

Long-Term Protein Synthesis with PURE in a Mesoscale Dialysis System

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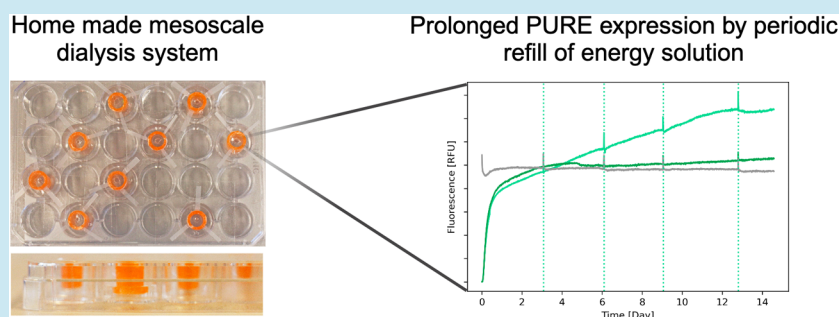
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ABSTRACT: Cell-free systems are powerful tools in synthetic biology with versatile and wide-ranging applications. However, a significant bottleneck for these systems, particularly the PURE cell-free system, is their limited reaction lifespan and yield. Dialysis offers a promising approach to prolong reaction lifetimes and increase yields, yet most custom dialysis systems require access to sophisticated equipment like 3D printers or microfabrication tools. In this study, we utilized an easy-to-assemble, medium-scale dialysis system for cell-free reactions using commercially available components. By employing dialysis with periodic exchange of the feeding solution, we achieved a protein yield of 1.16 mg/mL GFP in the PURE system and extended protein synthesis for at least 12.5 consecutive days, demonstrating the system's excellent stability.

KEYWORDS: cell-free expression, PURE, lysate, continuous dialysis, scale-up, extended expression

INTRODUCTION

Cell-free systems are an ideal chassis for engineering biomolecular systems due to their versatile, open, and well-defined nature.^{1–5} There are two main types of cell-free systems: lysate-based systems, where the cytoplasm is directly extracted from cells, and the fully recombinant PURE⁶ and OnePot PURE system.^{7,8} Current drawbacks of cell-free systems compared to cellular protein expression are a limited reaction time due to a lack of self-regeneration and cellular homeostasis,² and a limited protein production capacity. Therefore, there is a strong interest in prolonging reaction times to enhance protein yield in cell-free systems. There are generally two approaches to improve protein yield, one is to optimize the composition of the system, and the other is to extend the reaction time by supplying additional energy components and low molecular weight building blocks.

While it is generally the case that lysate systems have higher protein production capacities than PURE, cytoplasmic extracts render lysate systems ill-defined resulting in high batch-to-batch variability.^{1,9,10} In PURE, all components required for transcription-translation are produced separately, rendering the system well-defined. This is a considerable advantage over lysate systems for a variety of applications including synthetic cell - or therapeutic applications.

To our knowledge, the highest protein yield achieved to date using cell-free systems is 8 mg/mL of protein produced using semicontinuous expression with an optimized lysate system encapsulated in liposomes.¹¹ Using this lysate formulation, the authors not only improved the yield but also extended protein synthesis to 20 h. The highest protein yield achieved with PURE was reported 10 years ago at 3.8 mg/mL of GFP using a dialysis system. To achieve this yield the authors significantly altered the composition of the PURE system by increasing the concentration of protein and ribosomal components.¹²

Other approaches to prolong reaction times in both lysate¹³ and PURE systems^{14,15} are based on immobilization or encapsulation strategies. Using these approaches, protein expression was achieved for up to 16 days in PURE,¹⁵ and up to 28 days in lysate.¹³ These results demonstrated that cell-free systems can sustain protein synthesis for several days in confined and encapsulated systems. However, the hydrogels

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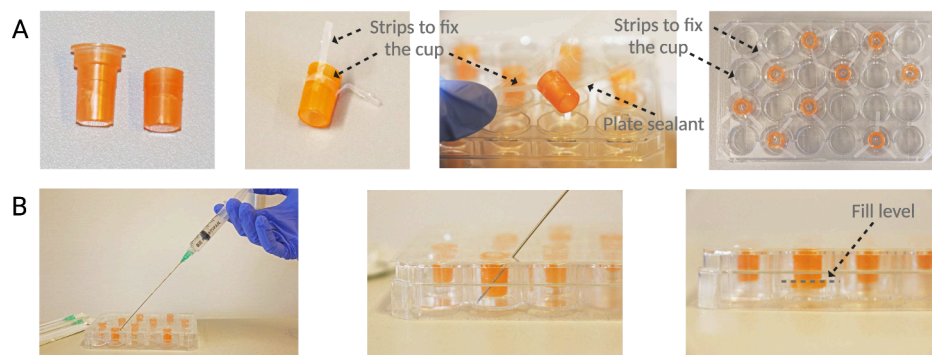


Figure 1. (A) Photographs of the assembly of the dialysis plate. From left to right: dialysis cup after and before being cut; fixing of the strips on the cup: three strips serve to attach the cup to the plate sealant, and the last one goes around the cup fixing the three strips perpendicularly; attachment of the cup to the sealing plate by using the three previously fixed strips; final appearance of the plate including the plate sealant. The cups were distributed randomly across the plate. Theoretically, 24 reactions can be run in parallel. (B) Photographs of the feeding solution refilling procedure and the fill level. A needle is used to pierce the plate sealant and exchange the feeding chamber. Finally, an extra plate sealant will be added to cover the holes.

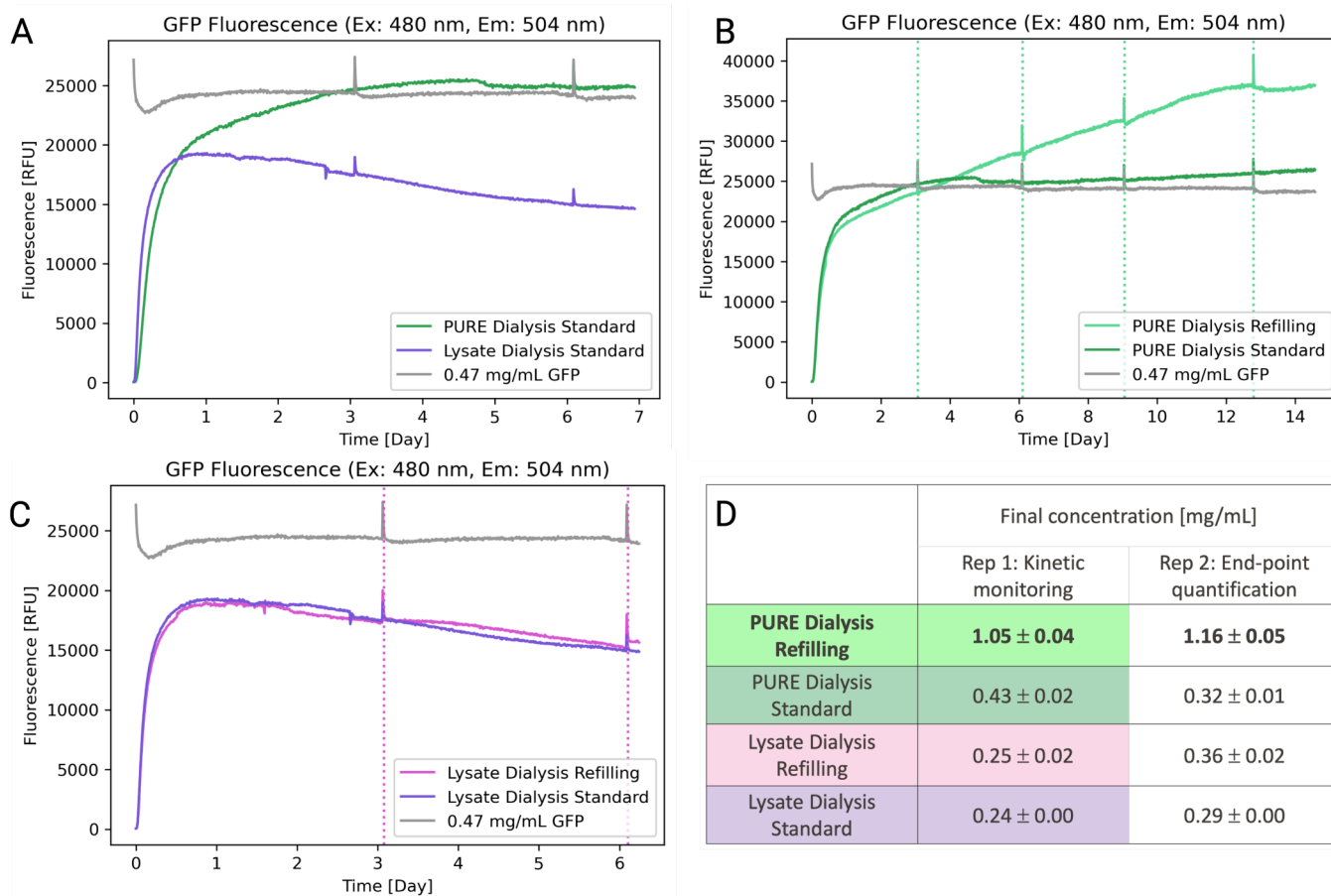


Figure 2. (A) Fluorescence signal from PURE (dark green) compared to lysate (purple) expressions when the dialysis compartment is not replenished, as well as a reference fluorescence measurement (grey) of a 0.47 mg/mL solution of GFP in H₂O, immersed in a feeding compartment containing 1 mL of H₂O. (B) Fluorescence from PURE reactions with replenishment of the feeding compartment (indicated by vertical dotted lines) in light green, and without replenishment of the feeding compartment, in dark green. (C) Fluorescence from lysate reactions in dialysis mode, with replenishment of the feeding compartment (indicated by vertical dotted lines) in pink, and without replenishment of the feeding compartment, in purple. (D) End point concentration calculations of the two replicates for each experiment indicating the mean and the standard deviation of technical duplicates for each replicate of the experiment. GFP concentrations were calculated via a GFP calibration curve (Supplementary Figure S1).

are labor-intensive to produce and the obtained yield of 200 $\mu\text{g/mL}$ ¹⁴ is fairly low. We previously implemented semi-permeable hydrogel membranes in a microfluidic chemostat.

Using a commercially available PURE system, we extended protein synthesis at a constant synthesis rate from two to at

least 30 h in this microscale dialysis system, and increased overall protein yield by 7-fold.¹⁶

The aforementioned examples require specialized equipment to fabricate microfluidic devices (nL scale)^{13–16} or microscale dialyzer plates (10–50 μL).^{12,17,18} This limits access to long-lived, high-yield dialysis based cell-free expression systems. For larger scale reactions of around 1 mL, commercially available dialysis devices exist, which often consist of dialysis cups inserted in tubes,¹⁹ and thus do not allow monitoring reaction kinetics using standard fluorescent plate readers. Even more problematic are the large reaction volumes, which make these reactions very costly. Commercially available or custom fabricated²⁰ dialysis plates exist. However, these are either too tall to fit into standard plate reader instruments, or they do not provide physical access for imaging the reaction.¹⁸

Here we created a simple DIY dialysis system for mesoscale (100 μL) cell-free expression that utilizes commercially available components and can be assembled and used with standard equipment. The main advantages are the open access of the reaction chambers during incubation, allowing feeding solution replenishment, and the possibility for real-time fluorescence monitoring using standard plate readers. Our dialysis system enabled sustained protein synthesis for 4 days using PURE. By periodically replacing the feeding solution, protein expression was extended to 12.5 days resulting in a protein yield of 1.15 mg/mL. To our knowledge, this represents the longest expression for PURE reported in nonencapsulated systems. Our results highlight the excellent stability of the PURE system and indicate a long protein and ribosome lifetime beyond what is currently harnessed in batch reactions and simple dialysis reactions without feeding solution replenishment. We anticipate that this system could be employed in mesoscale protein production in which protein yield is critical. Potential advanced applications in addition to protein production for research purposes could include the decentralized production of therapeutics²¹ as well as the possibility to develop continuous, long-term environmental monitoring,^{22,23} or diagnostic systems.²⁴

RESULTS

In this work we present a cell-free expression system complemented with a mesoscale dialysis chamber that can be prepared entirely from commercially available components. We used a 24-well plate as the basis for our DIY dialysis system. Each dialysis chamber consists of an independent feeding compartment, and a reaction chamber consisting of the dialysis cup. Dialysis cups have to fulfill two requirements. First, a molecular weight cutoff of 10 kDa is required, as this was shown to be ideal for coupling dialysis to cell-free protein expression.²⁵ Next, the geometry of the dialysis cup needs to allow the dialysis membrane to be fully immersed in the feeding solution, and the content of the reaction chamber needs to be accessible for imaging. Taking these considerations into account, we chose the Slide-A-Lyzer, 10K, with a 0.1 mL volume as our reaction chamber, which we cut to adjust its size to fit into the well plate (Figure 1A). This setup runs 100 μL cell-free reactions in 1000 μL feeding solution and allows easy exchange of the feeding solution (Figure 1B). We tested this dialysis with lysate reactions (RTS 500 *E. coli* HY (Biotech rabbit GmbH)) and PURE reactions (PURExpress (NEB)) combined with a homemade energy solution.

First, we assessed the influence of dialysis on GFP²⁶ expression in lysate and PURE reactions (Figure 2A). Protein

synthesis in a lysate reaction stops after less than 1 day, while PURE protein synthesis continues for about 4 days, leading to a higher overall protein yield for the PURE reaction of about 25300 RFU versus 19200 RFU for lysate. Interestingly, two synthesis rates can be clearly distinguished for the PURE dialysis reactions.

We then set out to investigate, whether replenishing the feeding solution can further prolong cell-free reactions. We replenished the feeding solution every three to four days, which we determined to be the lifetime of a standard PURE dialysis reaction. Experiments for both PURE and lysate were conducted in the same plate, and each condition was performed in duplicate. Expression of one of the replicates was monitored on the plate reader (Figure 2B,C), while the second replicate was incubated in parallel and used only for end point protein quantification. The PURE reactions were incubated for a total of 14 days and incubation was briefly interrupted four times to exchange the feeding solution for the PURE reactions. Periodic exchange of the feeding solution extended protein synthesis, maintaining a constant expression rate for eight additional days compared to a reaction without replenishment, leading to active protein synthesis for 12.5 days (Figure 2B). For lysate reactions, the feeding solution was only exchanged during the first two exchanges as protein synthesis did not recover after the first exchange (Figure 2C).

Lastly, total protein expression yield was determined for each sample. Reactions were recovered from the dialysis cups by dilution and resuspension with a defined volume of water leading to a ten time dilution of the recovered fraction. This ensured resuspension of potential protein precipitates caused by the long incubation time and the high overall protein concentration. Each solution was then introduced into the plate reader with technical duplicates together with a GFP calibration curve for quantification by fluorescence (Figure 2D, Figure S1 and Table S1). PURE reactions using dialysis without replenishment had an overall yield of 0.43 ± 0.02 mg/mL and 0.32 ± 0.01 mg/mL respectively. Replenishing the feeding solution every 3.5 days resulted in a 2.8-fold increase and a total protein yield of 1.05 ± 0.04 and 1.16 ± 0.05 mg/mL GFP, resulting in mean concentrations of 31.18 mM and 34.49 mM respectively. Protein yields for lysate reactions were considerably lower, and replenishing the feeding solution did not lead to an increase in protein yield. Final protein concentrations in lysate experiments ranged between 0.24 \pm 0.00 mg/mL and 0.36 ± 0.02 mg/mL.

DISCUSSION

In this work, we introduce a simple system for mesoscale cell-free protein expression augmented with dialysis. Using this dialysis system, we extended active protein synthesis in PURExpress from a few hours^{6–8} to around four days without exchanging the feeding solution in the feeding compartment. Exchanging the feeding solution every three to four days further extended active protein synthesis to 12.5 days, and an overall protein yield of 1 mg/mL. This presents an increase in total protein yield of at least five fold compared to concentrations of around 150 $\mu\text{g}/\text{mL}$ without dialysis.⁷ It needs to be mentioned that after 14 days of incubation, precipitate accumulation was observed on the dialysis membranes, although we could not determine at which point precipitate formation started. These precipitates might impede exchange of small molecules across the dialysis membranes, hindering the continued supply of small molecules and the

dilution of inhibitory molecules inside the PURE reaction, and could impact fluorescence imaging. It is thus not clear whether cessation of protein synthesis after 12.5 days occurred because of PURE component degradation, or due to obstructed dialysis. Using the same DIY dialysis system did not extend protein synthesis of a lysate-based system and the overall protein yield was substantially lower. We reason that cessation of protein synthesis could be due to degradation of lysate components.^{10,19} Recent findings by Ouyang and co-workers demonstrated active protein synthesis in lysate for 28 days using hydrogel beads.¹³ Encapsulation thus seems to prevent those degradation processes and seems to be required for prolonged protein synthesis in lysate systems. Interestingly, PURE sustains a stable synthesis rate without encapsulation for 12.5 days, which is comparable to the previously published value of 11–16 days using hydrogel encapsulation.^{14,15} This indicates that the PURE formulation is sufficiently free of proteases, which could negatively impact long-term protein synthesis. Protein expression using our dialysis system resulted in an increase in protein yield of about five fold compared to hydrogel based expression,^{14,15} rendering the open dialysis system more suitable for applications where high protein concentrations are beneficial, in addition to being simpler to use.

Kazuta and co-workers, have shown that commercial PURE formulations are not optimized for high yield protein expression.¹² It will be interesting to see, what yields can be achieved when combining optimized PURE compositions with simple mesoscale dialysis systems and periodic exchange of solutions. One avenue toward further increasing protein yield may be by reducing protein aggregation, for instance through the addition of chaperones.²⁷ An interesting phenomena is the decrease in synthesis rate approximately after 17 h, which we have previously reported using a different dialysis system with continuous exchange of the feeding solution.¹⁶ Further investigating this behavior and determining what limits protein synthesis rate during this phase might provide insights for further increasing protein yield in this system.

METHODS

Preparation of the Dialysis Plate. The dialysis cups (Slide-A-Lyzer MINI Dialysis Device, 10K MWCO, 0.1 mL) were cut below the rim to fit into the 24 well plate (Nunc, Thermo Fisher). The cups were then adhered to a transparent plate sealant (SealPlate film Z369659) while still empty, by securing them with three thin strips of tape. An additional strip of tape was wrapped around the entire perimeter of the cup for further reinforcement, and the cup-containing sealant was arranged on top of the plate ensuring the dialysis cup was centered in the well (Figure 1A). One mL of feeding solution (see below) was introduced into the feeding compartment by punching the seal with a needle. Subsequently, 100 μ L of the reaction solution (see below) were introduced into the dialysis cup. After the assembly of all reactions, plasmid DNA (see below) was introduced into each dialysis cup to initiate the reactions. The plate was then sealed with an additional layer of plate sealant and placed in the plate reader. The plate was incubated at 32 °C, monitoring the fluorescence over time (Excitation 480 nm, Emission: 504 nm).

To replenish the feeding solution, the plate was removed from the plate reader. Subsequently, the seal was perforated with a syringe and the spent feeding solution was aspirated. Using a fresh syringe, the feeding compartment was then

replenished with fresh solution, the plate was sealed with another layer of transparent plate sealant, and the plate was inserted back into the plate reader for another round of incubation (Figure 1B). A total volume of 1000 μ L of feeding solution in the feeding compartment was sufficient to entirely immerse the dialysis membrane in solution.

Energy and Feeding Solution Preparation for PURE.

The energy and feeding solution was prepared as previously published^{18,16} at 2.5 \times , but omitting tRNAs. The 2.5 \times energy and feeding solutions contained 125 mM HEPES, 250 mM potassium glutamate, 29.5 mM magnesium acetate, 5 mM ATP and GTP respectively, 2.5 mM UTP and CTP respectively, 50 mM creatine phosphate, 2.5 mM TCEP, 0.05 mM folic acid, 5 mM spermidine, and 0.75 mM of each amino acid. The solution was used at 2.5 \times concentration in the feeding solution, and was added as a 1 \times energy solution in the PURE reaction in the reaction compartment, supplemented with tRNAs.

The tRNAs were purified from *E. coli* BL21, slightly adapted from a previously described protocol.²⁸ Briefly, a culture of *E. coli* BL21 cells was grown at 37 °C for 6 h, and cells were harvested by centrifugation. The cell pellet was weighed and resuspended in five times the weight of the pellet in resuspension buffer (10 mM HEPES, 10 mM MgCl₂, pH 7.2), e.g., a 5 g cell pellet was resuspended in 25 mL resuspension buffer. The same amount of equilibrated phenol (Invitrogen) was added to achieve a 1:1 v/v ratio. The solution was mixed and incubated for 30 min at 4 °C on a lab rotisserie. Phases were separated by centrifugation for 10 min at 4000g and the aqueous phase was transferred to a new tube. Ultrapure isopropanol was added to achieve a 1:1 v/v ratio and the solution was incubated at –20 °C overnight. Nucleic acids were precipitated by centrifugation for 10 min at 4 °C and 4000g. The pellet was resuspended in lithium buffer (0.8 M LiCl, 0.8 M NaCl), and repelleted with another centrifugation step using the previous conditions. The supernatant was subsequently transferred to a new tube, isopropanol was added to achieve a v/v ratio of 1:1, followed by a precipitation step using the previous centrifugation parameters. The pellet was dissolved in ultrapure ethanol and subsequently pelleted for three rounds. After the third round, the pellet was dried with nitrogen and subsequently dissolved in as little final buffer volume as possible (40 mM NaCl, 10 mM HEPES in ultrapure water), typically resulting in a final concentration of around 25 mg/mL. One μ L of protector RNase inhibitor was added to approx 200 μ L of tRNA solution, and tRNAs were stored at –80 °C until further use.

Experimental Setup for PURE Expression with Dialysis.

For PURE protein expression, 1 \times PURExpress (NEB) solution B was supplemented with RNase inhibitor (2 U/ μ L), mScarlet, 10 mM TCEP, 0.47 mg/mL ampicillin, 1.5 mg/mL tRNAs, and 1 \times energy solution. The energy solution A from the commercially available kit was replaced by homemade energy solution, as the β -mercaptoethanol present in solution A is less stable than the TCEP used in the homemade solution, and was previously suspected to limit the lifetime of PURE reactions.¹⁶ The solution was assembled as a master mix for all reactions at a total reaction volume of 420 μ L. 100 μ L PURE solution were distributed to each dialysis cup, and the reaction was initiated by adding 5 nM of plasmid DNA. The feeding solution at 2.5 \times was supplemented with 2.5 mg/mL of ampicillin and applied to the feeding compartment. A 2.5 \times concentration has previously shown to be optimal for dialysis

reactions in PURE.¹⁶ It needs to be noted that, unlike in previous publications, the tRNAs were added solely to the reaction compartment, as their molecular weight would prevent them from diffusing via the 10 kDa cutoff dialysis membrane. Plasmid template DNA was used at a concentration of 5 nM.

Experimental Setup for Lysate Expression with Dialysis. Lysate reactions were assembled following the supplier's protocol (BR1400201 RTS 500 ProteoMaster *E. coli* HY Kit - Biotechrabbit GmbH), omitting the dialysis reaction device. The synthesis reaction was assembled as a master mix for all reactions in a total volume of 1030.5 μL using only freshly reconstituted components. The master mix included 525.5 μL of lysate, 225 μL of Reaction Mix, 250 μL of amino acids and 30 μL of methionine. The solution was mixed, and 100 μL were added into each dialysis cup. Remaining solution was either stored or directly used for further experiments. Plasmid DNA was added at a final concentration of 3.4 nM, as recommended by the supplier. The Feeding Mix was assembled by adding 2.65 mL of amino acids and 300 μL of methionine.

DNA Preparation. For all experiments, plasmid DNA encoding muGFP was used. The plasmid is a pET 29b(+) plasmid, and was purified from *E. coli* 10-beta (NEB) cells using a Zymo Miniprep kit according to the supplier's instructions. DNA elution was performed in water instead of elution buffer, as the latter contains EDTA, which complexes Mg^{2+} ions and is thus detrimental for cell-free reactions.

Experimental Setup for the GFP Control. GFP in water was used for the control well during the experiment, and for the mass calibration curve used for the end point quantification of the samples. It was obtained as follows. The same plasmid used for the experiments was transformed into BL21(DE3) (Lucigen) cells and plated. A colony of cells was grown overnight in LB media supplemented in kanamycin. For every liter of production culture, 20 mL of overnight culture were inoculated into Autoinduction TB (Formedium) media and grown at 37 °C. A total of 12 L culture was grown in batches of 2 L in 5 L flasks. After 4–5 h or an OD600 of >0.8, the incubator temperature was changed to 18 °C. The cultures were incubated overnight for at least 18 h. Cell pellets were harvested and stored at -20 °C until further use. Cell pellets were resuspended in Buffer A (700 mM NaCl, 20 mM HEPES 7.5), supplemented with glycerol to 10% v/v and 10 μL of Turbonuclease, then lysed by sonication. Sonication was performed for 2 min and 30 s in total, in pulses of 10 s on and 10 s off. For every 2 L of pellet, the approximate volume of lysate was 50 mL. Lysates were clarified by centrifugation, filtered through a 0.45 μm filter and supplemented with 25 mM imidazole. The sample was loaded onto a 25 mL NiNTA column (Cytiva or ProteinArk) and the protein was eluted on a gradient of 5–100% Buffer B (700 mM NaCl, 500 mM imidazole, 20 mM HEPES 7.5) on an AKTA system. Pooled eluted fractions were dialyzed twice in 5 L of Milli-Q water. The final concentration of the GFP solution was 1.87 mg/mL determined using a nanodrop at 280 nm.

The obtained solution was diluted to 0.47 mg/mL and introduced in the dialysis cup. The feeding compartment was filled with 1 mL of water. During the exchanges of the experiment reaction, the dialysis chamber was replenished with fresh water as well. The fluorescence was the monitored throughout the incubation period (Figure 1).

End Point Measurements. After 14 days of incubation, the plate was removed from the plate reader. The solutions in the reaction compartment were recovered by aspirating the content with a pipet, followed by several washing steps with water to recover all protein. This resulted in a final volume of 1 mL for each reaction, representing a 1:10 dilution. Duplicates of 40 μL for each reaction chamber were introduced in a 384 well plate for fluorescence measurement on the plate reader.

For the GFP calibration curve, the muGFP solution (see above) was diluted to 0.5, 0.1, 0.05, 0.01, and 0.005 mg/mL and applied to the same 384 plate in duplicates. The plate was centrifuged for 30 s at 10 000g and introduced into the plate reader. Readings were performed after 10 s shaking, and at room temperature. The calibration curve was obtained from a linear regression of the fluorescence counts averaged for the duplicates (Supplementary Figure S1). Sample concentrations were calculated from the measured RFU and the slope and intercept of the calibration curve. The obtained concentrations were averaged within the technical duplicates and the standard deviation was calculated (Supplementary Table S1).

The GFP control of our DIY dialysis system was used to assess the accuracy of the end point quantification method. By performing the same recovery method of the control well, we included duplicates of the GFP control to obtain the end point fluorescence of the latter, which allowed us to back-calculate the concentration. We obtained a value of 0.594 ± 0.009 mg/mL, indicating that the quantification method has an accuracy of about 25%.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acssynbio.4c00618>.

Fluorescence data of the samples; Fluorescence data of the calibrants; Calibration curve; Plasmid sequence (PDF)

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Author Contributions

[#]L.R. and L.G. contributed equally and performed experiments. L.R., L.G., F.S. and S.J.M. designed experiments, analyzed data, and wrote the manuscript.

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Notes

The authors declare no competing financial interest.

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