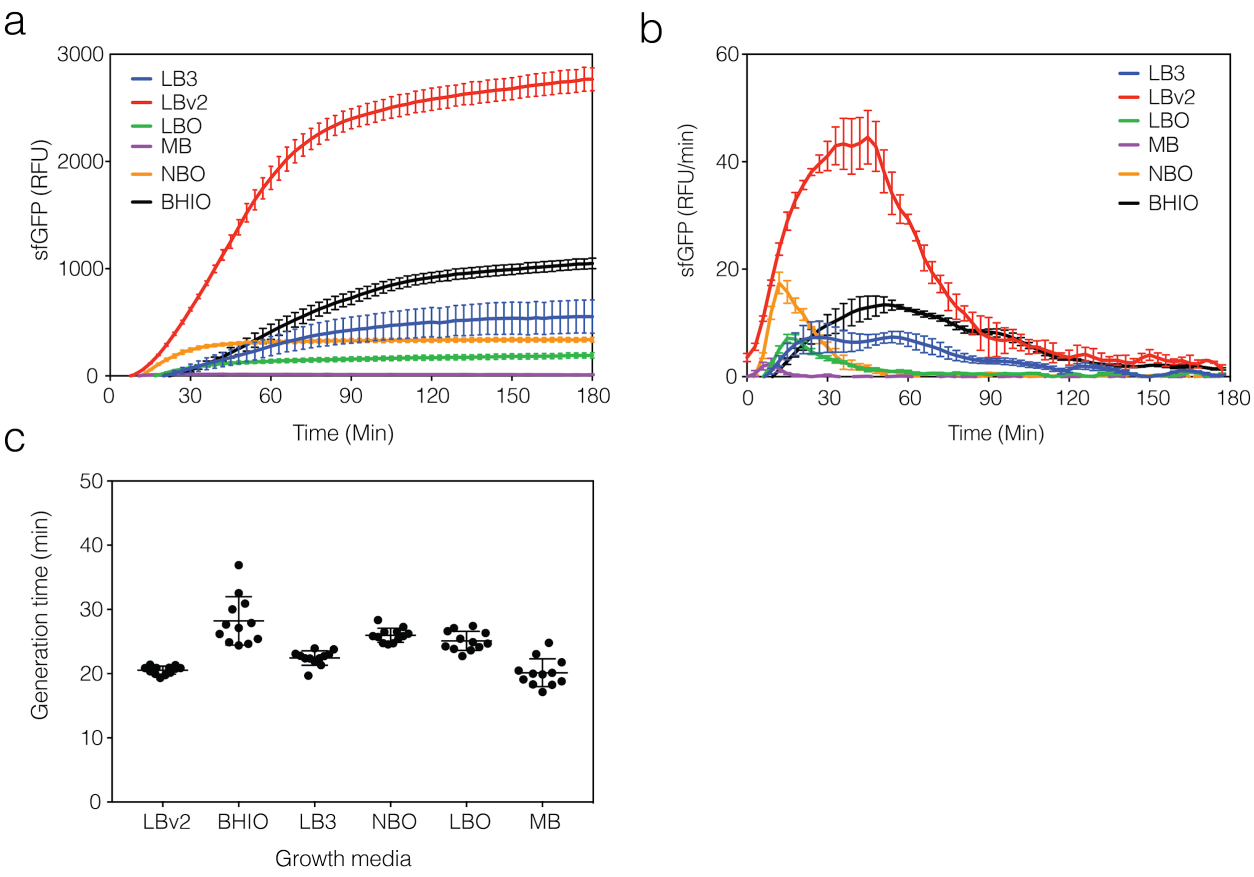
Quantification of Super Folder GFP Production: For CFPS kinetic assays, sfGFP fluorescence was continuously monitored over 180 minutes with measurements taken every 3 minutes on a Synergy HT Plate Reader (BioTek, Winooski, VT) set at the appropriate reaction temperature. The excitation and emission wavelengths for fluorescence of sfGFP were 485 nm and 528 nm, respectively. Endpoint CFPS reactions were performed in 384-well PCR plates at 10 µL volumes on a thermocycler (Veriti 384-well Thermocycler - Thermo Scientific) incubated at the desired temperature for 180 minutes. Upon completion, endpoint reactions were transferred to black, flat-bottom 384-well plates with a multi-channel pipette for subsequent quantification on a plate reader. For both CFPS kinetic assays and endpoint reactions, the yield of sfGFP was determined by comparing sample RFU at reaction completion to a standard curve consisting of known concentrations of purified sfGFP in MilliQ water. The stock concentration of sfGFP was determined using a micro BCA protein assay as per manufacturer's instructions (Thermo Scientific). A standard linear regression was then used to calculate the total yield of sfGFP. A negative control consisting of all CFPS reaction components with the exception of plasmid DNA was used to correct sfGFP yield. CFPS sfGFP synthesis was further visualized using a 16% Tris-Glycine (Thermo Scientific) protein gel under non-denaturing conditions and imaged on a Typhoon FLA 9500 (GE Healthcare Life Sciences - Pittsburgh, PA).

Cell-Free Protein Synthesis from Linear Template: sfGFP linear template containing the T7 promoter site was generated by PCR amplification from the pJL1 plasmid using Kapa HiFi polymerase using primers 5-GCCAGCAACGCGATCC and 5-GACCCGTTTAGAGGCCCC or phosphorothioated primers 5-A*A*CTCAGCTTCCTTTCGGGC and 5-A*C*GCCAGCAACGCGATCC. All PCR primers were purchased from Integrated DNA Technologies (Coralville, IA). The resultant material was cleaned and concentrated using Qiagen Quick PCR clean up spin columns, eluted in MilliQ water, and quantified with a Nanodrop 2000 UV-Vis spectrophotometer. Linear template CFPS reactions were performed as kinetic and endpoint reactions as previously described with 125 ng (5.3 pmol) of total material. The efficiency of CFPS from linear template was compared to reactions containing equimolar quantities of circular plasmid.

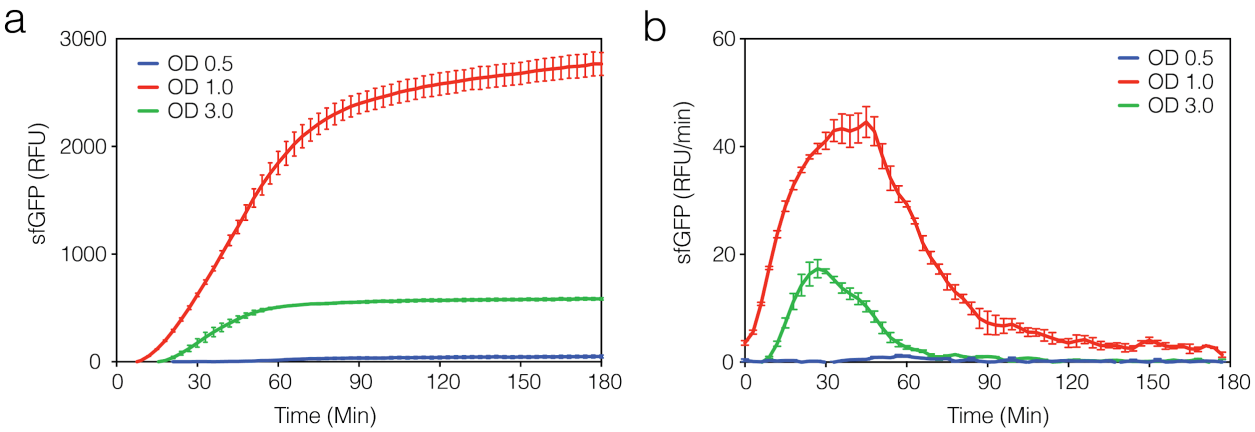
Linear Template Stability Assay: Linear template stability assays were performed as previously described [24]. Labeled T7-sfGFP linear template was generated by supplementing PCR reactions with Chromatide AlexaFluor 594 5-dUTP (Thermo Scientific) according to manufacturer instructions. The following PCR protocol was used: 94°C - 5 min, 55°C - 5 min, [72°C - 2 min, 94°C - 1 min, 55°C - 1 min] x 30 cycles, 72°C - 5min. All CFPS reactions were performed in 384-well PCR plates with 500 ng (0.9 pmol) of labeled linear template. At the predetermined time points, 2 uL of the CFPS reactions were transferred to a black, flat-bottom 384-well assay plate which was kept at 4°C for the duration of the stability assay. Once completed, all samples were brought to a volume of 10 µL with MilliQ water and the total sfGFP expression was determined on a plate-reader as previously described. Using a ZR-96 DNA Clean and Concentrator kit (Zymo Research - Irvine, CA), the linear template was extracted and purified from the CFPS reactions as per manufacturer's instructions and eluted into MilliQ water. The relative percentage of labeled linear template remaining was then determined with a plate reader measuring fluorescence at Ex: 590 nm/Em: 619 nm and normalizing to the initial time point at 0 minutes. Fluorescence for both sfGFP and the labeled linear template was corrected with negative controls consisting of all CFPS reaction components with the exception of labeled linear template (N=3).

Calculation of Reaction Rates: CFPS reactions rates (RFU/min) were calculated using a custom R script which fitted the raw kinetic data with a smoothing spline function and returned the slope at each time point by taking the first derivative of the fit. Data for replicates were processed with the script individually and then were averaged and plotted in Graphpad Prism (Graph Software - La Jolla, CA).

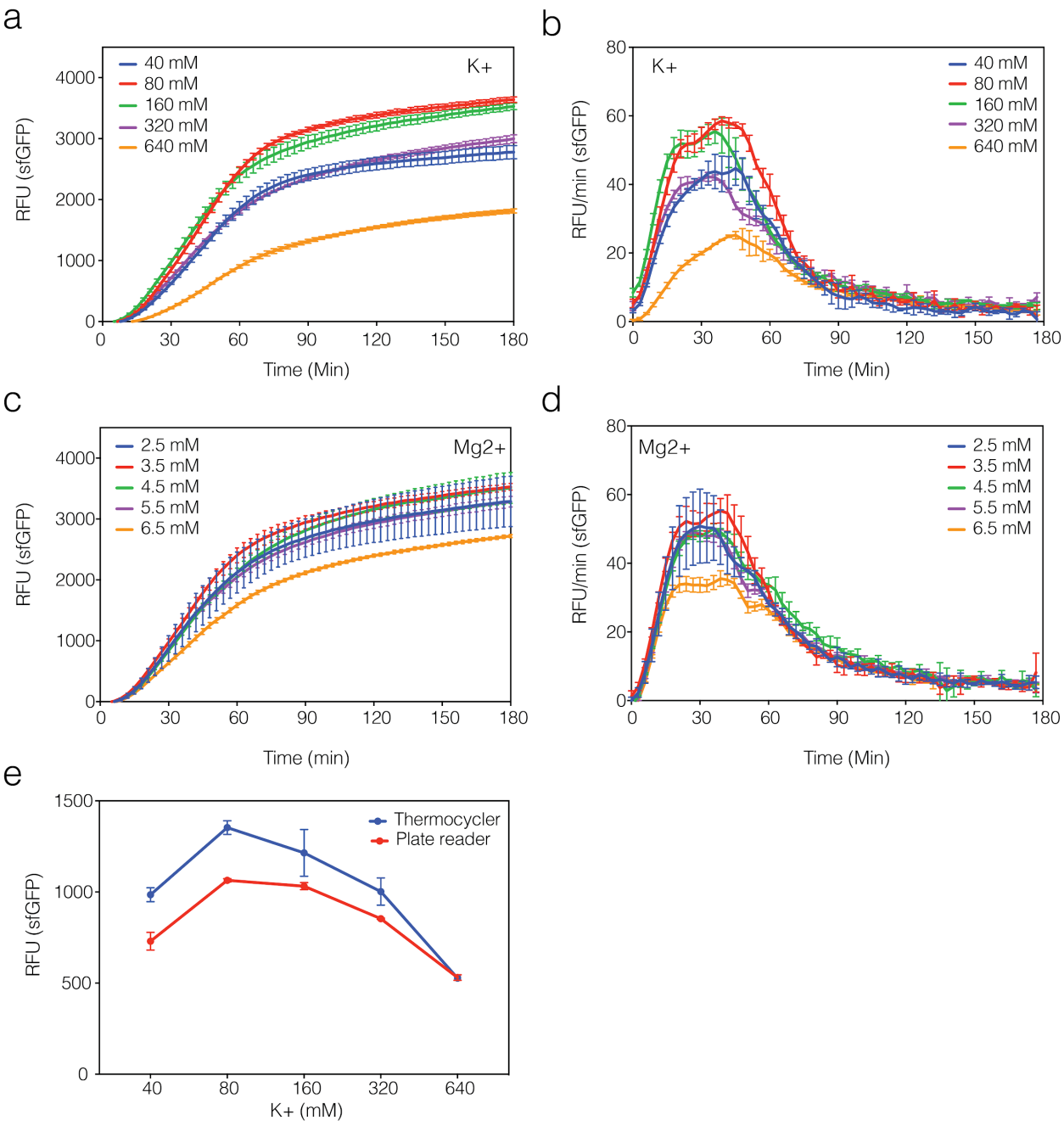
Measuring Growth Rates: *V. natriegens* was subcultured in each growth medium overnight at 30°C. Overnight cells were washed once in fresh growth media, then diluted 1:1000 into fresh media. Growth was measured by kinetic growth monitoring (Biotek H1, H4, or Eon plate reader) in 96-well plates with continuous orbital shaking and optical density (OD) measurement at 600nm taken every 2 minutes. The generation time for *V. natriegens* in each growth medium was calculated from the maximal growth rate using a custom Python script. Growth rates were calculated by linear regression of the log-transformed OD across at least 6 data points when growth was in exponential phase and the $OD \geq 0.015$.



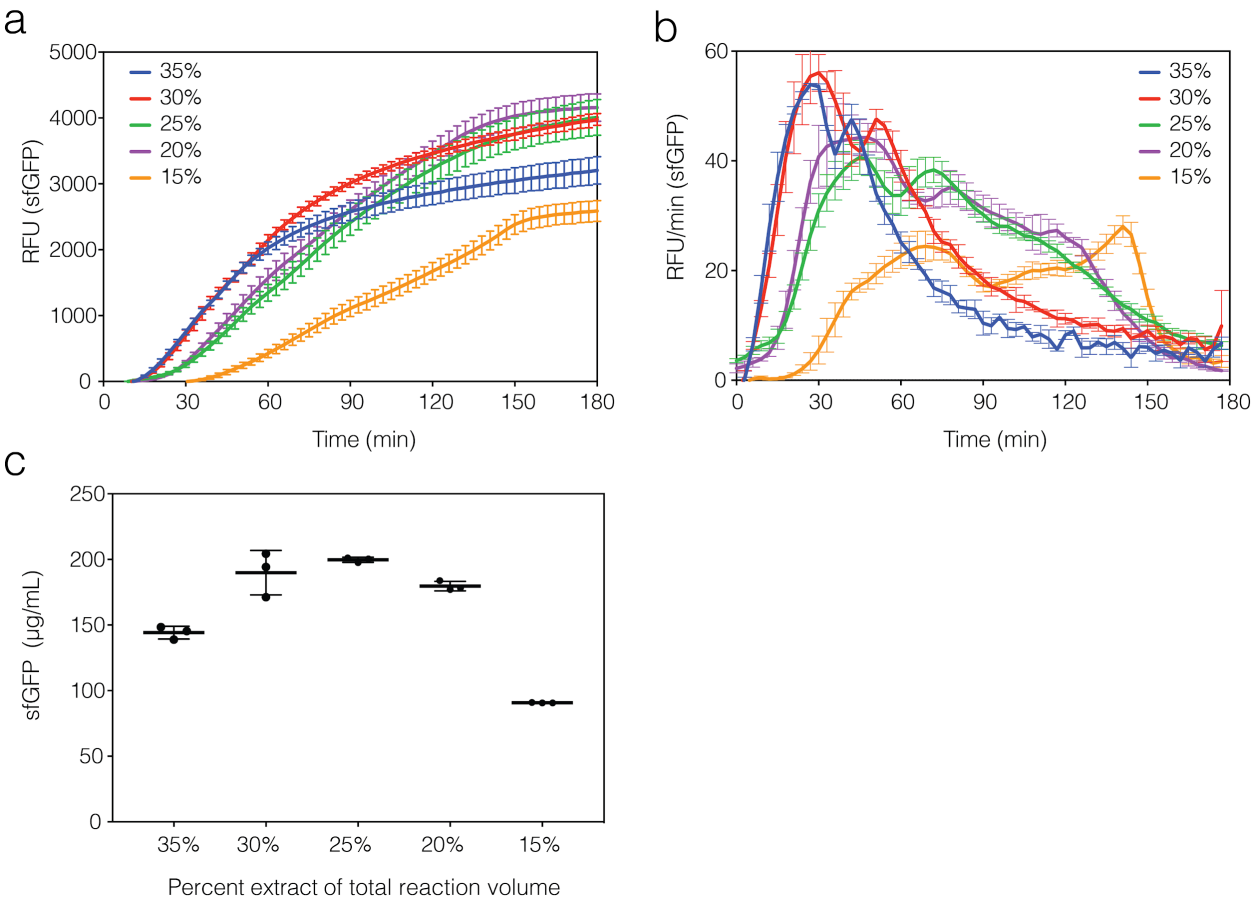
Supplementary Figure 1. Protein expression in *V. natriegens* extracts prepared from various culture media: (a) accumulation of sfGFP in extracts (b) rate of sfGFP expression in extracts. (c) Generation time measured in each media type. Crude extracts were prepared from 250 mL of *V. natriegens* cells grown at in the indicated medium at 30°C. Cells were harvested at OD_{600} = 1.0. The mean and standard deviations are shown (N≥3).



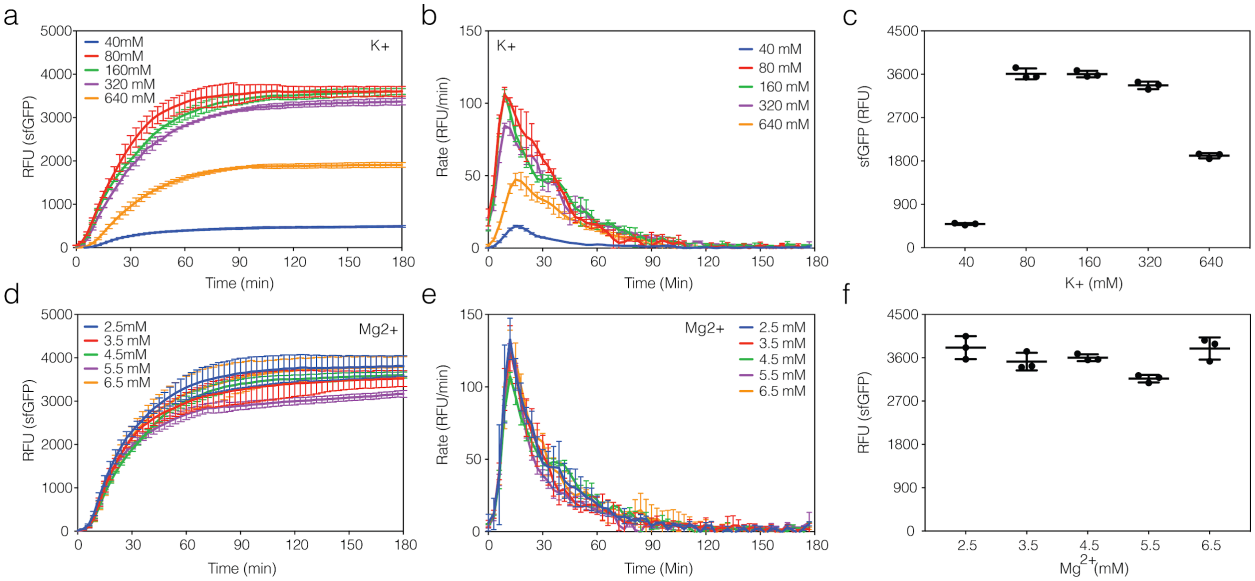
Supplementary Figure 2. Protein expression from *V. natriegens* extracts prepared from cultures at different phases of growth in LB-V2 medium at 30°C: (a) accumulation of sfGFP in extracts (b) rate of sfGFP expression in extracts. Crude extracts were prepared from 250 mL of *V. natriegens* cells grown at LB-V2 medium at 30°C. The mean and standard deviations are shown (N=3).



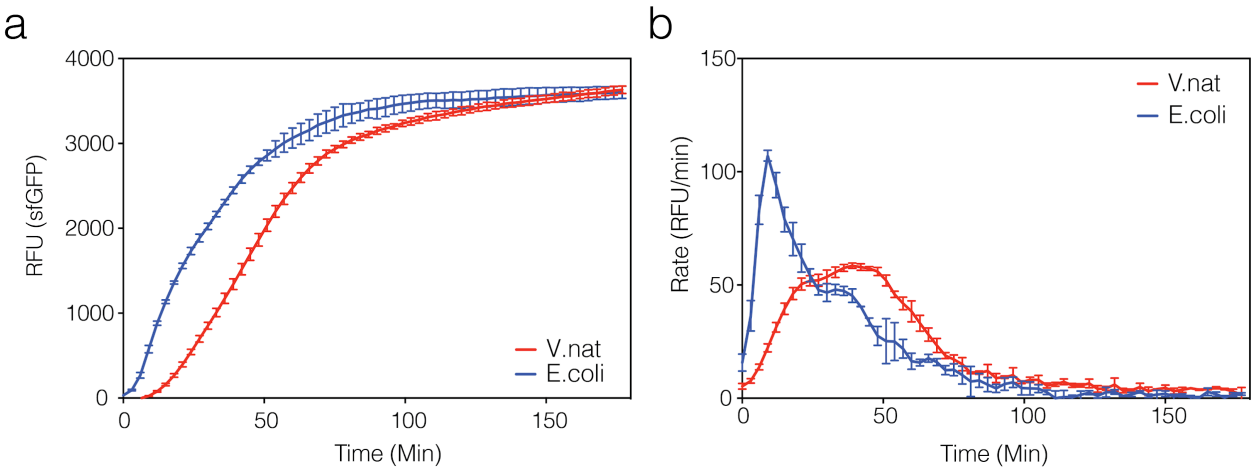
Supplementary Figure 3. Calibrating ion concentrations in *V. natriegens* cell free extracts. Effect of potassium ions on extract protein yield (a-b): accumulation of sfGFP (a) and expression rate (b) of sfGFP in extracts with varying potassium concentrations. Effect of magnesium ions on extract protein yield (c-d): accumulation of sfGFP (c) and expression rate (d) of sfGFP in extracts with varying magnesium concentrations. (e) comparison of protein yield when reactions were incubated in a thermocycler versus plate reader. Crude *V. natriegens* cell extracts were prepared from cultures grown in LB-V2 medium at 30°C. The mean and standard deviations are shown (N=3).



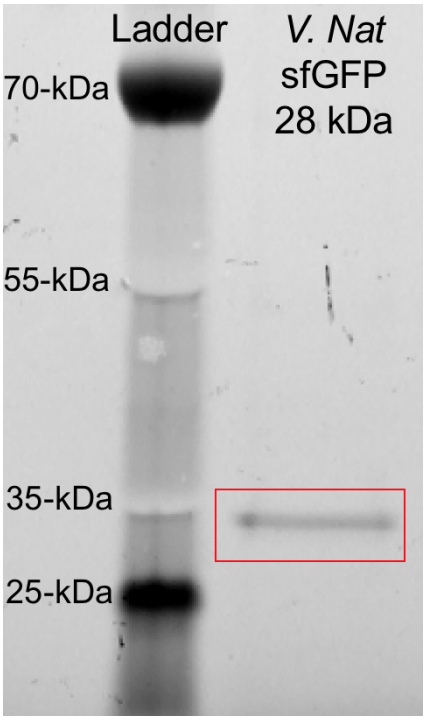
Supplementary Figure 4. Adjusting crude extract concentration in cell free reaction. The volume of cell extract relative to total reaction volume was varied (**Methods**): (a) accumulation of sfGFP (b) rate of sfGFP expression in extracts, and (c) yield of sfGFP after three hours. The mean and standard deviations are shown (N=3).



Supplementary Figure 5. Cell-free expression using crude extracts of *E. coli* A19. Cultures were grown in 2xYTP media at 37°C, harvested at OD_{600} = 1.0 and lysed to prepare crude cell extract. Small-scale batch reactions (10µL total) were incubated at 37°C for three hours using 500 ng plasmid DNA (pJL1) and sfGFP fluorescence was monitored over time. Effect of potassium ions on extract protein yield (a-c): accumulated sfGFP in extract (a), rate of sfGFP expression in extracts (b) and protein yield after three hours (c). Effect of magnesium ions on extract protein yield (d-f): accumulated sfGFP in extract (d), rate of sfGFP expression in extracts (e), and protein yield after three hours (f). The mean and standard deviations are shown (N=3).



Supplementary Figure 6. Cell-free extract expression for *E. coli* A19 and *V. natriegens* in each of their determined optimal conditions. (a) kinetic measurement of sfGFP in *E. coli* (red, 26°C, 80 mM K-glutamate, 3.5 mM Mg-glutamate) and *V. natriegens* (blue, 37°C, 160 mM K-glutamate, 3.5 mM Mg-glutamate) extracts. (b) rate of sfGFP expression in extracts. *E. coli* cultures were grown in 2xYTP media at 37°C and harvested at OD_{600} = 1.0. All experiments were performed using 500 ng plasmid DNA (pJL1). The mean and standard deviations are shown (N=3).



Supplementary Figure 7. Non-denaturing gel electrophoresis analysis of sfGFP (expected: 28 kDa) from *V. natriegens* cell free expression system. After 3 hours of incubation, 1 µL of the cell free reaction was prepared with the sample buffer as per manufacturer's instructions and loaded into a 16% Tris-Glycine protein gel. Samples were run for 45 minutes at 225V and fluorescence was directly imaged without staining on a Typhoon FLA 9500 imaging station at an excitation and emission wavelength of 488 nm and 510 nm, respectively. Sample weight was determined by comparing to a protein ladder (PageRuler Plus Prestained Ladder, 10 kDa to 250 kDa - Thermo Scientific).

References

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