The all-\textit{E. coli} TXTL Toolbox 3.0: new capabilities of a cell-free synthetic biology platform

David Garenne$^1$, Seth Thompson$^1$, Amaury Brisson$^1$, Aset Khakimzhan$^1$, Vincent Noireaux$^{1, \ast}$

$^1$University of Minnesota, School of Physics and Astronomy, 115 Union Street SE, Minneapolis, 55455 Minnesota, United States of America.

Corresponding Author:

\`{V}Vincent Noireaux (noireaux@umn.edu)

Abstract

The new generation of cell-free gene expression systems enables prototyping and engineering biological systems \textit{in vitro} over a remarkable scope of applications and physical scales. As the utilization of DNA-directed \textit{in vitro} protein synthesis expands in scope, developing more powerful cell-free transcription-translation (TXTL) platforms remains a major goal to either execute larger DNA programs, or improve cell-free biomanufacturing capabilities. In this work, we report the capabilities of the all \textit{E. coli} TXTL toolbox 3.0, a multipurpose cell-free expression system specifically developed for synthetic biology. In non-fed batch mode reactions, synthesis of the fluorescent reporter protein eGFP reaches 4 mg/ml. In synthetic cells, consisting of liposomes loaded with a TXTL reaction, eGFP is produced to concentrations of more than 8 mg/ml when the chemical building blocks feeding the reaction diffuse through membrane channels to facilitate exchanges with the outer solution. The bacteriophage T7, encoded by a genome of 40 kbp and about 60 genes, is produced at a concentration of $10^{13}$ PFU/ml. This
TXTL system extends the current cell-free expression capabilities by offering unique strength and properties, for either testing regulatory elements and circuits, biomanufacturing biologics, or building synthetic cells.

**Keywords:** Cell-free transcription-translation; gene circuits; bacteriophages; synthetic cell;
1. Introduction

The emerging enthusiasm for cell-free gene expression as a multipurpose bioengineering technology arises from several major improvements made in the last two decades. These advances have been predominantly made for cell-free expression systems from *E. coli*, the most common model organism (1–5). First, cell-free transcription-translation (TXTL; cell-free transcription-translation) systems have become powerful enough to enable executing genetic programs composed of several genes relevant to natural living systems (6, 7) and integrating new technologies like CRISPR (8–11). The current TXTL technology is characterized by a fast experimental turnover and high-throughput settings (12), which facilitates the rapid prototyping of regulatory elements and gene networks (13–22). Besides gene circuits, the strength of the current TXTL platforms is leveraged to develop new biomanufacturing methods that offer unique speed of delivery and portability (23–27). Second, the preparation of TXTL systems, from *E. coli* especially, has been streamlined and reported in detail (28–30), which considerably improved the affordability and accessibility to these systems. Third, TXTL carries by nature a high degree of safety, making it ideal for applications outside laboratories, such as education (11, 31).

Altogether, cell-free gene expression has been shaped into a versatile and user-friendly tool covering an ever-growing scope of applications (6, 7, 32–34). Although TXTL systems from a broad variety of organisms are being developed (35), cell-free gene expression based on *E. coli* lysate remains the major testbed due to its strength and the knowledge of this system.

Among the different *E. coli* platforms developed, the all-*E. coli* TXTL system, now commercially available under the name myTXTL, was devised to incorporate a broad transcription repertoire that comprises the seven *E. coli* sigma factors in addition to the routinely used T7 and T3 bacteriophage RNA polymerases and promoters (36, 37). In its second version (38), a new ATP regeneration was used to raise protein synthesis up to 2.3 mg/ml in batch mode (39). By means of its extensive transcription capabilities and its strength, this TXTL system has been employed in many different physical settings for synthetic biology purposes or
to address fundamental questions (Figure 1). This system has proven effective for prototyping short DNA (13–17) and gene circuits (18–20, 40, 41), biomanufacturing (38, 42–46), and building synthetic cells (38, 47–54). It is also a convenient platform to interrogate biological systems at a basic level, including reconstituting dynamical systems (19), emulating pattern formation (20, 40), showing decision making based on a few molecules in regulatory networks (55), and revealing the importance of molecular crowding in two dimensions for the self-assembly of cytoskeletal proteins (49, 50).

In this article, we present the major capabilities of the new version of the all-\textit{E. coli} TXTL toolbox, focusing on protein synthesis through the transcription repertoire, the synthesis of the phage T7 used as a reference for the processing of large DNA programs, and protein synthesis in synthetic cell systems. Other characteristics of this toolbox have been reported in version 2.0 (38) such as mRNA and protein degradation and are not discussed in this work. When appropriate, we highlight the main differences between the three versions of the system, toolbox 1.0 published in 2012 (36), toolbox 2.0 published in 2016 (38), and toolbox 3.0 reported in this article.

2. Materials and Methods

2.1 TXTL system and batch mode reactions.

The preparation of the cell-free expression system used in this work has been described thoroughly in several articles (28, 36, 38, 56). The toolbox 2.0 (38) is commercialized under the name myTXTL (Arbor Biosciences). Compared to the toolbox 2.0 (38), two major modifications were made. First, the cells (\textit{E. coli} strain BL21 Rosetta2, Millipore Sigma) were grown at 40 °C instead of 37 °C during lysate preparation. Second, cell-free reactions were supplemented with 60 mM maltodextrin (Sigma Aldrich 419672) and 30 mM d-ribose (Sigma Aldrich R-7500) instead of just maltodextrin. The Table S1 summarizes the references that describe, in details, the preparation of this system and the differences between the toolboxes versions. Cell-free
reactions were carried out in a volume of 2 µl to 20 µl at 29-30 °C. The reactions were either assembled by hand (10-20 µl) or dispensed on 96 well plates using a Beckman Labcyte Echo liquid dispenser (2-5 µl). Quantitative measurements were carried out with the reporter protein deGFP (25.4 kDa, 1 mg/ml = 39.37 µM). deGFP is a variant of the reporter eGFP that is more translatable in cell-free systems. The excitation and emission spectra, as well as fluorescence properties of deGFP and eGFP, are identical, as reported before (36). The fluorescence of deGFP produced in batch mode reaction was measured on an H1m plate reader (Biotek Instruments, 96-well plate). Endpoint measurements were carried out after 15-20 h of incubation. Pure recombinant eGFP with a His tag (either from Cell Biolabs Inc. or Biovision) was used for quantification (linear calibration of the plate reader and microscope as described before (38)). Error bars are the standard deviations from at least three replicates.

2.2 DNA part lists and plasmid preparation

The DNA parts used in this work are available at Arbor Biosciences and are reported in Supplementary Table S2 (plasmids) and Supplementary Table S3 (linear). GenBank files of the plasmids used in this work are available as supplementary material. Unless specified, the plasmids contain the highly efficient untranslated region named UTR1 (36). The plasmids were amplified using standard mini or midi prep kits and further cleaned up with a PCR purification kit and eluted in autoclaved water. The concentration of the DNA stock solutions was quantified on a Nanodrop. Linear DNA templates were amplified by standard PCR from the respective plasmids, cleaned up using a PCR purification kit, and eluted in autoclaved water. P70a-gamS was obtained from Twist Biosciences and amplified by PCR.

2.3 TXTL synthesis of phages

The T7 genomic DNA was purchased from Boca Scientific. The chi6 short DNA (Integrated DNA Technologies) was added at a concentration of 3 µM to prevent the degradation of the linear T7
DNA (57). dNTPs (Invitrogen) were added to a concentration of 0.1 mM each to enable genome replication, as described before (44). The PEG8000 (Sigma Aldrich) concentration was increased from 2% (2.5 mM) to 3.5% (4.3 mM) to emulate molecular crowding (43). Bacteriophages were counted by the standard plaque-forming assay using the *E. coli* strain B for T7. Cells were grown in Luria-Bertani (LB) broth at 37 °C. The plates were prepared as follows: each sample was added to a solution composed of 5 ml of 0.6% liquid LB-agar (45 °C) and 50 µl of cell culture, poured on a 1.1% solid LB-agar plate. Plates were incubated at 37 °C and plaques counted after 6 h.

2.4 TXTL-based synthetic cells

The cell-free reactions were encapsulated into large unilamellar phospholipid vesicles by the water-in-oil emulsion transfer method (48). Briefly, phospholipids (Avanti Polar Lipids, PC 840051, PE-PEG5000 880200) were dissolved in mineral oil (Sigma-Aldrich M5904) at a total concentration of 2 mg/ml (molar proportion: 99.33% PC and 0.66% PE-PEG5000). A few microliters of cell-free reaction were added to 0.5 ml of the phospholipid solution. This solution was vortexed for 5-10 s to create an emulsion. 100-250 µl of the emulsion were placed on top of 20 µl of feeding solution. The vesicles are formed by centrifugation of the biphasic solution for 20 seconds at 4000 rpm. The phospholipid vesicles were observed with a CCD camera mounted on an inverted microscope (Olympus IX-81) equipped with the proper set of fluorescence filters. The feeding solution contained the same components as the reaction except for the DNA and lysate that were replaced by water. Pure alpha-hemolysin was purchased from Sigma Aldrich.

2.5 Materials and resources availability statement
The list of plasmids used in this work (available at Arbor Biosciences) is summarized in the Supplementary Table S2. The protocols for the CFE system are available in the references provided and in the Table S1. Other materials are available on reasonable request.

3. Results and discussion

3.1 Overall picture of the all *E. coli* TXTL system

To build a versatile toolbox that does not rely only on the T7 promoter and polymerase, our original goal was to develop an all *E. coli* TXTL system that integrates a broad transcription repertoire so as to execute circuits composed of different regulatory elements. The primary transcription is achieved by the housekeeping sigma factor σ70 and the core RNA polymerase, both provided by the lysate, which also brings all the necessary components for translation. Transcription by the six other sigma factors and the two bacteriophage RNA polymerases T3 and T7 are performed via transcriptional activation cascades. The second goal was to achieve a protein synthesis level large enough to enable the expression of large genetic programs and biomanufacturing of biologics. To this end, the toolbox incorporates a chemical ATP regeneration based on a phosphate donor and a carbohydrate to exploit the glycolytic pathway of the lysate (38, 39). Several other functionalities were developed: (i) protein degradation via the ClpXP proteases (38), (ii) tunable mRNA degradation via the interferase MazF (58), (iii) protection of linear DNA templates such as PCR products via the GamS protein (28) and the chi6 short linear dsDNA (57). These functionalities, valid for the new version of the system reported here, have been already described thoroughly. Lysates are prepared on a regular basis and tested for leftovers of living *E. coli* cells by plating the equivalent of 100-200 µl of cell-free reaction on LB-agar petri dishes without antibiotics. As reported several times before, no colonies are observed when this control is done (Supplementary Figure 1), making it a true cell-free expression system.
Compared to the toolbox 2.0 (38), the toolbox 3.0 reported in this article incorporates two changes that enable protein synthesis greater than 3 mg/ml. First, during the lysate preparation, the cells are grown at 40 °C instead of 37 °C. It was demonstrated previously that increasing the temperature of *E. coli* cultures can improve cell-free protein synthesis yields (59). The second modification applies to the reaction. Rather than just adding maltodextrin as a carbohydrate source to exploit glycolysis in the lysate (39), a mixture of maltodextrin (60 mM) and d-ribose (30 mM) is added to the reaction. We assume that maltodextrin and d-ribose improve ATP regeneration but their role would need to be clarified by a study outside the scope of this work. It is the combination of these two changes (temperature and carbohydrate mixture) that enables cell-free protein synthesis to reach up to 4 mg/ml.

### 3.2 Protein synthesis yields and time course

We measured protein synthesis in batch mode for the seven *E. coli* transcription factors, the two bacteriophage RNA polymerases T3 and T7, linear PCR products for both σ70 and T7, and we compared the results to the two previous versions of the toolbox (Table 1). Except for σ70 already present in the lysate, the synthesis of the reporter protein deGFP was achieved through a transcriptional activation cascade and specific promoters for each transcription factor or RNA polymerase. Each transcription factor or RNA polymerase was expressed through the strong *E. coli* promoter P70a and the untranslated region UTR1 (36), which originates from the bacteriophage T7 (60). The performance of all the sigma factors and bacteriophage RNA polymerases was largely greater than for the toolboxes 1.0 and 2.0 (Table 1). For toolbox 3.0, the maximum protein synthesis concentration was observed for the T7 cascade and topped 4 mg/ml. The effects of varying maltodextrin and d-ribose shows the synergy produced by the two carbohydrates (Supplementary Figure S2). The effect of the temperature of cell growth cultures during the lysate preparation was measured in the case of the T7 cascade to show that...
a temperature of 40 °C also contributes to the increased performance of the Toolbox 3.0 (Supplementary Figure S3).

The concentration of plasmids was varied for each transcription factor to find the optimal settings (Supplementary Figure S4-S12). When linear PCR amplified DNA templates were used, protein synthesis reached 1.77 and 2.97 mg/ml for σ70 and T7 respectively (Table 1). To achieve such a level of protein synthesis with linear DNA, both chi6 (3 µM) and P70a-gamS (linear, 1 nM) were added to the reaction. The protein GamS is dynamically synthesized to inhibit linear DNA degradation (61), concurrently with the expression of the reporter gene. These results were obtained using a liquid dispenser for reactions of volume 2 µl incubated on a 96 well plate, sterile disposable plastic wares and freshly prepared solutions. When prepared by hand, the protein synthesis yield of the reactions is usually lower and can reach, in the case of the T7 cascade, 3-3.5 mg/ml (Supplementary Figure 13). The variability of cell-free protein synthesis yields across different batches of TXTL systems was small (Supplementary Figure 13). The differences in protein synthesis via the T7 transcriptional activation cascade for the three toolboxes appear clearly on SDS PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) (Supplementary Figure 14).

We measured similar increased productions of the reporter proteins deCFP and mCherry through the T7 transcriptional activation cascade (Supplementary Figure S15), thus showing that our observations are not specific and limited to deGFP. With a batch mode protein synthesis of 3.4 mg/ml and a time course of almost one day, semi-continuous TXTL reactions, based on dialysis, become less relevant to implement for several reasons. Semi-continuous TXTL reactions are not cost-effective, not easy to handle and generally less reproducible than non-fed batch mode reactions. Instead, semi-continuous TXTL was carried out in synthetic cells as discussed thereafter.

We measured the time course of protein synthesis through the T7 cascade for plasmids and linear DNA, and compared it to the toolbox 2.0 settings (Figure 2). Two major differences
appeared in the time course between the two versions of the system. For the toolbox 3.0, protein accumulation is characterized by a greater synthesis rate and a longer synthesis time, which explain the larger yield at the end of incubation. These results show that the toolbox 3.0 is a long-lived TXTL system that can express genes for periods of time up to 20 h instead of 12-15 hours or the toolbox 2.0. This was observed for both plasmids and linear DNA templates (Figure 2).

3.3 TXTL of the bacteriophage T7
We demonstrated previously that several E. coli bacteriophages can be synthesized from their genomes in both the toolboxes 1.0 and 2.0 (38, 44). To get a sense of phage synthesis in the toolbox 3.0 settings, we tested the synthesis of phage T7 as it also achieves genome replication, using the plaque assay as described before (44). We use the T7 genome as a benchmark to challenge the capabilities of the all-E. coli TXTL system for processing large gene sets and recapitulating self-assembly. The 40-kbp genome of T7 encodes for about 60 genes, including its own DNA replication genes. DNA replication is achieved by adding dNTPs to the cell-free reaction, as shown before (44). In the toolbox 3.0, we measured a concentration of $10^{13}$ PFU/ml (plaque forming unit per ml) (Figure 3A, B, C), which is on the order of 10000 and 100 times larger than in the toolboxes 1.0 ($\approx 10^9$ PFU/ml) and 2.0 ($\approx 10^{11}$ PFU/ml) respectively.

3.4 TXTL synthetic cells
TXTL is the most common approach to build, from the ground up, genetically programmed synthetic cells, minimal cells in particular (62–74). Minimal cells consist of a TXTL reaction encapsulated in a cell-sized compartment, such as a liposome made of a phospholipid bilayer. Plasmids or linear DNA are added to the reaction so as to construct biological functions by expressing specific gene sets inside liposomes. (Figure 4A). This approach to synthetic cells has proven effective to emulate several natural mechanisms found in living cells (71, 75–80). To
determine how large protein synthesis can be in synthetic cells in the toolbox 3.0 conditions, we
expressed the gene degfp via the T7 cascade with and without the membrane channel alpha-
hemolysin (AH) (Figure 4B). AH forms channels of 1.4 nm diameter into the phospholipid
bilayer, which corresponds to a molecular mass cut off of about 3-5 kDa (81). Thus AH enables
feeding the compartmentalized cell-free reaction with the necessary small nutrients, such as
ATP and amino acids, by diffusion through the membrane. To quantify the fluorescence of
deGFP, we made a calibration (Supplementary Figure 16) as described previously (38). Because it is difficult to get above 100 µM with pure eGFP, the calibration was carried out
between 10 µM and 100 µM and found to be linear in this range. Given that the synthesis of the
reporter in the liposomes was larger than 100 µM, we used a neutral density filter to verify that
above 100 µM the fluorescence intensity for eGFP concentrations larger than 100 µM is still
linearly proportional to the calibration (Supplementary Figure 17). This approach allowed us to
keep the same illumination intensity. We measured the average concentration of deGFP for
populations of 50-100 liposomes with diameters ranging from 1 to 20 µm. When AH was not
added to the reaction and external solution, we measured an average concentration of about
180-190 µM (≈ 4.7 mg/ml), which is slightly larger than our measurements in batch mode test
tube reactions (Figure 4C). When AH was added to the encapsulated cell-free reaction and the
external feeding solution at a concentration of 0.1 µM, we measured an average concentration
of 330 µM (≈ 8.4 mg/ml) (Figure 4C). The difference in protein synthesis with and without AH
was more pronounced for small liposomes. The variability in fluorescence intensity for a same
population was also slightly smaller when AH was used. We found that a concentration of 0.1
µM AH was optimal. The time course of protein synthesis in the liposomes shows that AH
enables a greater synthesis rate in the first hours of expression (Figure 4C).

4. Conclusion
Although major advances have been made in the optimization of cell-free gene expression systems, the exploration of their capabilities is far from complete and will be central to the development of new synthetic biology applications. In this work, we showed that an *E. coli*-based TXTL system is capable of producing a reporter protein at a concentration of 4 mg/ml in non-fed batch mode reactions, a concentration not observed before in prokaryotic TXTL. In a semi-continuous synthetic cell setting, the synthesis of deGFP attains more than 8 mg/ml. No strain engineering was required to get such synthesis yields. The strength of the toolbox 3.0 should facilitate expressing large DNA programs encoding for biosynthesis pathways or for biological functions to build synthetic cells.

**Supplementary Data**

Supplementary data are available online.

**Data Availability Statement**

Data are available on reasonable request.

**Author Contributions**

DG: experiments, manuscript editing.

ST: experiments, manuscript editing.

AB: experiments.

AK: experiments, manuscript editing.

VN: manuscript writing and editing.

**Funding**

This work was supported by the National Science Foundation (NSF MCB-1844152, EF-1934496, CBET-1916030).
Competing financial interest

The authors declare the following competing financial interest(s): Noireaux laboratory receives royalties from Arbor Biosciences, a distributor of myTXTL cell-free protein expression kit. Vincent Noireaux consults with Arbor on other cell-free expression topics.
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Figure legends

Figure 1. Overview of the all-*E. coli* TXTL system and its applications. References are given as examples of the toolbox usage.

- **prototyping - biomanufacturing**
  - parts, circuits, pathways, biosynthesis, un-natural chemistry
  - References: (9) (13) (14) (15) (17)

- **self-assembly - nanotechnology**
  - phage parts, infectious phages, controlled self-assembly
  - References: (38) (42) (43) (44) (46)

- **synthetic cells - biotic/abiotic interfaces**
  - cell mechanics, sensors, self-reproduction, microchip
  - References: (19) (20) (40) (50) (52)
Figure 2. Time course of deGFP synthesis via the T7 transcriptional activation cascade. A. Schematic of the experiment achieved in batch mode using either plasmids or linear DNA. B. Graph showing the time course of deGFP synthesis in the conditions of the Toolbox 2.0 (TB 2.0) (38) and Toolbox 3.0 (TB 3.0, this work). The variability envelop is shown for each curve. Plasmids: 0.2 nM $P_{70\alpha}^{a}$-$T7\text{map}$, 4 nM $T7p14$-$\text{degfp}$. Linear DNA: 1 nM $P_{70\alpha}^{a}$-$\text{gamS}$, 0.2 nM $P_{70\alpha}^{a}$-$T7\text{map}$, 4 nM $T7p14$-$\text{degfp}$. 
Figure 3. Cell-free expression and synthesis of the phage T7. A. Schematic of the experiment. The cell-free reaction also contained 3 µM of chi6 short DNA to prevent degradation of the linear T7 genome and 0.1 mM of each of the dNTPs for genome replication. B. Image of plaques. Top half: results from a TXTL reaction synthesizing the phage T7. Bottom half: negative control showing a lawn of *E. coli* cells. C. Plot of the measured PFU/ml for the three versions of the toolbox.
Figure 4. Cell-free expression and synthesis of deGFP in synthetic cells. A. Schematic of the experiment (plasmids: P70a-T7map fixed at 0.2 nM, T7p14-degfp fixed at 4 nM). B. Fluorescence images of the liposomes, with and without alpha-hemolysin (AH), taken after 20 h of incubation with a 40x objective. C. Fluorescence intensity (F.I.) as a function of the square of the radius for two populations of liposomes, one without alpha-hemolysin (- AH) and one with alpha-hemolysin (+ AH, 0.1 µM added to the inner and outer solutions). Linear fits: F.I. = - 2.29 x 10^6 + 77452 r^2 (- AH, R = 0.96968) and F.I. = - 6.42 x 10^5 + 1.379 x 10^5 r^2 (+ AH, R = 0.97959). D. Time course of deGFP synthesis in synthetic cells, without and with AH added to the solution.
Table 1. Endpoint measurements of the reporter protein deGFP concentration for each of the transcription (TX) factors and RNA polymerases of the all-\textit{E. coli} TXTL toolboxes 1.0 (36), 2.0 (38) and 3.0 (this work). The values shown in this table are the average maximum protein synthesis yields for each transcription. Variability in cell-free protein synthesis for each transcription is shown in the error bars of each histogram plots (Supplementary Figures). The transcription factor $\sigma_{70}$ and the core RNA polymerase are present in the lysate. The other TX factors and bacteriophages RNA polymerases (first column) are expressed from plasmids or PCR products, through the promoter $P_{70a}$. The reporter gene \textit{degfp} is expressed through two-stage transcriptional activation cascades via the respective promoters (second column). 1 mg/mL deGFP = 39.4 $\mu$M.

| TX factors plasmid | Reporter plasmid | TB 1.0 deGFP $\mu$M (mg/ml) | TB 2.0 deGFP $\mu$M (mg/ml) | TB 3.0 (this work) deGFP $\mu$M (mg/ml) |
|--------------------|------------------|------------------------------|------------------------------|------------------------------------------|
| $\sigma_{70}$      | $P_{70a}$-\textit{degfp} | 25 (0.63)                  | 81 (2.05)                  | 130 (3.3)                              |
| $P_{70a}$-$\sigma_{19}$ | $P_{19a}$-\textit{degfp} | 7 (0.18)                   | 35 (0.89)                   | 54 (1.27)                              |
| $P_{70a}$-$\sigma_{24}$ | $P_{24a}$-\textit{degfp} | 11 (0.28)                  | 70 (1.78)                  | 95 (2.41)                              |
| $P_{70a}$-$\sigma_{28}$ | $P_{28a}$-\textit{degfp} | 21 (0.53)                  | 77 (1.95)                  | 122 (3.1)                              |
| $P_{70a}$-$\sigma_{32}$ | $P_{32a}$-\textit{degfp} | 19 (0.48)                  | 89 (2.26)                  | 100 (2.54)                              |
| $P_{70a}$-$\sigma_{38}$ | $P_{38a}$-\textit{degfp} | 13 (0.33)                  | 75 (1.90)                  | 96 (2.43)                              |
| $P_{70a}$-$\sigma_{54}$/\textit{ntrC} | $P_{54a}$-\textit{degfp} | 5 (0.13)                   | 27 (0.68)                   | 58 (1.47)                              |
| $P_{70a}$-$T3$map | $T3p14$-\textit{degfp} | 27 (0.69)                  | 74 (1.88)                  | 129 (3.27)                              |
| $P_{70a}$-$T7$map | $T7p14$-\textit{degfp} | 29 (0.74)                  | 87 (2.20)                  | 160 (4.05)                              |
| $\sigma_{70}$      | $P_{70a}$-\textit{degfp} | NA                         | 50 (1.27)                  | 70 (1.77)                              |
| $P_{70a}$-$T7$map | $T7p14$-\textit{degfp} | NA                         | 36 (0.91)                  | 117 (2.97)                              |
# Data Availability

| Yes | No |
|-----|----|
|     | Does the manuscript use or report the following? If so, please provide details in a Data Availability statement below and in the manuscript. |
| ✓  | req Novel nucleic acid sequences including sequences of new plasmids.  
- Must deposit in EMBL / GenBank / DDBJ.  
- Must provide sequence names and accession numbers or upload GenBank files in the article online supplement. Plasmid sequences should NOT be embedded in Word or PDF files. |
| ✓  | req Synthetic nucleic acid oligonucleotides including PCR primers, gRNA, siRNAs or shRNAs  
- Manuscript should provide exact sequences, exact details of chemical modifications at any position, and source of reagent or precise methods for creation.  
- Sequences should be provided as multiple sequence FASTA file or comma-delimited text files.  
- These can be included in Supplementary Material. |
| ✓  | req Mass spectrometry proteomics  
- Must deposit to ProteomeXchange consortium and provide Dataset Identifier and reviewer account details. |
| ✓  | req New genome expression or sequencing data (ChIP-seq, RNA-seq...)  
- Must comply with ENCODE Guidelines, deposit data in GEO, and view data on the UCSC (eukaryotes) or Artemis (prokaryotes) sequence browsers.  
- Must provide GEO accession numbers and private tokens for Referees and UCSC or Artemis genome browser session links (even if GEO entries are publicly available). |
| ✓  | req Nextgen sequencing reads including Illumina, PacBio, Oxford Nanopore  
- Must Submit sequencing reads to SRA, ArrayExpress or GEO. |
| Requirement | Details |
|-------------|---------|
| - | Must provide accession numbers and private tokens (GEO) or login details (ArrayExpress). |
| ✓ | Microscopy images including series of time-lapse microscopy images should be deposited with The Cell Image Library.  
- Must provide accession number. |
| ✓ | Novel nucleic acids structures  
- Must deposit to NDB (via PDB if possible) and provide accession details. |
| ✓ | Novel protein sequences including sequences of engineered proteins  
- Must submit to UniProt using the interactive tool SPIN and provide sequence names and accession number. |
| ✓ | Quantitative PCR  
- Must comply with the MIQE Guidelines.  
- Details should be supplied in Materials and Methods section of manuscript. |
| ✓ | Flow Cytometry experiments  
- Must deposit in FlowRepository.  
- Must provide Repository ID and secret code for Referees. |
| ✓ | Source code of open source software  
- Should be deposited in a publicly available repository (GitHub, SourceForge, BitBucket).  
- Source code should include an OSI-approved license.  
- Non-open source software including commercial software should be accessible anonymously and at no cost to the reviewers. |
| ✓ | Gel images, micrographs, graphs, and tables may be included in the article online supplement. Optionally, they may be deposited in a general-purpose repository such as Zenodo, Figshare, or Dryad. |
Supporting Data

The all *E. coli* TX-TL Toolbox 3.0: new capabilities of a cell-free expression system for synthetic biology

David Garenne, Seth Thompson, Amaury Brisson, Aset Khakimzhan and Vincent Noireaux*
School of Physics and Astronomy, University of Minnesota, 115 Union Street SE, Minneapolis, 55455 Minnesota, United States of America.

*Corresponding author: noireaux@umn.edu

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Table S1. References providing the detailed preparation of the cell-free expression system. From the Toolbox 1.0 to 2.0, 30 mM of maltodextrin (Mdx) is added to the reaction. From the Toolbox 2.0 to 3.0, the Mdx is added to the reaction at a concentration of 60 mM, d-ribose is added at a concentration of 30 mM and the lysate is prepared from cells grown at 40 °C, using the procedure described in reference (1).

| Components                  | TB 1.0  | TB 2.0  | TB 3.0  |
|-----------------------------|---------|---------|---------|
| Lysate                      | (1)     | (1)     | (1)     |
| Energy mix                  | (1)     | (1)     | (1)     |
| Amino acid mix              | (2)     | (2)     | (2)     |
| Reaction composition        | (1, 3)  | (1, 4)  | (1, 4)  |
| Other components            |         | Mdx at 30 mM | Mdx at 60 mM |
|                             |         |         | d-ribose at 30 mM |
|                             |         |         | 40 °C lysate |

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Table S2. List of plasmids used in this work. The right column shows the product number of each plasmid available at Arbor Biosciences. All these plasmids are also available as GenBank files in the Supplementary Data.

| plasmid name     | reference | product number |
|------------------|-----------|----------------|
| P\textsubscript{70a}-\sigma\textsubscript{19} | (3, 4)    | 502067         |
| P\textsubscript{70a}-\sigma\textsubscript{34} | (3, 4)    | 502069         |
| P\textsubscript{70a}-\sigma\textsubscript{28} | (3, 4)    | 502072         |
| P\textsubscript{70a}-\sigma\textsubscript{32} | (3, 4)    | 502075         |
| P\textsubscript{70a}-\sigma\textsubscript{38} | (3, 4)    | 502077         |
| P\textsubscript{70a}-\sigma\textsubscript{54} | (3, 4)    | 502079         |
| P\textsubscript{70a}-ntrC   | (3, 4)    | 502071         |
| P\textsubscript{70a}-T3map   | (3, 4)    | 502081         |
| P\textsubscript{70a}-T7map   | (3, 4)    | 502082         |
| P\textsubscript{70a}-degfp   | (3, 4)    | 502086         |
| P\textsubscript{19a}-degfp   | (3, 4)    | 502001         |
| P\textsubscript{24a}-degfp   | (3, 4)    | 502010         |
| P\textsubscript{28a}-degfp   | (3, 4)    | 502019         |
| P\textsubscript{32a}-degfp   | (3, 4)    | 502040         |
| P\textsubscript{38a}-degfp   | (3, 4)    | 502041         |
| P\textsubscript{44a}-degfp   | (3, 4)    | 502048         |
| T3p14-degfp   | (3, 4)    | 502111         |
| T7p14-degfp   | (3, 4)    | 502104         |
| T7p14-decfp   | This work |                |
| T7p14-mcherry | This work |                |
The promoters are highlighted in yellow.
The *degfp* reporter gene is highlighted in green.
The other genes are highlighted in blue.
The transcription terminator is highlighted in grey.
The primers used for amplification are underlined.
Table S3. List and sequences of linear DNA used in this work.
Figure S1. Plating of different lysates on agar plates. All the LB-agar plates (no antibiotic) were incubated at 37°C for 16 h. The equivalent of 100 µl of cell-free reaction was plated on each plate. Top left: as a positive control, about 50 E. coli cells were added to the lysate before plating. Top right and bottom: 100 µl of three different batches of cell-free reaction (three different lysates) were plated on each of the three plates. No colonies were observed.
Figure S2. Two-stage transcriptional activation cascade using the T7 RNA polymerase. The reactions were prepared on a liquid dispensing machine. Top: schematic of the circuit (only plasmids were used: 0.2 nM $P_{70a}$-$T7rnap$, 4 nM $T7p14$-$degfp$). Bottom: endpoint measurement of deGFP synthesis when the maltodextrin (Mdx) and ribose concentrations are varied in the reaction.
Figure S3. Two-stage transcriptional activation cascade using the T7 RNA polymerase. The reactions were prepared on a liquid dispensing machine. **Top**: schematic of the circuit (only plasmids were used: 0.2 nM P$_{70}$a-T7rnap, 4 nM T7p14-degfp). **Bottom**: endpoint measurement of deGFP synthesis for a lysate prepared from cells grown at 37 °C and for a lysate prepared from cells grown at 40 °C.
Figure S4. Cell-free synthesis of deGFP via an E. coli promoter specific to the housekeeping σ70. Top: Schematic of the gene circuit. Both plasmid and PCR product of the plasmid can be used as template. Bottom left: endpoint measurement of the concentration of synthesized deGFP vs plasmid concentration. Response typically saturates above 5-10 nM. Bottom right: endpoint measurement of the concentration of synthesized deGFP vs PCR product concentration. Response saturates or even decreases above 10 nM.
Figure S5. Two-stage transcriptional activation cascade using *E. coli* $\sigma_{19}$. **Top:** schematic of the circuit (only closed circular plasmids were used). **Bottom:** endpoint measurement of the concentration of synthesized deGFP as a function of the concentration of the two plasmids.
Figure S6. Two-stage transcriptional activation cascade using *E. coli* σ24. Top: schematic of the circuit (only closed circular plasmids were used). Bottom: endpoint measurement of the concentration of synthesized deGFP as a function of the concentration of the two plasmids.
Figure S7. Two-stage transcriptional activation cascade using *E. coli* $\sigma_28$. **Top:** schematic of the circuit (only closed circular plasmids were used). **Bottom:** endpoint measurement of the concentration of synthesized deGFP as a function of the concentration of the two plasmids.
Figure S8. Two-stage transcriptional activation cascade using *E. coli* $\sigma_{32}$. **Top**: schematic of the circuit (only closed circular plasmids were used). **Bottom**: endpoint measurement of the concentration of synthesized deGFP as a function of the concentration of the two plasmids.
Figure S9. Two-stage transcriptional activation cascade using *E. coli* σ₃₈. **Top:** schematic of the circuit (only closed circular plasmids were used). **Bottom:** endpoint measurement of the concentration of synthesized deGFP as a function of the concentration of the two plasmids.
Figure S10. Two-stage transcriptional activation cascade using *E. coli* $\sigma_{54}$ and NtrC. **Top:** schematic of the circuit (only closed circular plasmids were used). **Bottom:** endpoint measurement of the concentration of synthesized deGFP as a function of the concentration of the two plasmids.
Figure S11. Two-stage transcriptional activation cascade using the T7 RNA polymerase. **Top:** schematic of the circuit (only closed circular plasmids were used). **Bottom:** endpoint measurement of the concentration of synthesized deGFP as a function of the concentration of the two plasmids.
Figure S12. Two-stage transcriptional activation cascade using the T3 RNA polymerase. **Top:** schematic of the circuit (only closed circular plasmids were used). **Bottom:** endpoint measurement of the concentration of synthesized deGFP as a function of the concentration of the two plasmids.
Figure S13. Two-stage transcriptional activation cascade using the T7 RNA polymerase, experiments carried by hand for two different lysates. The experiments consisted of adding the plasmids and water to a reaction mix, all done by hand. **Top:** schematic of the circuit (only plasmids were used). **Bottom:** endpoint measurement of the concentration of synthesized deGFP as a function of the concentration of the two plasmids.
Figure S14. Two-stage transcriptional activation cascade using the T7 RNA polymerase. The reactions were prepared on a liquid dispensing machine. Concentrations of deGFP synthesized: (i) Toolbox 1.0 (3): 25 µM, Toolbox 2.0 (4): 80 µM, Toolbox 3.0 (this work): 160 µM. Top: schematic of the circuit (only plasmids were used). Bottom: image of the SDS PAGE. C: control (CFE reaction no plasmid), M: marker, TB1, TB2, TB3 toolboxes 1.0, 2.0, 3.0. The bands for deGFP are shown by the arrow on the left.
Figure S15. Synthesis of deCFP and mCherry through the T7 transcriptional activation cascade for the three different toolboxes conditions. The experiments were prepared by a liquid dispenser. Top: schematic of the circuits (only plasmids were used). Bottom: endpoint measurement of the concentration of synthesized deCFP and mCherry for the three toolboxes. The fluorescence intensity was scaled to 100% (toolbox 3.0 conditions) for each reporter.
Figure S16. Calibration of fluorescence in liposomes. **Top:** pure eGFP (10 µM, 33.33 µM or 100 µM) was added to a TXTL reaction (no plasmid) that was encapsulated into cell-sized liposomes. **Bottom left:** fluorescence intensity (F.I.) of liposome populations for the three different eGFP concentrations (10, 33.33, 100 µM) as a function of the square of the radii. **Bottom right:** linear fit for the three curves.
**Figure S17.** Fluorescence measured with a neutral density filter to reduce light intensity. 

**Top:** Schematic of the experiment (plasmids: P70a-T7map fixed at 0.2 nM, T7p14-degfp fixed at 4 nM). AH was added to the external and internal solutions at a concentration of 0.1 µM. **Bottom left:** fluorescence intensity (F.I.) of liposome populations without and with a neutral density filter reducing the light intensity by a factor of 4. Note that the scattered plot without filter is the same as in Figure 4, with AH. **Bottom right:** linear fits for the two scattered plots. We effectively measured a factor of 4 between the two linear fit coefficients.
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