Chapter 4

Synthetic Biology with an All *E. coli* TXTL System: Quantitative Characterization of Regulatory Elements and Gene Circuits

Ryan Marshall and Vincent Noireaux

Abstract

Over the past decade, a new generation of cell-free transcription-translation (TXTL) systems has been devised for emerging multidisciplinary applications. The DNA-dependent in vitro protein synthesis technology has been developed to tackle applications in synthetic biology, biological and chemical engineering, as well as quantitative disciplines such as biophysics. In addition to being convenient at the biosafety level, the new TXTL platforms are user-friendly; more affordable; more versatile at the level of transcription, with a TX repertoire covering hundreds of parts; and more powerful, with protein production reaching a few mg/mL in batch and continuous modes. As a consequence, TXTL is rising up as a popular research tool and is used by a growing research community. While TXTL is proving reliable for an increasing number of applications, it is important to gain appropriate TXTL skills, especially for quantitative applications. TXTL has become particularly useful to rapidly prototype genetic devices, from single regulatory elements to elementary circuit motifs. In this chapter, we describe the basic procedures to develop appropriate TXTL practices for the characterization of such genetic parts. We use an all *E. coli* TXTL system developed in our lab, now commercialized by Arbor Biosciences under the name myTXTL.

Key words TXTL (cell-free transcription-translation), *E. coli*, Prototyping, Regulatory elements, Gene circuits, Quantitative biology, CRISPR

1 Introduction

Until the 1990's, cell-free expression systems had been developed and primarily used as a tool to produce proteins outside living organisms [1]. In the past 15 years, a new generation of TXTL systems has been devised to address a broad scope of post-genomic multidisciplinary applications. TXTL systems are now used in many engineering disciplines, including synthetic biology [2, 3], biotechnologies and chemical engineering [4, 5], as well as for nanosciences [6] and medicine [7]. The TXTL technology has been reshaped to respond to the increasing interest for constructing complex biochemical systems in vitro through the execution

Jeffrey Carl Braman (ed.), *Synthetic Biology: Methods and Protocols*, Methods in Molecular Biology, vol. 1772,
https://doi.org/10.1007/978-1-4939-7795-6_4,

of genetic information [8]. Fostered by other techniques, for instance microfluidics and DNA synthesis, TXTL has recently proven useful as a quantitative platform to rapidly prototype regulatory elements and to recapitulate complex biological processes in vitro by expressing synthetic or natural gene circuits [9–15].

While TXTL systems can be prepared from different organisms, *E. coli* extract-based platforms are the most utilized because they are more affordable and their preparation has been well described [16–18]. Besides being convenient at the biosafety level, the new *E. coli* TXTL systems have been improved so as to present several advantages compared to the first generation and to other methods used to construct biological systems in test tube reactions. First, novel metabolisms have been found to fuel cell-free expression up to 2 mg/mL in batch mode and more than 5 mg/mL in semi-continuous mode [10]. Such performances allow the execution of larger and larger DNA programs in vitro, as demonstrated by the complete synthesis of the 40-kbp phage T7 [19]. Second, the scalability of TXTL and the variety of setups accommodating TXTL reactions have been considerably improved. TXTL reactions are performed from femtoliter scale, in cell-sized compartments [20, 21], up to tens of liters [22], and in test tubes, microfluidics, well plates, or on paper [13, 15, 23]. Third, TXTL has become modular so as to incorporate new functions, such as non-natural biochemistries [24–26]. The TXTL system used in this chapter, also named myTXTL, integrates all these advances [10]. In addition, myTXTL possesses three unique features. First, it has the most versatile TX of all the systems currently available. The TX is based on the *E. coli* core RNA polymerase and primary sigma factor 70 [27], thus expanding the transcription repertoire to hundreds of regulatory elements, as opposed to the traditional hybrid TXTL systems based on the T7 RNA polymerase and promoter. myTXTL can be easily transformed into the traditional T7 system by simply expressing the T7 RNA polymerase through an *E. coli* promoter. Second, two methods have been developed to tune the mRNA and protein degradation rates [28]. In myTXTL, synthesis and degradation rates can be easily varied so as to explore the dynamics of gene circuits in test tube reactions.

While TXTL systems are becoming as popular as rapid bioengineering platforms, it is essential to understand the basic operating procedures of such systems. That is the objective of this chapter, where we walk through the very basic protocols to set up a TXTL reaction and perform elementary gene circuits quantitatively. Most of the genetic parts and circuits used in this chapter have been reported in one of our recent publications [10]. Plasmid design is, first and foremost, the critical step before going into some TXTL experiments, especially for circuit engineering. myTXTL was specifically developed so as to use the housekeeping TX machinery from *E. coli*. Consequently, all the circuitries are booted up from *E. coli* promoters specific to sigma factor 70. One of the strongest

E. coli promoters is P70a [10], derived from the lambda phage and repressible by the CI repressor. Any of the six other *E. coli* sigma factors can be expressed, as well as the T7 and T3 RNA polymerases, through a sigma 70 promoter, and used with their respective promoters [10]. The untranslated region (UTR) containing the ribosome-binding site is also critical to adjust the strength of protein expression. The strongest *E. coli* UTR so far reported is the leader sequence of the g10 gene from the bacteriophage T7, which we named UTR1. When P70a and UTR1 are associated, the synthesis of eGFP can reach up to 2 mg/mL (80 μM) in batch mode reactions, comparable to using a T7 promoter.

2 Materials

2.1 Genetic Part List

See Table 1.

Table 1
Genetic part lists used in this chapter

Promoter	Description	Source - references
P_{70a}	Lambda phage promoter OR_2-OR_1-Pr specific to *E. coli* σ_{70}	GenBank: J02459.1 [27]
P_{70b}	Mutated version of P_{70a}	Unpublished
P_{70c}	Mutated version of P_{70a}	Unpublished
P_{70d}	Mutated version of P_{70a}	Unpublished
P_{19a}	Promoter of the *E. coli fec* transporter genes specific to σ_{19}	GenBank: U00096.2 [29]
P_{24a}	Promoter of the *htrA* gene (*E. coli*) specific to σ_{24}	GenBank: U00096.2 [30]
P_{28a}	Promoter of the *tar* gene (*E. coli*) specific to σ_{28}	GenBank: U00096.2 [31]
P_{28a} -tetO1	P_{28a} promoter with *tetO1* operator sites upstream of −35 and downstream of −10.	Unpublished
P_{32a}	Promoter of the *groE* gene (*E. coli*) specific to σ_{32}	GenBank: U00096.2 [32]
P_{38a}	Promoter of the *osmY* gene (*E. coli*) specific to σ_{38}	GenBank: U00096.2 [33]
P_{54a}	Promoter of the *glna* gene (*E. coli*) specific to σ_{54}	GenBank: U00096.2 [34]
$P_{L\text{-}tetO1}$	Promoter specific to *E. coli* σ_{70} regulated by the *tetR* gene	[35]

UTRs	Description	Source – References
UTR1	The untranslated region containing the T7 g10 leader sequence for highly efficient translation initiation	GenBank: M35614.1 [27, 36]
UTR2	UTR1 with mutation in RBS	Unpublished
UTR3	UTR1 with mutation in RBS	Unpublished
UTR4	UTR1 with mutation in RBS	Unpublished

(continued)

Table 1
(continued)

TX term.	Description	Source – References
T500	Transcription terminator for *E. coli* RNA polymerase	[37]

Genes	Description	Source – References
deGFP	eGFP truncated and modified in N- and C- terminus	[27]
Broccoli	Fluorescent RNA aptamer (requires the cofactor DFHB1-T)	[27]
σ_{19}	*fecI* (*E. coli* σ_{19})	GenBank: U00096.2
σ_{24}	*rpoE* (*E. coli* σ_{24})	GenBank: U00096.2
σ_{28}	*rpoF* (*E. coli* σ_{28})	GenBank: U00096.2
σ_{28}-*ssrA*	*rpoF* tagged with *ssrA*	[9]
σ_{32}	*rpoH* (*E. coli* σ_{32})	GenBank: U00096.2
σ_{38}	*rpoS* (*E. coli* σ_{38})	GenBank: U00096.2
σ_{54}	*rpoN* (*E. coli* σ_{54})	GenBank: U00096.2
clpXP	*E. coli* clpXP tandem proteins (same operon)	GenBank: J05534.1, L18867.1
ntrC	Co-activator of σ^{54} promoters	GenBank: U00096.2
mazF	*E. coli* interferase MazF (*chpA* gene)	GenBank: CDJ74047.1
T7 RNAP	T7 bacteriophage RNA polymerase	GenBank: FJ881694.1
tetR	Tet operon regulatory gene	GenBank: BAG71042.1
Cl	Lambda phage repressor protein Cl	GenBank: CAB96428.1
ssrA	SsrA peptide for degradation by AAA+ proteases	[38]
sgRNA-1	Single guide RNA under the promoter J23119, targeting P70a-deGFP	[39]
sgRNA-2	Single guide RNA under the promoter J23119, targeting P70a-deGFP	[39]
sgRNA-3	Single guide RNA under the promoter J23119, targeting P70a-deGFP	[39]
sgRNA-4	Single guide RNA under the promoter J23119, targeting P70a-deGFP	[39]
sgRNA-NT	Non-targeting single guide RNA under the promoter J23119	[39]
dCas9	Deactivated (catalytically dead) Cas9 protein from *S. pyogenes*	[39]
MGapt	Fluorescent RNA aptamer (requires the cofactor malachite green)	Unpublished

2.2 Plasmid List

All the plasmids and linear DNA listed below can be found on Benchling (www.benchling.com) using the following link: https://benchling.com/noireaux/f_/Cg9KQ3ps-mmb-ssb-txtl-2017-part-list/

1. P70a-deGFP.
2. P70a-deGFP-ssrA.
3. P70a-Broccoli.
4. P70b-Broccoli.
5. P70c-Broccoli.
6. P70d-Broccoli.
7. P70a-UTR2-deGFP.
8. P70a-UTR3-deGFP.
9. P70a-UTR4-deGFP.
10. Spy-dCas9.
11. P70a-S19.
12. P70a-S24.
13. P70a-S28.
14. P70a-S32.
15. P70a-S38.
16. P19a-deGFP.
17. P24a-deGFP.
18. P28a-deGFP.
19. P32a-deGFP.
20. P38a-deGFP.
21. P70a-cI.
22. PL-tetO1-tetR.
23. PL-tetO1-deGFP.
24. PL-tetO1-deGFP-ssrA
25. P28a-tetO1-deGFP-ssrA.
26. P28a-tetR-ssrA.
27. P70a-S28-ssrA.
28. P70a-ClpXP.

2.3 Linear DNA List

1. T7-Broccoli-s oligo.
2. T7-Broccoli-as oligo.
3. T7-MGapt-s oligo.
4. T7-MGapt-as oligo.
5. Chi6-s oligo.
6. Chi6-as oligo.

7. Spy-sgRNA-2.
8. Spy-sgRNA-3.
9. Spy-sgRNA-4.
10. Spy-sgRNA-5.
11. Spy-sgRNA-NT.

2.4 Measurements

1. Nunc 384 flat-bottom well plate.
2. Well plate sealing tape.
3. V-bottom 96-well plate.
4. Caps for 96 V-bottom well plate.
5. Plate reader (Biotek H1m).
6. reGFP (CellBio Labs).
7. DFHB1-T dye (Lucerna).
8. Malachite Green.
9. PBS.

2.5 Cell-Free Reaction

1. Composition of the split system: Table 2.
2. MYtxtl kit (MYcroarray).
3. Midi prep kit.
4. PCR cleanup kit.
5. 1.7 mL Posiclick tubes.
6. T7 in vitro TX kit.
7. RNA clean and concentrator kit.
8. Mg-Glutamate.

Table 2
Split system reaction conditions

Reaction component	Final concentration in TXTL
Cell extract	~1/3 of reaction volume
Water	Fill to 100% reaction volume
Potassium glutamate	60–100 mM
Magnesium glutamate	3–5 mM
Maltodextrin	30 mM
Energy buffer	18×
Amino acid mix	3 mM
PEG 8000	1.5% weight/volume
DNA, cofactors	Add desired concentrations

9. K-Glutamate.
10. S30B buffer: 14 mM Mg-glutamate, 150 mM K-glutamate, buffered to pH 8.2 with a 2 M Tris solution.
11. IPTG.
12. PEG8000.
13. Maltodextrin.
14. RNase A.
15. Rifampicin.
16. 6His-eGFP-ssrA [28].

2.6 SDS PAGE

1. 1.5 M Tris buffered to pH 8.8 with HCl.
2. 1 M Tris buffered to pH 6.8 with HCl.
3. 20% w/v SDS in water.
4. 10% w/v ammonium per sulfate (APS) in water.
5. 40% w/v acrylamide.
6. Temed.
7. Bromophenol blue.
8. 2× Sample buffer: 100 mM Tris–HCl pH 6.8, 200 mM DTT, 4% w/v SDS, 0.1% w/v bromophenol blue, 20% glycerol.
9. Running buffer: 25 mM Tris, 192 mM Glycine, 0.1% w/v SDS.
10. Hoefer Mighty Small II Mini Vertical Electrophoresis System.
11. Hoefer Dual Gel Caster.
12. Power supply: Bio-Rad PowerPac.
13. SimplyBlue Safestain.
14. Scanner for gel (any basic office type of scanner).

3 Methods

3.1 About myTXTL and Some Numbers

myTXTL is an *E. coli* extract-based cell-free TXTL system developed in the Noireaux lab between 2010 and 2016, specifically optimized for rapid characterization of genetic elements and gene circuits (Fig. 1). The preparation of this system has been reported in several publications [40, 41], and the last version of the system, the toolbox 2.0, was published recently [10]. The amino acid mixture now used with this system [42] contains equimolar concentrations of the 20 canonical amino acids. The energy mixture that fuels TXTL is composed of an ATP regeneration system and a carbon source: maltose or maltodextrin. The TX is based on the *E. coli* core RNA polymerase and primary sigma factor 70 (gene *rpoD*). Consequently, expression is initiated through promoters specific to sigma factor 70, with consensus sequence −35 (-TTGACA-) and −10 (-TATAAT-). One of the strongest promoters is P70a, derived from the lambda

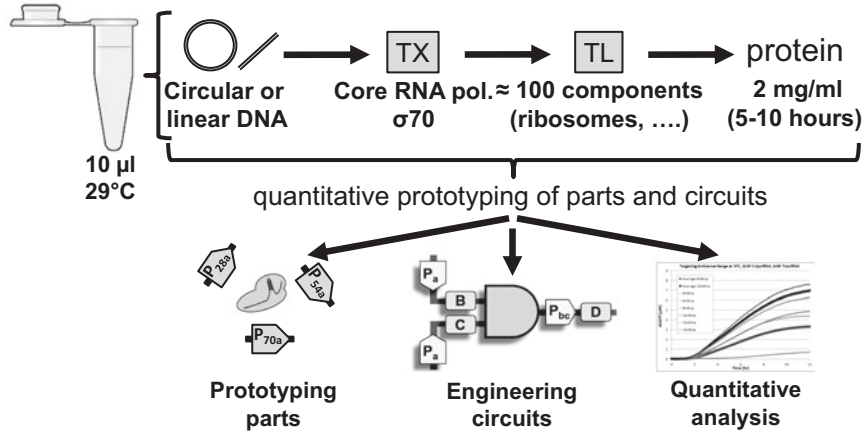

Fig. 1 Schematic of the all *E. coli* TXTL range of applications for circuit prototyping. TXTL reactions, carried out around 29 °C in volumes of 2–12 μL, can be used to rapidly test genetic parts and elementary gene circuits by quantitative analysis, using either linear or circular DNA templates

phage, which can be repressed by the CI repressor. The strongest UTR so far reported is from the phage T7 [27], which we named UTR1. When P70a and UTR1 are associated, expression of a reporter gene can reach up to 2 mg/mL in batch mode reactions (80 μM eGFP). As a scale, the average cytoplasmic protein concentration in *E. coli* is about 500 nM [43], and 1 nM is about 1 molecule in 1 μm^3, the volume of *E. coli*. Cell-free reactions with MYtxtl are scalable over 12 orders of magnitude (femtoliter to milliliter). A typical batch mode reaction is performed in volumes of 2–10 μL. Fluorescence is the most used technique for reporting. Reporters that are typically used with myTXTL include: fluorescent reporter proteins (*see* SI Fig. S3–S5 in [10]), Firefly and Renilla luciferase, fluorescent RNA aptamers malachite green and broccoli.

3.2 Plasmid Preparation

It is essential to prepare high quality DNA templates for cell-free expression with myTXTL. One can use either plasmids or linear DNA. For plasmids, mini, midi, and maxiprep kits from any company are appropriate for isolation and as a first purification step. The quality of plasmids, however, is not optimum. We strongly suggest adding a PCR cleanup step. The kit PureLink from Invitrogen, for example, is adequate as a second step for plasmids and as a single purification step for linear DNA (PCR product). To work quantitatively, it is essential to quantify each DNA template stock. We recommend using a Nanodrop because it is fast, but the Picogreen or Quantifluor assays are also appropriate. It is essential to use plasmid concentrations in molar units. Using mass concentration is obsolete and not useful to work quantitatively. There are many free websites to calculate the molar mass of

plasmids, any basic software does it. Whenever possible, we recommend preparing at least 100 nM plasmid and PCR template stocks.

3.3 TXTL Reaction Working Conditions

Like other TXTL systems, the performances of myTXTL reactions are sensitive to several conditions. The optimum temperature for maximum protein synthesis with myTXTL is 29 °C. If a temperature optimization has to be done, we suggest testing room temperature, 29 °C, 33 °C, and 37 °C. Oxygenation is another very important aspect of cell-free expression. Without proper oxygenation, the rate of protein synthesis is low. The surface area:volume ratio should be maximized to ensure efficient diffusion of oxygen from the environment into the reaction. Therefore, for a given compartment (tube, well plate, etc.) there is a maximum volume that should be used for optimum protein synthesis. Posiclick 1.7 mL tubes from Denville are appropriate. In these tubes, not more than 12 μL in the bottom of the tube should be used for optimum protein synthesis. In a standard 384-well plate, 5 μL should be used for kinetics and 10 μL for endpoint, and for a V-bottom 96-well plate 1–5 μL (kinetics and endpoint). With these settings, no shaking is required. Quantitative measurements can be done as long as the TXTL reaction keeps a fixed volume and shape. In a V-bottom 96-well plate, gravity and capillarity help to make the droplet uniform in size and shape, facilitating reproducibility of measurements.

3.4 Fluorescence Calibration of Plate Readers

Plate readers are typically used to measure TXTL reactions because cell-free expression systems are particularly well adapted for high-throughput experiments in low volumes. One can easily work quantitatively with myTXTL by performing fluorescence calibrations. Each calibration is specific to the exact settings used (plate reader, plate type, volume of reaction, gain, lamp energy, excitation/emission wavelengths, optics position: top or bottom, etc.) Calibrations for eGFP and the broccoli aptamer using a Biotek H1m are performed as follows:

1. For GFP calibration, obtain 1 mg/mL recombinant reGFP (29 kDa). This corresponds to 34.5 μM.
2. Re-quantify the stock of reGFP by measuring on Nanodrop. The 488 nm molar extinction coefficient is 55,000 M^{-1} cm^{-1}. Use this measured concentration in the subsequent steps.
3. Create a standard curve from the stock of reGFP by using serial dilutions: dilute reGFP into PBS 1× to make stocks of reGFP at 0, 0.1, 0.33, 1, 3.3, 10, 33 μM (*see* **Note 1**).
4. Pipette this series into the plate that you are calibrating in at least three locations on the plate (top left corner, middle, bottom right corner of the plate). Seal the plate with caps or sealing tape.

5. Measure the fluorescence of the wells on the plate reader, using the exact settings to be used for experiments (*see* **Note 2**).
6. For each concentration, measure the intensity from each well 3–5 times.
7. Average the intensity counts measured to get one plot of fluorescence intensity vs reGFP concentration.
8. Fit a line to the data and extract the slope of the fit line. This slope is the scale factor to be used for later experiments (Fig. 2). For example, [reGFP] μM = (Fluorescence Intensity)/(Slope) (*see* **Notes 3** and **4**).
9. Repeat calibration for all the plate types to be calibrated.
10. Re-calibrate plate readers for GFP every 3 months.
11. For Broccoli RNA aptamer calibration, anneal oligos T7-Broccoli-s with T7-Broccoli-as (*see* **Note 5**).
12. Amplify the Broccoli RNA using a T7 in vitro transcription kit and the dsDNA-annealed oligos as the DNA template. In a 30 μL reaction, add 1 μg DNA template, 10 μL NTP Buffer Mix (6.7 mM each NTP), 2 μL RNA polymerase mix, and complete with water. Incubate at 37 °C (dry air incubator) for 4–16 h.
13. Purify the RNA using a kit. Typically, this yields 80 ng of purified Broccoli RNA (15 μL at 333 μM). This should be enough to complete the calibration.

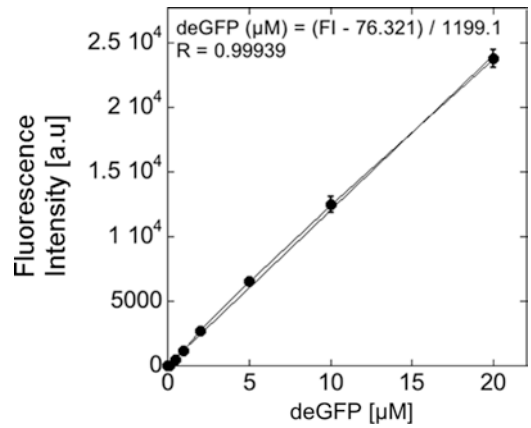

Fig. 2 Calibration of a plate reader for deGFP fluorescence. The fluorescence intensity for a range of concentrations of the fluorescent reporter is determined. In this example, plate reader Biotek H1m, 96 V-bottom well plate, 5 μL volume, 485/528 nm Ex/Em, gain 60. The calibration is linear from 0.1 μM to 20 μM

14. Create RNA stocks for a standard curve from the stock of Broccoli RNA purified in step 13 at ten times the concentrations to be calibrated by using serial dilutions. For example, dilute the RNA with water to make 10 μL volume stocks at 0, 1, 3.3, 10, 33 μM (*see* **Note 6**).
15. Assemble a 90% volume cell-free reaction, substituting the cell extract with S30B buffer, and including 40 μM DFHB1-T dye final concentration (concentration at 100% volume).
16. Split the reaction into 5 tubes of 21.6 μL volume (*see* **Note 7**).
17. Fill the remaining 10% volume (2.4 μL) with the 10× dilution series of Broccoli RNA, so that the final concentration of RNA in each tube is the desired concentration for the standard curve (0, 0.1, 0.33, 1, 3.3 μM).
18. Pipette the dilution series into the plate to be calibrated in at least three locations.
19. Measure the fluorescence of the wells on the plate reader, using the exact settings to be used for the experiments (*see* **Note 8**).
20. For each concentration of Broccoli RNA, average the intensity counts measured to get one plot of fluorescence intensity vs Broccoli RNA concentration.
21. Fit a line to the data and extract the slope of the fit line. This slope is the scale factor to be used for later experiments. For example, [Broccoli RNA] μM = (Fluorescence Intensity)/(Slope) (*see* **Notes 4** and **9**).
22. Repeat calibration for all plate types to be calibrated.
23. Re-calibrate plate readers for RNA aptamers every 3 months.

3.5 Assembling a Reaction: Prepackaged System

The prepackaged system is the simplest and easiest to use. It consists of a master mix solution frozen at −80 °C that contains all the necessary components at 75% of the final reaction volume to execute a TXTL reaction. The remaining 25% of the volume is used to add DNA template, any other specific cofactors (such as IPTG for working with the lac system, for example), and water. The prepackaged system is optimized for the expression of P70a-deGFP (strong S70 promoter and strong UTR). These settings are also compatible with other transcriptions such as T7 or any other *E. coli* sigma factors.

1. Calculate the volumes of DNA, cofactors, and water to be added to the TXTL reaction (*see* **Note 10**). The master mix is 75 μL, 75% reaction volume for 100 μL total volume (*see* **Note 11**). Typical individual reaction volumes are 5–12 μL, so one master mix aliquot can be used for 8–18 reactions. In the following example, we want to prepare seven 12 μL reactions, one with each of seven DNAs: A, B, C, D, E, F, G. We

also want 300 μM IPTG in all the reactions. Therefore, in future steps we need to add 6 μL of 5 mM IPTG and 9 μL water to the master mix to reach 90 μL (90% volume). Then we would split the reaction into 7 tubes of 10.8 μL and add the respective DNA to each tube, 1.2 μL at 10× concentration (*see* **Note 12**).

2. Retrieve master mix from −80 °C and thaw on ice.
3. Pipette directly into the master mix tube all of the components that are in every reaction. In our example, this is 6 μL of 5 mM IPTG, and 9 μL water. Mix by vortexing gently (*see* **Note 13**).
4. Split the reaction into the desired volume and number of tubes. In our example, this is seven tubes of 10.8 μL.
5. Add the DNA and other components that are unique to each reaction. In our example, this is 1.2 μL of respective 10× DNA stocks.
6. For endpoints, incubate for 10–24 h at 29 °C.
7. For kinetics, pipette into a well plate and begin measuring on the plate reader (*see* Subheading 3.10).

3.6 Assembling a Reaction: Multi-Component System

The multi-component system allows for fine-tuning the concentrations of components that make up the master mix: magnesium, potassium, energy mix, amino acid mix, PEG, carbon source. As an example, this section uses standard conditions for optimal deGFP synthesis from P70a-deGFP.

1. Calculate the volumes of all the reaction components before getting out anything on the bench.
2. Make necessary stocks of DNAs and other components by making dilutions. Convenient concentrations for components are: 3 M potassium glutamate, 180 mM magnesium glutamate, 500 mM maltodextrin, 40% w/v PEG.
3. Retrieve frozen components and thaw on ice. Cell extract, amino acid mix, and energy mix are stored at −80 °C. Magnesium, potassium, and PEG are stored at 4 °C. DNA is stored at −20 °C. Maltodextrin is prepared fresh.
4. Add the components directly to the cell extract tube, in the following order: 33 μL cell extract, 3 μL 3 M potassium glutamate, 2.22 μL 180 mM magnesium glutamate, 6 μL 500 mM maltodextrin, 23 μL 17 mM amino acid mix, 5.56 μL 18× energy mix, 3.75 μL 40% w/v PEG, and then any other component that is in every reaction. If we were using the same example from Subheading 3.5, we would add 6 μL 5 mM IPTG and 6.6 μL water. Vortex gently.
5. Split the reaction into the desired volume and number of tubes. In our example, this is 7 tubes of 10.8 μL.

6. Add the DNA and other components that are unique to each reaction. In our example, this is 1.2 μL of respective DNA at 10× concentration.
7. For endpoints, incubate for 10–24 h at 29 °C.
8. For kinetics, pipette into a well plate and begin measuring on the plate reader (*see* Subheading 3.10).

3.7 Maximizing eGFP Synthesis by Optimizing Concentration of Reaction Components in the Multi-Component System

Depending on what is being expressed in TXTL, a quick component optimization should be done. Components that should be optimized are (typical optimum concentrations in parenthesis): magnesium glutamate (2–8 mM), potassium glutamate (20–150 mM), amino acids (1–4 mM), and PEG (1–4% w/v). As an example, this section is a procedure for optimizing the concentration of magnesium glutamate, but it can be used for all of the components, substituting them in for magnesium. This protocol is similar to Subheading 3.6 but here, DNA is in the master mix, and magnesium glutamate (or other components) is specific to each reaction. This protocol should be done in triplicate to get error bars, and the three trials can be done simultaneously or one after another, such that they all incubate at the same time and are measured on the plate reader at the same time.

1. Calculate the volumes of all the reaction components before getting out anything on the bench. The master mix should be filled with components and water to 90% reaction volume, and stocks of magnesium should be made at 10× desired final concentrations in the reaction. Here, we do a magnesium range of 1, 2, 3, 4, 5, 6, 7, 8 mM final concentration in reaction, using stocks of 10, 20, 30, 40, 50, 60, 70, and 80 mM magnesium glutamate.
2. Make necessary dilutions of DNAs and other components to get stocks at desired concentrations.
3. Retrieve frozen components and thaw on ice. Cell extract, amino acid mix, and energy mix are stored at −80 °C. DNA is stored at −20 °C.
4. Add the components directly to the cell extract tube, in the following order: cell extract, water, potassium glutamate, maltodextrin, amino acid mix, energy mix, PEG, and DNA. Mg-glutamate is omitted because we are doing an Mg-Glutamate range.
5. Split the reaction into 8 tubes of 10.8 μL. This is 90% volume, for 12 μL 100% volume reaction.
6. Add 1.2 μL of 10× Mg-glutamate to the tubes.
7. For endpoints, incubate for 10–24 h at 29 °C.
8. After incubation, pipette 10 μL from each reaction into a well in the 384 flat bottom well plate.

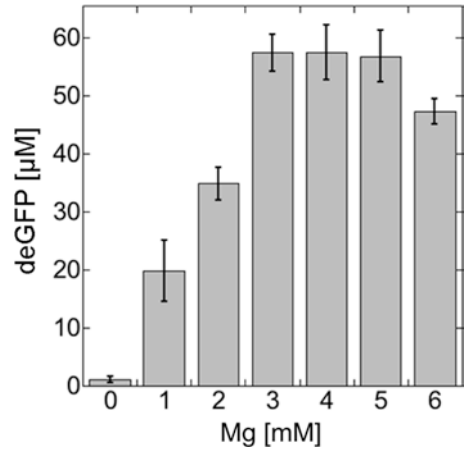

Fig. 3 Endpoint measurements for a magnesium concentration range of TXTL reactions containing 5 nM of the plasmid P70a-deGFP (constitutive expression of deGFP). The reactions were incubated overnight at 29 °C. Magnesium is the most important biochemical component to optimize in a TXTL reaction. Error bars are standard deviations from at least three repeats

9. Measure on the plate reader. The greatest signal corresponds to the optimum concentration of Mg-glutamate in the reaction (Fig. 3).

3.8 Working with Linear DNA

In *E. coli* extract-based TXTL, the RecBCD exonuclease complex quickly degrades linear DNA templates. However, a dsDNA oligonucleotide containing chi sites, an 8-base sequence specifically recognized by RecBCD (5′-GCTGGTGG-3′), can be added to the reaction to sequester the RecBCD and stabilize linear DNA templates [44]. This allows for PCR products and other linear templates to be used directly in a TXTL reaction (Fig. 4). Use standard oligonucleotide annealing to make a dsDNA chi6 containing 6 repeats of chi:

1. Mix equimolar chi6-s and chi6-as oligos.
2. Heat the mixed oligos to 95 °C for 5 min, then cool by 1 °C every 30 s to 15 °C.
3. In the TXTL reaction, add 2 μM final concentration chi6 dsDNA and mix by vortexing gently BEFORE the addition of any linear DNA template.

3.9 Endpoints: Plate Reader Measurements and Data Analysis

After an endpoint reaction has been incubated for 10–24 h, when using a fluorescent reporter, the fluorescence is measured and the concentration of fluorescent reporter is quantified (Fig. 5). At this point, the cell-free reactions should be 12 μL in volume, in 1.7 mL tubes.

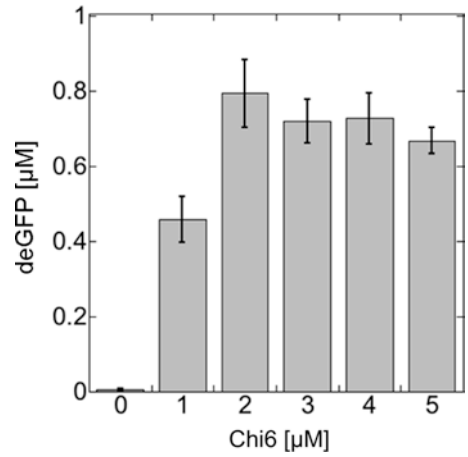

Fig. 4 Endpoint measurements for a Chi6 concentration range of TXTL reactions containing 0.5 nM of a PCR amplification of P70a-deGFP (constitutive expression of deGFP). The PCR contains 200–300 bp of hangovers upstream and downstream P70a-deGFP. The reactions were incubated overnight at 29 °C. Chi6 inhibits linear DNA degradation in TXTL reactions [44]. Error bars are standard deviations from at least three repeats

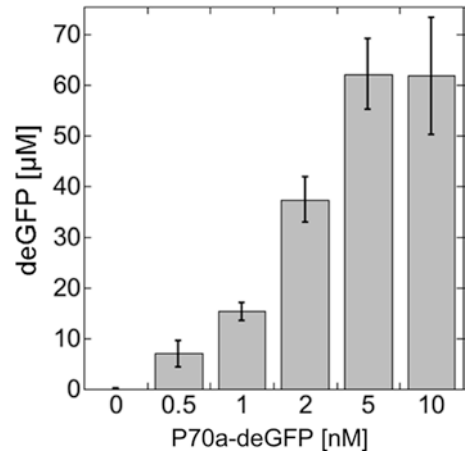

Fig. 5 Endpoint measurements for a P70a-deGFP plasmid concentration range (constitutive expression of deGFP). The reactions were incubated overnight at 29 °C. Error bars are standard deviations from at least three repeats

1. Pipette 10 μL of one reaction into a well in a 384-well plate. Go back and forth in the pipette tip a couple of times (in the 1.7 mL tube) before pipetting into the well. This will ensure that the reaction is homogenized.
2. Repeat **step 1** for all the reactions, including the background reaction, with no fluorescent protein template DNA.

3. Measure the fluorescence of each well with the reaction on a plate reader.
4. Using the plate reader calibration (from Subheading 3.4), calculate the molar concentration of fluorescent reporter for all the reactions, including the background reaction.
5. Subtract the background reaction concentration from all the other reactions. The background reaction typically measures the equivalent of ~20–80 nM deGFP, which corresponds to the autofluorescence of the cell-free reaction.
6. Take the average and standard deviation (for error bars) of each set of repeat reactions (if multiple repeats were done).

3.10 Kinetics: Plate Reader Measurements and Data Analysis

Immediately after a cell-free reaction is assembled, the fluorescence kinetics can be measured. Set up the plate reader kinetics settings before assembling the cell-free reaction, to minimize the time between finishing assembling the reaction and the first measurement (time zero point) on the plate reader.

1. Set up the kinetics protocol on the plate reader. For example, measure for 16 h with a 3-min increment, at gain 60.
2. Preincubate the well plate to be used at 29 °C (or whatever temperature to be used during kinetic). Plates should preincubate at least 30 min before reactions are added into the plate.
3. Assemble the cell-free reaction.
4. Pipette reactions into the now-warm well plate. For example, pipette 5 μL from each reaction into a 96 V-bottom well plate. With standard 12 μL reactions, a replicate can be made, so that there are two wells for each reaction (*see* **Note 14**).
5. Place the plate in the plate reader and start the measurement.
6. When the measurement is complete, the data can be analyzed (Fig. 6).
7. Using the plate reader calibration (from Subheading 3.4), calculate the molar concentration of fluorescent protein for all the reactions, including the background reaction, and for all time points. There should now be a table, where each column is one reaction kinetic, and each row is an increasing time point.
8. Subtract the background reaction concentration from all other reactions, for all time points.
9. Take the average and standard deviation (for error bars) of each set of repeat reactions (if multiple repeats were done), for all time points.

3.11 Expression of a Fluorescent Reporter

Fluorescent reporters are extremely useful in TXTL to measure the concentrations of mRNA and protein. To express a fluorescent reporter protein, simply assemble a reaction by following

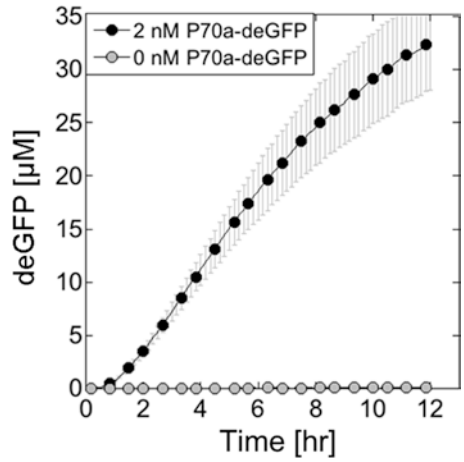

Fig. 6 Kinetics of deGFP expression (P70a-deGFP plasmid) in TXTL reactions. The reactions were incubated at 29 °C, in a Biotek H1m plate reader (96 V-bottom well plate, 5 μL reactions). The negative control consisted of a TXTL reaction with no plasmid added to it. Error bars are standard deviations from at least three repeats

Subheading 3.5 or 3.6, and add the reporter protein DNA template, for example, P70a-deGFP. To probe the mRNA concentration, RNA aptamers can be added (like Broccoli and MG aptamer). RNA aptamers bind to specific dyes, which only fluoresce when they are bound to the mRNA. To express fluorescent RNA aptamers, assemble a reaction by following Subheading 3.5 or 3.6, and add the RNA aptamer DNA template and the corresponding dye.

3.12 Prototyping Regulatory Elements: E. coli P70 Promoters

We named the series of promoters specific to S70 (sigma 70) the P70 series. These promoters require sigma 70-RNAP holoenzyme for transcription. The promoter consensus sequence is: TTGACA for −35 and TATAAT for −10. The consensus sequence is not necessarily the strongest in terms of transcription. Mutating one or more bases in either recognition sequence can dramatically change the strength of the promoter. Using Broccoli RNA aptamer is the simplest way to prototype *E. coli* P70 promoters, in particular, to get their strength. This section outlines how to determine the strength of a set of P70 promoters, relative to P70a, which is one of the strongest reported (TTGACA for −35 and GATAAT for −10). As an example, we compare P70a with three other promoters: P70b, P70c, and P70d. Use a 96 V-bottom well plate.

1. Follow Subheading 3.10, **steps 1** and **2**.
2. Make 10 nM stocks of each DNA plasmid template (P70a-Broccoli, P70b-Broccoli, P70c-Broccoli, P70d-Broccoli).

These stocks are 10× of desired final reaction concentration of 1 nM (*see* **Note 15**).

3. Assemble a reaction (either prepackaged or multi-component), adding 40 μM final concentration DFHBI-1T, to 90% reaction volume (*see* **Note 16**).
4. Vortex gently to mix and split the reaction into 5 tubes of 10.8 μL.
5. Add 1.2 μL of respective DNA to each of four tubes. To the fifth tube, add 1.2 μL of water. This fifth tube is used as background.
6. Pipette 5 μL from each reaction into two wells in the well plate.
7. Seal the wells with caps, place the well plate into the plate reader, and begin measurement.
8. When the kinetics are done, use the Broccoli aptamer calibration from Subheading 3.4 to calibrate the raw data.
9. Subtract the background reaction from all other reactions for all time points.
10. Compare the maximum slope of the RNA kinetics between each promoter. Relative strengths of promoters can now be seen.
11. Repeat as necessary for error bars.

3.13 Prototyping Regulatory Elements: *E. coli* UTRs

The main determining factor of the strength of an *E. coli* untranslated region (UTR) is the ribosome-binding site (RBS). The consensus RBS in *E. coli* is the Shine-Dalgarno sequence: 5′-AGGAGGU-3′. The consensus sequence is not necessarily the strongest in terms of translation. Mutating one or more bases in the RBS can dramatically change the strength of the UTR. A reporter protein must be used to determine UTR strength because it is a measure of translation. This section outlines how to determine the strength of a set of UTRs, relative to the UTR in P70a-deGFP, which has UTR1, the strongest reported (AGGAGAU for RBS). This UTR is the UTR of the gene 10 of bacteriophage T7. As an example, we compare UTR1 with three other UTRs: UTR2, UTR3, and UTR4. Use a 96 V-bottom well plate.

1. Follow Subheading 3.10, **steps 1** and **2**.
2. Make 10 nM stocks of each DNA plasmid template (P70a-deGFP, P70a-UTR2-deGFP, P70a-UTR3-deGFP, P70a-UTR4-deGFP). These stocks are 10× of desired final reaction concentration of 1 nM (*see* **Note 15**).
3. Assemble a reaction (either prepackaged or multi-component), completing with water to 90% volume (*see* **Note 17**).
4. Vortex gently to mix and split the reaction into 5 tubes of 10.8 μL.

5. Add 1.2 μL of respective DNA to each of four tubes. To the fifth tube, add 1.2 μL of water. This fifth tube is used as background.
6. Pipette 5 μL from each reaction into two wells in the well plate.
7. Seal the wells with caps, place the well plate into the plate reader, and begin measurement.
8. When the kinetics are done, use the GFP calibration from Subheading 3.4 to calibrate the raw data.
9. Subtract the background reaction from all other reactions for all time points.
10. Compare the slope of the deGFP kinetics between each UTR template. Relative strengths of UTRs can now be seen.
11. Repeat as necessary for error bars.

3.14 Prototyping CRISPRi sgRNA for dCas9 from S. pyogenes

We have recently installed CRISPR technology into myTXTL [39]. The deactivated Cas9 protein (dCas9) from *S. pyogenes* is expressed concurrently with single guide RNAs (sgRNA) targeting a 20 bp sequence within a template plasmid to repress transcription on that template plasmid (Fig. 7). This section outlines how to determine the effectiveness of an sgRNA in TXTL. As an example, we compare four different sgRNA (sgRNA-2, sgRNA-3, sgRNA-4, sgRNA-5) targeting different locations on the template plasmid P70a-deGFP, all within the promoter-UTR-gene section, as well as one control sgRNA that is non-targeting (sgRNA-NT). The sgRNA-NT has a random 20 bp sequence for its spacer instead

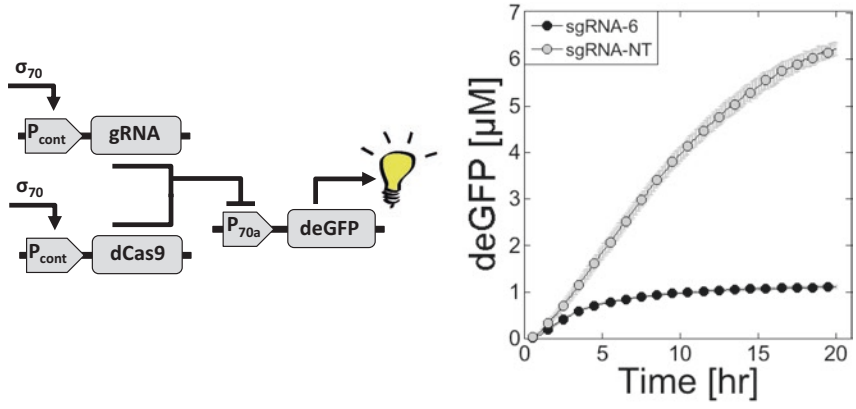

Fig. 7 Kinetics of CRISPR dCas9 repression in TXTL reactions. Three plasmids were added to the TXTL reaction, expressions occur all concurrently: dCas9 is expressed from a constitutive promoter, a gRNA targeting the promoter P70a is expressed from a constitutive promoter and deGFP is expressed through P70a. The negative control consisted of expressing a non-targeting gRNA (sgRNA-NT). Error bars are standard deviations from at least three repeats

of a sequencing matching the target template. sgRNA templates can be ordered as gBlocks from IDT and PCR amplified.

1. Follow Subheading 3.10, **steps 1** and **2**.
2. Make 10 nM stocks of each DNA PCR product (sgRNA-2, sgRNA-3, sgRNA-4, sgRNA-5, sgRNA-NT). These stocks are 10X of desired final reaction concentration of 1 nM.
3. Assemble a reaction (either prepackaged or multi-component), adding 2 μM chi6, 2 nM Spy-dCas9 plasmid, 1 nM sgRNA-NT, and completing with water to 100% volume. This reaction has no reporter protein template and is used as background.
4. Assemble a second reaction (either prepackaged or multi-component), adding 2 μM chi6, 1 nM P70a-deGFP plasmid, 2 nM Spy-dCas9 plasmid, and completing with water to 90% volume.
5. Vortex gently to mix and split the reaction into five tubes of 10.8 μL.
6. Add 1.2 μL of respective sgRNA DNA template (sgRNA-2, sgRNA-3, sgRNA-4, sgRNA-5, sgRNA-NT) to each of the five tubes.
7. Pipette 5 μL from each reaction into two wells in the well plate. This includes the background reaction from **step 3** and the five reactions from **steps 4–6**.
8. Seal the wells with caps, place the well plate into the plate reader, and begin measurement.
9. When the kinetics are done, use the GFP calibration from Subheading 3.4 to calibrate the raw data.
10. Subtract the background reaction from all other reactions for all time points.
11. The slopes and endpoints can now be calculated, and the repression factors for each sgRNA can be determined.

3.15 Prototyping Circuits: Transcription Cascade

Transcriptional activation cascades are simple genetic circuits that require the expression of a transcription factor to activate the expression of another protein. The major *E. coli* transcription scheme is composed of seven sigma factors: sigma 19, 24, 28, 32, 38, 54, and 70; each activating specific promoters. The numbers refer to the molecular weight of the protein in kDa. Sigma 70 is the essential housekeeping sigma factor, present in myTXTL. When grown in ideal conditions, like during an extract preparation (rich medium, 37 °C), the other six sigma factors are not expressed. Consequently, they have a very low or zero concentration in TXTL. This section outlines how to do a transcription cascade reaction (Fig. 8). As an example we show how to determine the strength of each of six *E. coli* sigma factor.

1. Follow Subheading 3.10, **steps 1** and **2**.

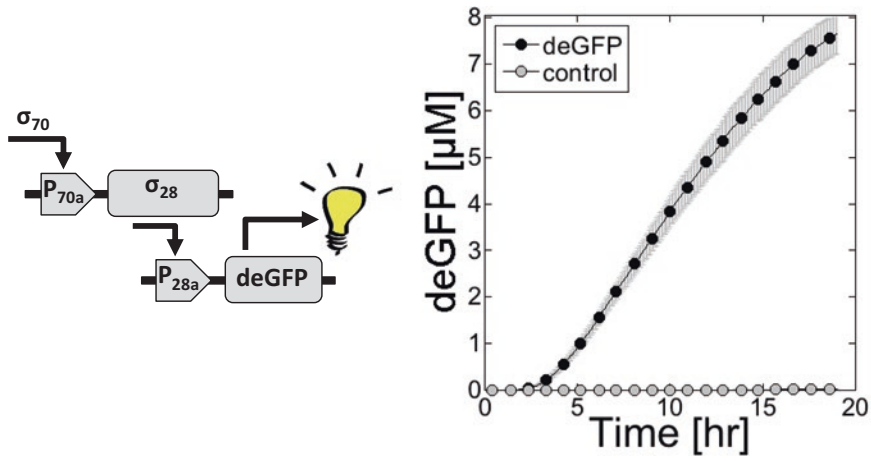

Fig. 8 Expression of deGFP through a transcriptional activation cascade in TXTL reaction. deGFP is expressed through the promoter P28a (P28a-deGFP, 1 nM) specific to the *E. coli* sigma factor 28, expressed from the constitutive promoter P70a (P70a-S28, 0.05 nM). The negative control included the plasmid P28a-deGFP only. Error bars are standard deviations from at least three repeats

2. Make 5 nM stocks of each DNA plasmid template encoding transcription factors (P70a-S19, P70a-S24, P70a-S28, P70a-S32, P70a-S38), and 10 nM stocks of each DNA plasmid template encoding for deGFP (P70a-deGFP, P19a-deGFP, P24a-deGFP, P28a-deGFP, P32a-deGFP, P38a-deGFP). These stocks are 10× of desired final reaction concentrations.
3. Assemble a reaction (either prepackaged or multi-component), completing with water to 80% volume.
4. Vortex gently to mix and split the reaction into seven tubes of 9.6 μL.
5. Add 1.2 μL of respective plasmid encoding sigma factors under P70a promoter to each of six tubes (for S70, because it is already in the reaction, add 1.2 μL water). Add 1.2 μL of the corresponding deGFP plasmid to each tube to complete to 12 μL. To the seventh tube, add 2.4 μL of water. This seventh tube is used as background. Gently mix all the tubes.
6. Pipette 5 μL from each reaction into two wells in the well plate.
7. Seal the wells with caps, place the well plate into the plate reader, and begin measurement.
8. When the kinetics are done, use the GFP calibration from Subheading 3.4 to calibrate the raw data.
9. Subtract the background reaction from all other reactions for all time points.
10. Compare the slope of the deGFP kinetics between each transcription cascade (*see* **Note 18**).
11. Repeat as necessary for error bars.

3.16 Prototyping Circuits: Negative Feedback Loop

The strength, versatility, and tunability of TXTL reactions are adequate to rapidly prototype circuit motifs in TXTL. One such example is a negative feedback loop (NFL). An NFL is a circuit whose output represses its own transcription. One example of an NFL is the circuit using the plasmid P70a-cI. cI is the repressor from bacteriophage lambda, which binds to operator sites around P70a (OR2 and OR1), repressing expression from P70a (Fig. 9). Another example is PL-tetO1-tetR, where tetR binds to tetO1 operator sites. For both of these NFLs, a reporter can be used to track the expression levels from the promoter, such as P70a-deGFP and PL-tetO1-deGFP, respectively. If degradable versions of the reporter are used (with ssrA tags), a pulse signal of fluorescent GFP can be generated (Fig. 9).

3.17 Prototyping Circuits: Incoherent Feed-Forward Loop

Another possible circuit in TXTL is an incoherent feed-forward loop (IFFL) (Fig. 10). One example of an IFFL is the circuit using plasmids P28a-tetO1-deGFP-ssrA, P28a-tetR-ssrA, and P70a-S28-ssrA. Sigma 70 activates the expression of sigma 28 through the P70a promoter. Then sigma 28 activates the expression of the output deGFP-ssrA through one pathway, and activates the expression of the repressor tetR through another pathway. tetR then represses the production of the output deGFP-ssrA by binding to the tetO1 operator sites. As with the NFL, if a degradable deGFP (with an ssrA tag) is used, a pulse signal of fluorescent GFP can be generated (Fig. 10).

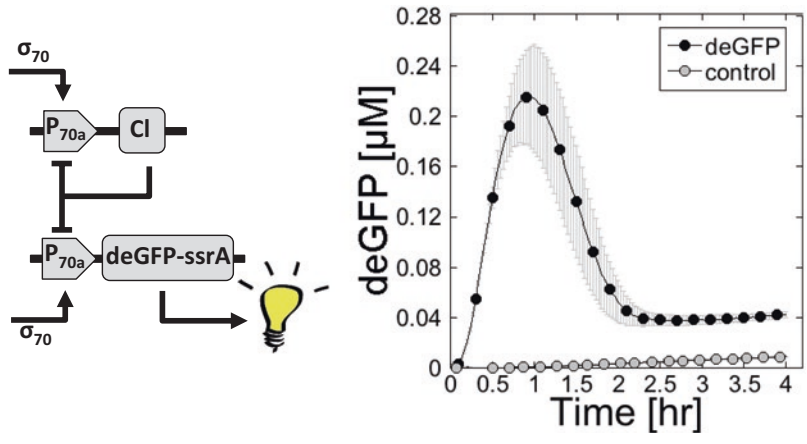

Fig. 9 Expression of deGFP through a transcriptional activation cascade in TXTL reaction. deGFP is expressed through the promoter P28a (P28a-deGFP, 1 nM) specific to the *E. coli* sigma factor 28, expressed from the constitutive promoter P70a (P70a-S28, 0.05 nM). The negative control included the plasmid P28a-deGFP only. Error bars are standard deviations from at least three repeats

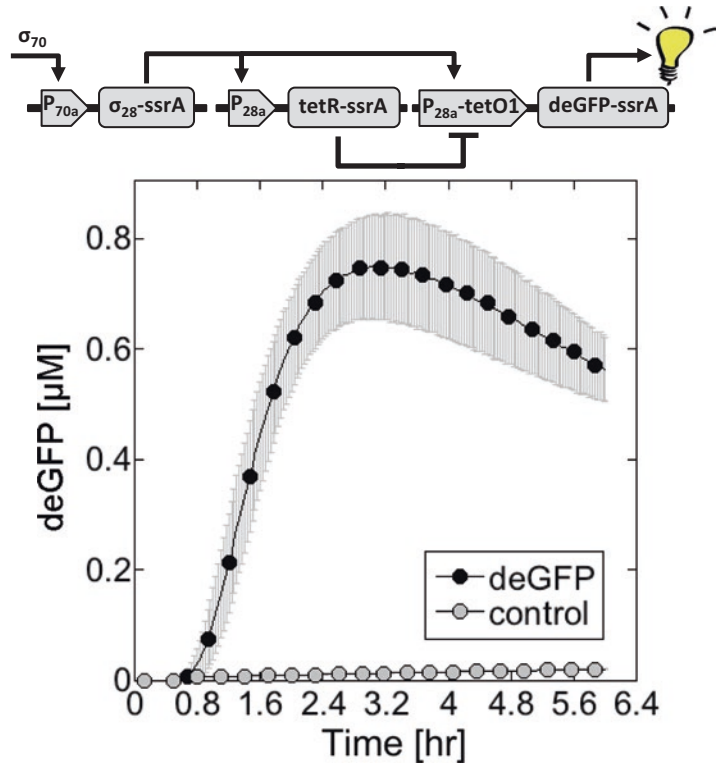

Fig. 10 An incoherent feed-forward loop circuit consisting of three plasmids: P28a-tetO1-deGFP-ssrA (5 nM), P28a-tetR-ssrA (0.1 nM), P70a-S28-ssrA (0.5 nM). The negative control only included the P28a-tetO1-deGFP-ssrA plasmid. Error bars are standard deviations from at least three repeats

3.18 SDS-PAGE for Protein Synthesis Analysis

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) is a technique used to verify size and relative concentrations of proteins. In TXTL, fluorescent reporters are easily measured. However, many experiments require synthesizing proteins that are not fluorescent reporters. In these cases, SDS-PAGE is used to make sure the protein is expressed. This protocol assumes that a TXTL endpoint reaction (and a blank reaction without DNA template) has already been incubated for at least 8 h.

1. Thoroughly wash all materials (gel cast and holder, clips, well comb, etc.).
2. Prepare fresh at least 200 μL 10% w/v ammonium per sulfate (APS) (*see* **Note 19**).
3. Assemble the gel cast.
4. Prepare the gel (14% acrylamide) by adding the following components to a 15 mL tube: 1.25 mL Tris–HCl 1.5 M pH 8.8, 25 μL 20% w/v SDS, 50 μL 10% w/v APS, 1.9 mL water, 1.75 mL 40% w/v acrylamide, 5 μL Temed (*see* **Note 20**).

5. Mix by inverting multiple times.
6. Gently pipette the mixture into the gel cast, leaving 1 cm (measured vertically) for the stacking gel (*see* **Note 21**).
7. Incubate for 3 min, then pipette water on the top of the gel to fill up the last 1 cm (*see* **Note 22**).
8. To prepare the stacking gel, add the following components to a 15 mL tube: 1.8 mL water, 0.2 mL 0.1% w/v bromophenol blue in water, 250 μL 40% w/v acrylamide, 250 μL 1 M Tris–HCl pH 6.8, 25 μL 10% w/v APS, 2.5 μL Temed.
9. Gently tilt the SDS PAGE 14% gel cast and absorb the water on the top of the gel with a lint-free wipe (Kimwipe).
10. Pipette the stacking gel mix into the gel cast on the top of the SDS PAGE 14% gel (where the water was).
11. Slide the comb into the gel to create wells in the gel.
12. Incubate for 30 min. Meanwhile, prepare the TXTL sample to be analyzed.
13. For each sample to be analyzed add to a 1.5 mL tube: 25 μL 2× sample buffer, 5 μL TXTL reaction, 20 μL water. Make sure to include one tube for the blank TXTL reaction (*see* **Note 23**).
14. Put the samples in a 95 °C heat block for 3 min to denature the proteins.
15. Remove the gel from the caster and clamp it to the electrophoresis device. Pipette the running buffer into the gel holder such that the top and bottom of the gel are in the running buffer.
16. Remove the comb from the stacking gel.
17. Pipette 5 μL of each sample, blank and protein ladder marker into their own well.
18. Connect the power cables to gel holder and run at 110 V for 10 min.
19. After the 10 min, run at 170 V for 65 min (*see* **Note 24**).
20. Turn off the power supply and disconnect the cables.
21. Remove the gel from the cast and cut off the stacking gel. Place the gel in a small container (pipette tip box for example).
22. Wash the gel three times in water (5 min each time) by placing the box on a rocker.
23. Discard water, incubate the gel for at least 1 h at room temperature in 20 mL of SimplyBlue Safestain.
24. Wash the gel with water multiple times, keep the gel in water.
25. Scan an image of the gel (for this you can use any standard office scanner: place the gel on the glass, add a white background onto it such as a piece of flat Teflon, scan).

3.19 Measuring the Maturation Rate of deGFP

When deGFP is first translated, the protein is not fluorescent. The protein must fold and the chromophore must mature for the protein to be functional and fluoresce. To accurately model gene circuits using a fluorescent reporter like deGFP, the maturation rate of the protein must be determined. This section describes the assay to determine the maturation rate of deGFP in TXTL [10]. This assay assumes that RNAse A immediately stops all translation, and that there is no fully translated protein before 2 min of incubation.

1. Follow Subheading 3.10, **steps 1** and **2**.
2. Assemble two cell free reactions to 100% volume, with 5 nM P70a-deGFP. To the first reaction, immediately add 1 μL RNAse A diluted 300 X. This is the background reaction (*see* **Note 25**).
3. After 2 min of incubation, add 1 μL RNAse A diluted 300× to the second reaction.
4. Gently mix and immediately pipette 5 μL from each reaction into a 96 V-bottom well plate.
5. Measure deGFP expression kinetics for 3 h with a one-minute interval.
6. Use the GFP calibration from Subheading 3.4 to calculate the concentration of deGFP.
7. Subtract the background reaction concentrations from the second reaction.
8. Fit the data to the following equation:

$$[\text{deGFP}] = [\text{endpoint}]\left(1 - e^{-kt}\right) \tag{1}$$

where [deGFP] is the concentration of fluorescent deGFP protein, [endpoint] is the concentration of deGFP at saturation (endpoint, at 3 h should be plateaued), k is the maturation rate of deGFP, and t is time. The maturation rate of deGFP should be the only parameter to be fit.

3.20 Measuring the deGFP mRNA Degradation Rate

In TXTL, mRNA degradation follows a first-order kinetic (the degradation rate of mRNA is proportional to the concentration of mRNA). It is important to estimate the rate of degradation, or the average lifetime of mRNA, for gene circuit prototyping. This assay consists of three steps: starting a TXTL reaction with P70a-deGFP, stopping transcription with an RNAP inhibitor Rifampicin, and modeling the saturation of deGFP fluorescent protein. This work requires the maturation time of deGFP (*see* Subheading 3.19). The equation to be fit is

$$[\text{deGFP}_{\text{mat}}]_t = [\text{deGFP}_{\text{mat}}]_0 + [\text{deGFP}_{\text{dark}}]_0\left(1 - e^{-kt}\right) + \frac{am_0}{b(b-k)}\left[b\left(1 - e^{-kt}\right) + k\left(e^{-bt} - 1\right)\right] \tag{2}$$

where $[deGFP_{mat}]_t$ is the mature, fluorescent deGFP protein as a function of time, $[deGFP_{mat}]_0$ is the initial mature deGFP protein, measured at transcription arrest, $[deGFP_{dark}]_0$ is the initial dark, non-fluorescent deGFP, corresponding to the concentration of dark deGFP at transcription arrest, k is the deGFP maturation rate, b is the mRNA inactivation rate, a is a protein translation constant, and m_0 is the initial mRNA concentration at transcription arrest. The derivation of this equation can be found in [10] (supplementary information).

1. Assemble a reaction with 2 nM P70a-deGFP, and measure kinetics at 29 °C for 2 h.
2. Calculate the slope of the kinetic at the 2 h point using the plate reader calibration. This slope value is used as the parameter am_0 (*see* **Note 26**).
3. Assemble a reaction with 2 nM P70a-deGFP. Incubate for 2 h at 29 °C.
4. Add 50 μM Rifampicin to the reaction and immediately begin measuring kinetics every minute for 2 h.
5. Using the deGFP maturation rate from Subheading 3.20 as parameter k, the initial fluorescent deGFP measure as $[deGFP_{mat}]_0$, fit the kinetic data to Eq. 2, above (using the computer to get best fits for parameters b and $[deGFP_{dark}]_0$.
6. The fitted parameter b, is the mRNA inactivation rate.

3.21 Tuning mRNA Lifetime with MazF

The versatility of TXTL allows for the tuning of mRNA lifetime with the interferase MazF. First, a TXTL loaded with MazF is prepared [28]. The MazF concentration in this extract is estimated to be around 2 μM. The mRNA turnover in a TXTL reaction is done by adding different amounts of the MazF-loaded extract to a standard TXTL reaction. For a direct measurement of the average deGFP mRNA lifetime with MazF, Subheading 3.20 can be followed, with MazF added to the reaction. In Garamella et al. [10], Figure S12 shows how the mRNA lifetime changes with increasing concentrations of MazF. This protocol shows how to simply tune mRNA lifetime in a standard TXTL reaction using MazF.

1. Follow Subheading 3.10, **steps 1** and **2**,
2. Make stocks of MazF at concentrations of 0, 100, 250, 500, 750, 1000 nM by diluting in water.
3. Assemble a reaction to 90% volume with 5 nM P70a-deGFP. Mix gently.
4. Split the reaction into six tubes of 10.8 μL.
5. Add 1.2 μL of each concentration of MazF to a tube, for reactions with 0, 10, 25, 50, 75, 100 nM MazF.
6. Continue with **steps 4–9** of Subheading 3.10.

3.22 Measuring eGFP-ssrA Degradation Rate

For this section, a stock of 6His-eGFP-ssrA is required [28]. 6His-eGFP-ssrA is overexpressed in *E. coli* and purified using standard procedures by affinity chromatography. The TXTL system is capable of achieving degradation of proteins tagged with peptides recognized by ClpXP proteases. The most efficient degradation tag is ssrA (amino acid sequence: ANDENYALAA). This section outlines how to measure the degradation rate of eGFP-ssrA in a TXTL reaction.

1. Follow Subheading 3.10, **steps 1** and **2**.
2. Make stocks of eGFP-ssrA at concentrations of 0, 10, 20, 50, 100 μM.
3. Assemble a reaction to 90% volume, without DNA. Mix gently.
4. Split the reaction into 5 tubes of 10.8 μL.
5. Add 1.2 μL of each concentration of eGFP-ssrA to a tube, for reactions with 0, 1, 2, 5, 10 μM eGFP-ssrA.
6. Mix gently and pipette 5 μL from each tube into two wells of a 96 V-bottom plate (*see* **Note 27**).
7. Begin measuring the fluorescence of GFP on the plate reader, measuring every minute for 6 h.
8. When the kinetic is over, calibrate and analyze the data, calculating GFP concentration in time, as well as the slope, which is the degradation rate.
9. Confirm that the degradation is zeroth order (independent of eGFP-ssrA concentration) by plotting the concentration vs. the slope.

3.23 Tuning the Degradation Rate with ClpXP

ClpXP can be added to TXTL to increase the degradation rate of ssrA-tagged proteins. ClpXP can be expressed from a plasmid in a TXTL reaction, or the protein complex can be added. This section outlines how to add ClpXP to a reaction to increase the degradation rate. In this example, we add different concentrations of the plasmid P70a-ClpXP [10] to a reaction with eGFP-ssrA.

1. Follow Subheading 3.10, **steps 1** and **2**.
2. Make stocks of P70a-ClpXP at 0, 5, 10, 20, 30 nM.
3. Assemble a reaction to 90% volume with 10 μM eGFP-ssrA. Mix gently.
4. Split the reaction into 5 tubes of 10.8 μL.
5. Add 1.2 μL of P70a-ClpXP at each concentration, to give reactions with 0, 0.5, 1, 2, 3 nM P70a-ClpXP. Mix gently.
6. Pipette 5 μL from each reaction into two wells in the 96 V-bottom well plate, and begin measuring fluorescence.
7. The data can be analyzed to see how the degradation rate is affected by expressing ClpXP (*see* **Note 28**).

4 Summary

The work described in this chapter has been developed over the past 10 years in our laboratory and gathers the achievements of several graduate students and postdocs. The methods presented are the very basics in TXTL reaction executions and data analysis. TXTL systems have become more accessible and have proven useful to help engineering and accelerate the prototyping of gene circuits. However, artifacts are easily produced in TXTL reactions. One must be careful in executing TXTL reactions, in choosing relevant controls, and in analyzing data, especially at the quantitative level. Cell-free expression is a growing research area. New TXTL platforms, from other bacteria and eukaryotic cells, are being developed [45, 46] beyond the standard *E. coli* TXTL.

5 Notes

1. The range of concentrations for which the calibration is linear depends on the plate reader and its gain settings. For example, for a Biotek H1m, only concentrations within the range of 0–33 μM provide a linear fit with gains 50–60. Above, the response is not linear.
2. For Biotek H1M plate readers using 485/528 nm Ex/Em, bottom optics position, high lamp energy, Nunc 384 flat bottom plate, and 10 μL volume, a typical gain for the range of 0–33 μM reGFP is 50–60.
3. The calibration equation does not factor in background subtraction, which should be done with every experiment.
4. The same plate can be used for multiple plate readers and plate reader settings if it is all done within a few hours, and the plate is sealed to prevent evaporation. The reaction must also maintain the same shape in the well. If 5 μL is being used in a V-bottom 96-well plate, it is possible for the droplet to leave the bottom-center of the well if the plate is hit or shaken hard enough. Therefore, be careful with the plate.
5. The Broccoli aptamer calibration can be generalized for any RNA aptamer, like MG aptamer. The RNA aptamer calibration assumes that the concentration of dye is always in excess to RNA (typically 40 μM of the dye DFHB1-T for Broccoli RNA aptamer). The MG aptamer calibration would use oligos T7-MGapt-s and T7-MGapt-as. The dye for MGapt is malachite green, which should be used at 20 μM. The optimal Ex/Em wavelengths are 625/655 nm. A typical gain for MGapt is 150–160 (on a Biotek H1M).

6. Before starting, determine how much volume will be needed of each concentration of Broccoli RNA. This depends on how many wells and volume per well being used.
7. For the calibration of multiple plates, more volume may be required. Scale volumes as needed.
8. For Biotek H1m plate readers using 470/505 nm Ex/Em, bottom optics position, high lamp energy, 96-well plate, 5 μL volume, 40 μM DFHB1-T dye, a typical gain for the range of 0–3.33 μM Broccoli RNA is 90–100.
9. The calibration does not factor in a background subtraction, which should be done with every experiment, especially for RNA quantification because a high gain (high background) must be used. A background reaction would be the exact same conditions except for without template expressing Broccoli RNA.
10. This should be done prior to starting the reaction so that, once the components are thawed, the reaction can be assembled as quickly as possible.
11. Multiple tubes of cell extract or master mix can be combined together to do more cell-free reactions. The aliquot size is somewhat arbitrary and for convenience.
12. DNA stocks should be made at concentrations such that the volume added to the reaction is never less than 0.5 μL, to limit pipette error.
13. At no point should the reaction be mixed so vigorously that any liquid touches the caps of the 1.7 mL tubes.
14. It is essential that the drops all be similarly shaped and in the bottom of the well, so that they can be properly calibrated. It is important to work quickly to minimize the time between assembling the reaction and beginning measurements; however, it is more important to be precise with pipetting.
15. For PCR product template, a higher concentration is recommended, like 30–50 nM.
16. If promoters are being tested from PCR product, 2 μM chi6 must be added (*see* Subheading 3.8). Promoters from PCR product templates should not be directly compared to promoters from plasmid template because transcription initiation is more efficient on a plasmid.
17. If UTRs are being tested from PCR product, 2 μM chi6 must be added (*see* Subheading 3.8). UTRs from PCR product templates should not be directly compared to UTRs from plasmid template because transcription initiation is more efficient on a plasmid.

18. Relative critical concentrations of sigma factors can also be qualitatively determined by analyzing the time before maximum slope is reached. The longer the time, the more sigma factor is required for optimum protein synthesis.
19. APS can be stored at 4 °C and reused for up to 1 week.
20. Temed and APS catalyze the polymerization of acrylamide, so it is important to work quickly after adding them.
21. The gel cast can be rocked back and forth gently to ensure a flat horizontal gel border.
22. The water should also get rid of any bubbles, so none should be left.
23. This is a 10× dilution, ideal for most of the TXTL reactions. Based on the concentration of protein, a smaller or larger dilution factor may be desired.
24. This time depends on the size of the protein being analyzed. If the protein is much smaller or larger than 25 kDa, a shorter or longer time may be desired.
25. For this protocol, it is essential to work quickly and accurately. A digital timer is necessary.
26. *See* ref. 10 for derivation.
27. Work quickly after adding eGFP-ssrA to get as close to a time zero measurement as possible.
28. The concentration of ClpXP is not constant in time for each reaction because we continuously express. Two other approaches can be used if we want a constant level of ClpXP [10]. The first one consists of purifying the ClpXP complex and adding it to a cell-free reaction. The second one, more convenient, consists of producing first ClpXP in a TXTL reaction using the plasmid P70a-ClpXP. After the reaction is complete, a restriction enzyme is added to inhibit expression from P70a-ClpXP. Various amounts of this reaction can be added to a TXTL reaction to accelerate protein degradation.

Acknowledgments

This material is based upon work supported by the Defense Advanced Research Projects Agency (contract HR0011-16-C-01-34) and the Office of Naval Research (award N00014-13-1-0074).

References

1. Katzen F, Chang G, Kudlicki W (2005) The past, present and future of cell-free protein synthesis. Trends Biotechnol 23(3):150–156. https://doi.org/10.1016/j.tibtech.2005.01.003
2. Hodgman CE, Jewett MC (2011) Cell-free synthetic biology: thinking outside the cell. Metab Eng. S1096-7176(11)00092-9 [pii] 14(3):261–269. https://doi.org/10.1016/j.ymben.2011.09.002
3. Takahashi MK, Hayes CA, Chappell J, Sun ZZ, Murray RM, Noireaux V, Lucks JB (2015) Characterizing and prototyping genetic networks with cell-free transcription-translation reactions. Methods. https://doi.org/10.1016/j.ymeth.2015.05.020
4. He M (2008) Cell-free protein synthesis: applications in proteomics and biotechnology. New Biotechnol 25(2–3):126–132. https://doi.org/10.1016/j.nbt.2008.08.004. S1871-6784(08)00072-1 [pii]
5. Liu CC, Schultz PG (2010) Adding new chemistries to the genetic code. Annu Rev Biochem 79(79):413–444. https://doi.org/10.1146/annurev.biochem.052308.105824
6. Daube SS, Arad T, Bar-Ziv R (2007) Cell-free co-synthesis of protein nanoassemblies: tubes, rings, and doughnuts. Nano Lett 7(3):638–641. https://doi.org/10.1021/nl062560n
7. Pardee K, Slomovic S, Nguyen PQ, Lee JW, Donghia N, Burrill D, Ferrante T, McSorley FR, Furuta Y, Vernet A, Lewandowski M, Boddy CN, Joshi NS, Collins JJ (2016) Portable, on-demand biomolecular manufacturing. Cell 167(1):248–259.e212. https://doi.org/10.1016/j.cell.2016.09.013
8. Noireaux V, Bar-Ziv R, Libchaber A (2003) Principles of cell-free genetic circuit assembly. Proc Natl Acad Sci U S A 100(22):12672–12677. https://doi.org/10.1073/pnas.2135496100. 2135496100 [pii]
9. Shin J, Noireaux V (2012) An E. coli cell-free expression toolbox: application to synthetic gene circuits and artificial cells. ACS Synth Biol 1(1):29–41. https://doi.org/10.1021/sb200016s
10. Garamella J, Marshall R, Rustad M, Noireaux V (2016) The all E. coli TX-TL toolbox 2.0: a platform for cell-free synthetic biology. ACS Synth Biol. https://doi.org/10.1021/acssynbio.5b00296
11. Sun ZZ, Yeung E, Hayes CA, Noireaux V, Murray RM (2014) Linear DNA for rapid prototyping of synthetic biological circuits in an Escherichia coli based TX-TL cell-free system. ACS Synth Biol 3(6):387–397. https://doi.org/10.1021/Sb400131a
12. Takahashi MK, Chappell J, Hayes CA, Sun ZZ, Kim J, Singhal V, Spring KJ, Al-Khabouri S, Fall CP, Noireaux V, Murray RM, Lucks JB (2015) Rapidly characterizing the fast dynamics of RNA genetic circuitry with cell-free transcription-translation (TX-TL) systems. ACS Synth Biol 4(5):503–515. https://doi.org/10.1021/sb400206c
13. Karzbrun E, Tayar AM, Noireaux V, Bar-Ziv RH (2014) Programmable on-chip DNA compartments as artificial cells. Science 345(6198):829–832. https://doi.org/10.1126/Science.1255550
14. Tayar AKE, Noireaux V, Bar-Ziv R (2015) Propagating gene expression fronts in a one-dimensional coupled system of artificial cells. Nat Phys. https://doi.org/10.1038/NPHYS3469
15. Niederholtmeyer H, Sun ZZ, Hori Y, Yeung E, Verpoorte A, Murray RM, Maerkl SJ (2015) Rapid cell-free forward engineering of novel genetic ring oscillators. eLife 4. https://doi.org/10.7554/eLife.09771
16. Kigawa T, Yabuki T, Matsuda N, Matsuda T, Nakajima R, Tanaka A, Yokoyama S (2004) Preparation of Escherichia coli cell extract for highly productive cell-free protein expression. J Struct Funct Genom 5(1–2):63–68. https://doi.org/10.1023/B:JSFG.0000029204.57846.7d
17. Calhoun KA, Swartz JR (2005) An economical method for cell-free protein synthesis using glucose and nucleoside monophosphates. Biotechnol Prog 21(4):1146–1153. https://doi.org/10.1021/bp050052y
18. Spirin AS, Swartz JR (2008) Cell-free protein synthesis: methods and protocols. Wiley-VCH, Weinheim
19. Shin J, Jardine P, Noireaux V (2012) Genome replication, synthesis, and assembly of the bacteriophage T7 in a single cell-free reaction. ACS Synth Biol 1(9):408–413. https://doi.org/10.1021/Sb300049p
20. Ishikawa K, Sato K, Shima Y, Urabe I, Yomo T (2004) Expression of a cascading genetic network within liposomes. FEBS Lett 576(3):387–390. https://doi.org/10.1016/j.febslet.2004.09.046. S0014579304011743 [pii]
21. Noireaux V, Libchaber A (2004) A vesicle bioreactor as a step toward an artificial cell assembly. Proc Natl Acad Sci U S A 101(51):17669–17674. https://doi.org/10.1073/pnas.0408236101. 0408236101 [pii]

22. Swartz J (2006) Developing cell-free biology for industrial applications. J Ind Microbiol Biotechnol 33(7):476–485. https://doi.org/10.1007/s10295-006-0127-y
23. Pardee K, Green AA, Ferrante T, Cameron DE, DaleyKeyser A, Yin P, Collins JJ (2014) Paper-based synthetic gene networks. Cell 159(4):940–954. https://doi.org/10.1016/j.cell.2014.10.004
24. Chemla Y, Ozer E, Schlesinger O, Noireaux V, Alfonta L (2015) Genetically expanded cell-free protein synthesis using endogenous pyrrolysyl orthogonal translation system. Biotechnol Bioeng 112(8):1663–1672. https://doi.org/10.1002/bit.25587
25. Iwane Y, Hitomi A, Murakami H, Katoh T, Goto Y, Suga H (2016) Expanding the amino acid repertoire of ribosomal polypeptide synthesis via the artificial division of codon boxes. Nat Chem 8(4):317–325. https://doi.org/10.1038/NCHEM.2446
26. Hong SH, Kwon YC, Jewett MC (2014) Non-standard amino acid incorporation into proteins using Escherichia coli cell-free protein synthesis. Front Chem 2. https://doi.org/10.3389/Fchem.2014.00034. Artn 34
27. Shin J, Noireaux V (2010) Efficient cell-free expression with the endogenous E. coli RNA polymerase and sigma factor 70. J Biol Eng 4:8
28. Shin J, Noireaux V (2010) Study of messenger RNA inactivation and protein degradation in an Escherichia coli cell-free expression system. J Biol Eng 4:9. https://doi.org/10.1186/1754-1611-4-9. 1754-1611-4-9 [pii]
29. Enz S, Braun V, Crosa JH (1995) Transcription of the region encoding the ferric dicitrate-transport system in Escherichia coli: similarity between promoters for fecA and for extracytoplasmic function sigma factors. Gene 163(1):13–18. doi:037811199500380O [pii]
30. Lipinska B, Sharma S, Georgopoulos C (1988) Sequence analysis and regulation of the htrA gene of Escherichia coli: a sigma 32-independent mechanism of heat-inducible transcription. Nucleic Acids Res 16(21):10053–10067
31. Arnosti DN, Chamberlin MJ (1989) Secondary sigma-factor controls transcription of flagellar and chemotaxis genes in Escherichia-coli. Proc Natl Acad Sci USA 86(3):830–834
32. Wang Y, deHaseth PL (2003) Sigma 32-dependent promoter activity in vivo: sequence determinants of the groE promoter. J Bacteriol 185(19):5800–5806. https://doi.org/10.1128/Jb.185.19.5800-5806.2003
33. Yim HH, Brems RL, Villarejo M (1994) Molecular characterization of the promoter of Osmy, an Rpos-dependent gene. J Bacteriol 176(1):100–107
34. Reitzer LJ, Magasanik B (1985) Expression of Glna in Escherichia-Coli is regulated at tandem promoters. Proc Natl Acad Sci USA 82(7):1979–1983
35. Lutz R, Bujard H (1997) Independent and tight regulation of transcriptional units in Escherichia coli via the LacR/O, the TetR/O and AraC/I-1-I-2 regulatory elements. Nucleic Acids Res 25(6):1203–1210
36. Olins PO, Devine CS, Rangwala SH, Kavka KS (1988) The T7 phage gene 10 leader RNA, a ribosome-binding site that dramatically enhances the expression of foreign genes in Escherichia coli. Gene 73(1):227–235. doi:0378-1119(88)90329-0 [pii]
37. Larson MH, Greenleaf WJ, Landick R, Block SM (2008) Applied force reveals mechanistic and energetic details of transcription termination. Cell 132(6):971–982. https://doi.org/10.1016/j.cell.2008.01.027. S0092-8674(08)00127-X [pii]
38. Flynn JM, Neher SB, Kim YI, Sauer RT, Baker TA (2003) Proteomic discovery of cellular substrates of the ClpXP protease reveals five classes of ClpX-recognition signals. Mol Cell 11(3):671–683. doi:S1097276503000601 [pii]
39. Marshall R, Maxwell C, Collins SP, Jacobsen T, Luo ML, Begemann MB, Gray BN, January E, Singer A, He Y, Beisel CL, Noireaux V (2018) Rapid and scalable characterization of CRISPR technologies using an E. coli cell-free transcription-translation system. Mol Cell. 69:146–157. https://doi.org/10.1016/j.molcel.2017.12.007
40. Caschera F, Noireaux V (2014) Synthesis of 2.3 mg/ml of protein with an all Escherichia coli cell-free transcription-translation system. Biochimie 99:162–168. https://doi.org/10.1016/j.biochi.2013.11.025
41. Sun ZZ, Hayes CA, Shin J, Caschera F, Murray RM, Noireaux V (2013) Protocols for implementing an Escherichia coli based TX-TL cell-free expression system for synthetic biology. J Vis Exp 79:e50762. https://doi.org/10.3791/50762
42. Caschera F, Noireaux V (2015) Preparation of amino acid mixtures for cell-free expression systems. BioTechniques 58(1):40–43. https://doi.org/10.2144/000114249
43. Ishihama Y, Schmidt T, Rappsilber J, Mann M, Hartl FU, Kerner MJ, Frishman D (2008) Protein abundance profiling of the Escherichia coli cytosol. BMC Genomics 9:102. https://

doi.org/10.1186/1471-2164-9-102. 1471-2164-9-102 [pii]

44. Marshall R, Maxwell CS, Collins SP, Beisel CL, Noireaux V (2017)Short DNA containing chisites enhances DNA stability and gene expression in E. coli cell-free transcription-translation systems. Biotechnol Bioeng. https://doi.org/10.1002/bit.26333
45. Kelwick R, Webb AJ, MacDonald JT, Freemont PS (2016) Development of a Bacillus subtilis cell-free transcription-translation system for prototyping regulatory elements. Metab Eng 38:370–381. https://doi.org/10.1016/j.ymben.2016.09.008
46. Gan R, Jewett MC (2014) A combined cell-free transcription-translation system from Saccharomyces cerevisiae for rapid and robust protein synthesis. Biotechnol J 9(5):641–651. https://doi.org/10.1002/biot.201300545