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TITLE:

Rapid Characterization of Genetic Parts with Cell-free Systems

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SUMMARY:

Well-characterized genetic parts are necessary for the design of novel genetic circuits. Here we describe a cost-effective, high-throughput method for rapidly characterizing genetic parts. Our method reduces cost and time by combining cell-free lysates, linear DNA to avoid cloning, and acoustic liquid handling to increase throughput and reduce reaction volumes.

ABSTRACT:

Characterizing and cataloging genetic parts are critical to the design of useful genetic circuits. Having well-characterized parts allows for the fine-tuning of genetic circuits, such that their function results in predictable outcomes. With the growth of synthetic biology as a field, there has been an explosion of genetic circuits that have been implemented in microbes to execute functions pertaining to sensing, metabolic alteration, and cellular computing. Here, we show a

rapid and cost-effective method for characterizing genetic parts. Our method utilizes cell-free lysate, prepared in-house as a medium to evaluate parts via the expression of a reporter protein. Template DNA is prepared by PCR amplification using inexpensive primers to add variant parts to the reporter gene, and the template is added to the reaction as linear DNA without cloning. Parts that can be added in this way include promoters, operators, ribosome binding sites, insulators, and terminators. This approach, combined with the incorporation of an acoustic liquid handler and 384-well plates, allows the user to carry out high-throughput evaluations of genetic parts in a single day. By comparison, cell-based screening approaches require time-consuming cloning and have longer testing times due to overnight culture and culture density normalization steps. Further, working in cell-free lysate allows the user to exact tighter control over the expression conditions through the addition of exogenous components and DNA at precise concentrations. Results obtained from cell-free screening can be used directly in applications of cell-free systems or, in some cases, as a way to predict function in whole cells.

INTRODUCTION:

A core effort of synthetic biology is to develop genetic tool kits containing well-characterized parts, which can be used to construct genetic circuits[1] that carry out useful functions when deployed in microbes or cell-free lysates. Areas in which such genetic circuits have gained traction are sensing[2–4], human performance[5,6], biofuels[7,8], materials production[9,10], and cellular computing[11]. Registries of standardized genetic parts have been established[12] to catalog new and existing parts into categories such as promoters, operators, coding sequences, and terminators, to name just a few. Efforts such as the iGEM (international Genetically Engineered Machines) competition[13] have been instrumental in characterizing and cataloging these genetic parts. Many methods have been developed to facilitate the rapid assembly of these parts into useful genetic circuits[14,15]. Software has even been developed to automate the composition of well-characterized parts into circuits that achieve a desired function[16]. However, the assembly of useful genetic circuits with predictable functions rests on the presumption that the genetic tool kits contain well-characterized genetic parts. Due to the necessity of these tool kits toward the advancement of synthetic biology, numerous efforts to better catalog circuits and parts with appropriate characterization data have been described[17–21].

One approach to characterizing genetic components makes use of cell-free protein synthesis (CFPS) systems, which reconstitute cellular functions such as transcription and translation *ex vivo*[22]. Several studies have demonstrated the potential of CFPS for prototyping genetic components[23–32] whether for direct applications in cell-free systems or to predict the function of genetic constructs in cells, such as the relative activity of parts within a library[29], metabolic pathway optimization[27], and cellular burden[30]. Advantages to prototyping in CFPS versus cells highlighted by these studies include avoiding time-consuming cloning, precise control over the concentration of DNA and other reaction components, and the ability to easily mix and match multiple DNA constructs. The advantage of avoiding cloning is especially apparent when using linear DNA templates, which enables new constructs to be assembled by *in vitro* methods that take hours instead of days[33]. The ability to manipulate the concentration of DNA constructs and other components simply by pipetting makes the approach even more attractive by enabling high-throughput experimentation powered by liquid handling robots[34,35]. While successes using

CFPS for prototyping have been reported, it is important to note that it remains to be seen under what contexts CFPS results can reliably predict functionality in cells.

Here, we present a method for CFPS prototyping that emphasizes the advantages in speed, throughput, and cost compared to traditional cell-based approaches. The approach is derived from our previous work where we used CFPS to rapidly characterize a library of T7 promoter variants regulated by the transcription factor TetR[32], significantly expanding on the small handful of regulated T7 promoter variants that were available in the literature at the time[36,37]. Others have, since then, further expanded the range of such promoters[38]. In our method, genetic construct assembly is accelerated by using PCR to amplify template DNA via primers that add variant genetic parts to a reporter gene. Acoustic liquid handling in 384-well plates is used to increase throughput and decrease the volume of materials required. Previous work has demonstrated successful use of acoustic liquid handling at significantly lower volumes[39,40] with variability comparable to manual pipetting of larger volumes[41]. In addition to the method, we provide troubleshooting information and an assessment of potential cost and time savings. Note that while we include a protocol for producing cell-free lysates based on Sun et al.[42] here, numerous other commercial kits and protocols[43,44] should also work. Similarly, while we demonstrate the method for the characterization of promoter variants[32], other parts can be interchanged by PCR amplification, such as riboregulators, Ribosome Binding Sites (RBSs), insulators, protein tags, and terminators. We hope that this methodology can help the synthetic biology community continue to grow the number of characterized parts for the assembly of predictable genetic circuits with useful function.

PROTOCOL:

1. Preparation of cell extract

1.1. Preparation of media

1.1.1. For 2xYT media: Add 16 g of tryptone, 10 g of yeast extract, and 5 g of NaCl to 900 mL of deionized water and adjust the pH to 7.0 with 5 M NaOH. Raise the solution volume to 1 L using deionized water and autoclave or filter sterilize. Alternatively, purchase 2xYT media.

1.1.2. For S30B buffer: Prepare a solution of 14 mM Mg-glutamate, 60 mM K-glutamate, and 5 mM Tris in 2 L of deionized water. Use 2 M acetic acid to adjust the pH to 8.2 and store at 4 °C. Complete the solution by adding dithiothreitol (DTT) to a final concentration of 1 mM just before the use.

1.2. Preparation of cells

1.2.1. Streak *Escherichia coli* BL21(DE3) Rosetta2 cells or other cell line of choice (see Cole et al.[44] for a recent comprehensive review) onto an LB (Lysogeny Broth) agar plate and incubate at 37 °C for 10–14 h.

1.2.2. Use a single *E. coli* colony to inoculate 3 mL of 2xYT medium in a 10 mL culture tube. Incubate this tube at 37 °C with shaking at 250 rpm for 8 h.

1.2.3. Use 50 µL from the 3 mL culture to inoculate 50 mL of 2xYT medium in a 500 mL flask. Incubate this flask at 37 °C with shaking at 250 rpm for 8 h.

1.2.4. Use 7.5 mL from the 50 mL culture to inoculate each of the four 4 L baffled flasks containing 0.75 L of 2xYT medium. Incubate these flasks at 37 °C with shaking at 220 rpm until they have reached an optical density at 600 nm of 2 to 4, after approximately 3–4 h.

1.2.5. Harvest the cells from each flask by transferring them to 1 L containers and centrifuging at 5,000 x *g* for 12 min. Discard the supernatant by decanting into a waste container.

1.2.6. Wash each cell pellet with 150 mL of ice-cold S30B buffer by completely resuspending them using a pipette to disrupt the cell mass, and then collect the cells again by centrifugation at 5,000 x *g* for 12 min. Discard the supernatant.

1.2.7. Wash each cell pellet again in 40 mL of ice-cold S30B buffer by completely resuspending them and disrupting the cell mass using a pipette. Transfer the cells to pre-weighed 50 mL conical tubes and collect the cells again by centrifugation at 2,000 x *g* for 8 min. Discard the supernatant by decanting.

1.2.8. Weigh the wet cell pellets. Flash-freeze the cell pellets by placing the tubes directly into liquid nitrogen and store at -80 °C.

1.3. Cell lysis

1.3.1. Thaw the cell pellets on ice.

1.3.2. Resuspend each cell pellet in 1.4 mL of S30B buffer per 1 g of the cell pellet by vortexing.

1.3.3. Lyse the cells by French pressure cell at 640 psi at 4 °C. Collect the lysate in microcentrifuge tubes on ice and add 3 µL of 1 M DTT per 1 mL of lysate immediately after lysis.

NOTE: It is best to tap the French press release valve with a small metal rod to maintain even pressure and avoid sudden drops in pressure.

1.3.4. Clear the lysate by centrifugation at 30,000 x *g* for 30 min at 4 °C and discard the pellet after pipetting the supernatant to a new ice-cold microcentrifuge tube, taking care not to disrupt the pellet.

1.3.5. Centrifuge the supernatant a second time at 30,000 x *g* for 30 min at 4 °C. Pipette the resulting supernatant into an ice-cold microcentrifuge tube. Discard the pellet.

1.3.6. Incubate the supernatant in a 37 °C water bath for 1 h.

1.3.7. Clear the supernatant by centrifugation at 15,000 x *g* for 15 min at 4 °C and transfer the resulting supernatant to an ice-cold microcentrifuge tube, taking care not to disrupt the pellet.

1.3.8. Centrifuge the supernatant a second time at 15,000 x *g* for 15 min at 4 °C and transfer the resulting supernatant to an ice-cold microcentrifuge tube, taking care not to disrupt any remaining pellet.

1.3.9. Distribute the supernatant in 100 µL aliquots into 1.5 mL microcentrifuge tubes and flash freeze them by placing directly into liquid nitrogen. Store the supernatant at -80 °C.

2. Linear template preparation

2.1. Primer design

2.1.1. Choose a core sequence as the PCR template. Include at a minimum a reporter sequence, such as sfGFP (superfolder Green Fluorescent Protein), LacZ, or Spinach aptamer. Include other parts that will be fixed across screened variants, such as terminators, promoters or RBSs, as appropriate for the design.

NOTE: Inclusion of a terminator is not always required for expression from linear DNA in cell-free systems.

2.1.2. For the forward primers, choose a minimum of 20 bp matching the 5′ end of the core sequence as the 3′ end of the primer. If adding parts to the 5′ end of the construct, design the remainder of the 5′ end of the primer to add the genetic parts of interest to the core sequence via PCR amplification (**Figure 1A** and **Figure 2**).

NOTE: Since primers above ~60 bp frequently increase dramatically in cost, multiple overlapping primers can be designed to add longer sequences or multiple parts. While multiple primers can be used in a single PCR reaction, perform multiple rounds of PCR.

2.1.3. For the reverse primer, choose a minimum of 20 bp to match the 3′ end of the core sequence as the 5′ end of the primer. If adding parts to the 3′ end of the construct, design the remainder of the 3′ end of the primer to add the genetic parts of interest to the core sequence via PCR amplification (**Figure 1A** and **Figure 2**). Ensure that the reverse primer's annealing temperature is within 5 °C of the annealing temperature of the entire forward primer.

2.2. Linear template amplification

2.2.1. Determine the number of PCR reactions to be performed based on the number of core sequences and calculate the amount of each component required using **Table 1**.

2.2.2. Prepare the master mix according to **Table 1** and store it on ice. Aliquot 30 or 40 µL (see **Table 1**) of the master mix into the determined number of PCR tubes and add 10 µL of each variable primer (i.e., primers encoding a part change, see **Table 1**) at 5 µM to appropriately labeled PCR tubes.

2.2.3. Place the PCR tubes into the thermocycler and run the following PCR program: 98 °C for 3 min; 30 cycles of 98 °C for 15 s, XX °C for 20 s, 72 °C for YY min, final extension at 72 °C for 10 min. Then, hold the reaction at 4 °C.

Where, XX represents the annealing temperature for the primer with the lower annealing temperature and YY represents the extension time calculated for the length of the amplicon based on the manufacturer's recommendations for the high-fidelity polymerase used. Optimize these conditions as needed for different primers and/or templates.

2.2.4. (Optional) Add 1 µL of DpnI restriction enzyme to digest the original template. Incubate the reaction at 37 °C for 1 h. Perform this step only if the original template is plasmid DNA.

2.2.5. Analyze 5 µL of each PCR product by gel electrophoresis. Separate the product using a 1% agarose gel at 180 V for 20 min. Check for the correct band size, which will vary with the chosen core sequence and the length of the parts added.

2.3. Purify the linear template using a commercial PCR purification kit or by the preferred PCR cleanup method. If multiple bands were present by gel electrophoresis analysis, either optimize the PCR conditions or purify the correct molecular weight bands using a commercial gel extraction kit as per the manufacturer's recommendation.

2.4. Quantify each DNA template using a spectrophotometer. Assess the DNA template quality by checking that the 260 nm/280 nm ratio is approximately 1.8.

2.5. (Optional) Again separate a portion of the DNA template using a 1% agarose gel at 180 V for 20 min and ensure that any unwanted bands were removed during template purification.

2.6. Use purified DNA templates immediately or store at -20 °C.

3. Purified protein preparation

3.1. Protein expression

3.1.1. For each protein to be expressed, assemble an appropriate expression construct. Codon-optimize the gene for expression in *E. coli*. Insert the gene into a pET-22b expression vector or other appropriate expression vector via the preferred plasmid assembly method. Transform the expression plasmid into BL21(DE3) Rosetta2 expression cells or other appropriate cell line.

3.1.2. For each protein, use a single colony to inoculate 3 mL of the LB medium in a 10 mL culture

tube. Incubate these tubes at 37 °C with shaking at 250 rpm overnight.

3.1.3. Inoculate a 2 L flask containing 750 mL of LB medium with 1 mL of the overnight culture. Incubate these flasks at 37 °C with shaking at 250 rpm until they reach an OD_{600} of 0.6–1.0.

3.1.4. Induce protein expression by adding 0.75 mL of 1 M isopropyl β-D-1-thiogalactopyranoside (IPTG) in water to each flask and continue to incubate these flasks at 37 °C with shaking at 250 rpm for 4 h.

3.1.5. Harvest the cells from each flask, using a 1 L centrifuge bottle, by centrifugation at 5,000 x *g* for 12 min. Discard the supernatant.

3.1.6. Transfer the pellets to a 50 mL conical tube and weigh each pellet. Flash-freeze the cells in liquid nitrogen and store them at -80 °C or proceed to step 3.2.

NOTE: 2–5 g of protein per 0.75 mL are expected to result from this step.

3.2. Protein purification by nickel affinity column chromatography

3.2.1. Prepare the lysis buffer by combining 50 mM Tris-Cl, 500 mM NaCl, and 5 mM imidazole. Adjust to pH 8.0.

3.2.2. Prepare the wash buffer by combining 50 mM Tris-Cl, 500 mM NaCl, and 25 mM imidazole. Adjust to pH 8.0.

3.2.3. Prepare the elution buffer by combining 50 mM Tris-Cl, 500 mM NaCl, and 250 mM imidazole. Adjust to pH 8.0.

3.2.4. Prepare the dialysis buffer by combining 50 mM $NaHPO_4$, 100 mM NaCl, and 2% DMSO. Adjust to pH 7.5.

3.2.5. Thaw the cell pellets by placing the tubes in room temperature water. Add 5 mL of the lysis buffer per 1 g of the cell pellet and resuspend by vortexing.

3.2.6. Lyse the cells by sonication. Separate the cell homogenate so that there is no more than 30 mL per 50 mL conical tube and keep each tube on ice. Lyse the cells using a sonicator with a 0.16 cm diameter probe in 15 s rounds with 30 s breaks, 10 times.

NOTE: Avoid foaming, as this denatures the protein. The formation of foam can be avoided by keeping the sonicator tip at least 2/3 submerged in the lysate while it is operational. Other methods of cell lysis besides sonication are also possible[44].

3.2.7. Clear the lysate by centrifugation at 15,000 x *g* for 30 min at 4 °C and place the supernatant into a new 50 mL conical tube.

3.2.8. Add 1 mL of nickel-nitrilotriacetic acid (Ni-NTA) resin for each 5 mL of supernatant. Divide the cell lysate/Ni-NTA slurry so that there is no more than 36 mL per 50 mL conical tube. Incubate at 4 °C on a tube rotator at 10 rpm for 1 h.

3.2.9. Load the resin by decanting the cell lysate/Ni-NTA slurry into a 2 mL of bed volume chromatography column and collect the eluant if needed for further analysis, otherwise, discard. Wash the resin with 10 resin bed volumes of wash buffer.

3.2.10. Collect the protein by adding three resin bed volumes of elution buffer to the column and concentrate the volume to 1.5 mL using a centrifugal concentrator with the appropriate molecular weight cut-off membrane for each protein.

3.2.11. Dialyze the protein against 2 L of dialysis buffer at 4 °C for 1 h. Dialyze the protein again against 2 L of dialysis buffer overnight at 4 °C.

3.2.12. Quantify the protein using its molar extinction coefficient and absorbance at 280 nm. Analyze the protein for purity by separating it using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Store the protein at -80 °C.

4. Cell-free protein synthesis

4.1. Preparation of CFPS reaction mixture

4.1.1. Prepare the Supplement Mix by following the Amino Acid Solution Preparation, Energy Solution Preparation, and Buffer Preparation steps in Sun et al.[42]. Store separately or combined at -80 °C in aliquots. Ensure that final concentrations match those described in Sun et al.[42] in the Experimental Execution of a TX-TL Reaction section.

4.1.2. Prepare an additive to protect linear DNA from degradation. If using GamS[33,45], prepare via steps in section 3 above, or obtain from a commercial vendor; for other approaches, check the corresponding literature[46–48]. Alternatively, use a CFPS system that does not require additives[49].

4.1.3. Prepare T7 polymerase, repressor proteins, and other additives using the steps in section 3 above or obtain from a commercial vendor.

4.1.4. Determine the number of CFPS reactions to be performed and calculate the amount of each component required using **Table 2**. Modify the concentrations of the components, including adding or removing components as needed, and adjust the amount of water such that the final volume of each reaction mixture is always 10 µL. Similarly, modify the master mix to facilitate dispensing of other components by acoustic liquid handling as desired (see the **Discussion** section).

4.1.5. Thaw all the components on ice and prepare a master mix by mixing each component as

calculated above. Mix all the components thoroughly by pipette. Pay careful attention to avoid precipitation, especially for the amino acid mixture. Keep the master mix on ice.

4.1.6. Chill a 384-well plate on ice and distribute the master mix in 9 µL aliquots into each well using an electronic repeater pipette.

NOTE: It is possible to distribute these components by acoustic liquid handling, though care should be taken to ensure proper dispensing (see the **Discussion** section for troubleshooting).

4.2. Distribution of additional components by acoustic liquid handling

4.2.1. Calculate the amount of repressor protein (and other optional components) required for all the CFPS reactions.

4.2.2. Thaw the repressor protein on ice and distribute it into an acoustic liquid handling source plate or other appropriate plate. Ensure that the appropriate amount of dead volume required for the type of source plate used is included.

4.2.3. Distribute the repressor protein in 1 µL volumes into the appropriate wells via the liquid handler. For more information on distribution troubleshooting, see the **Discussion** section.

4.3. Standard curves

4.3.1. Include a serial dilution of the purified reporter (see section 3 for protein purification)[41] or appropriate chemical standard[50] on the plate to enable comparison of results with other studies and other labs. Choose a range of concentrations appropriate for the reporter used and expected expression range of the experiments.

4.4. Running CFPS reactions

4.4.1. Pre-warm the plate reader to 30 °C. Set the plate reader to read at settings appropriate for the reporter used in the core sequence without the shaking steps.

NOTE: While 30 °C is used here, 29 °C and 37 °C are also commonly used and work well with this protocol. Other temperatures may be preferred for alternative cell-free reaction preparations. For read intervals, 10 min is sufficient to achieve a good resolution for the representative data presented here; however, other resolutions may be better depending on the reporter protein and the particular CFPS recipe.

4.4.2. (Optional) Run a test reaction first to set the appropriate gain or sensitivity setting to capture the change in fluorescence without signal overflow.

4.4.3. Seal the 384-well plate with an impermeable plastic sealable lid to prevent evaporation. If possible, on the instrument, set a 1 °C vertical temperature gradient to limit condensation on the

seal. Place the 384-well plate on the plate holder and begin reading.

REPRESENTATIVE RESULTS:

To demonstrate the utility of our methods, we present results that describe the effects of proximity of the *tetO* sequence to the T7 promoter on the regulation of T7 RNAP-driven expression. The full results and their implications can be found in the work of McManus et al.[32]. The workflow is described in **Figure 1**. Fifteen linear templates, varying only in the distance of the T7 promoter relative to the *tetO* sequence, were prepared by PCR-amplifying the sfGFP reporter with primers designed to add each promoter variant (**Figure 2**) as described in section 2 of the protocol. CFPS reaction components and reactions were prepared following the protocol. The expression of sfGFP was measured from each template with a titration of 12 different concentrations of the TetR protein, in triplicate, using an acoustic liquid handler. At 36 CFPS reactions per template and 15 templates, a total of 540 reactions for the entire set of T7-*tetO* combinations were performed. The entire evaluation was carried out on two plates in two plate readers. Analysis of this data showed that the T7 RNAP downregulates T7-driven expression equally up through 13 bp downstream from the start of the T7 transcript (**Figure 3**). This result has implications for the future design of regulatable T7-driven gene circuits by describing a putative window for an effective repression of T7 by other repressors. Comparison of results from the protocol described here with DNA prepared by traditional cloning revealed a small but statistically significant difference in the degree of TetR repression between formats. We hypothesized that non-specific binding of TetR to the vector DNA could explain the observed difference. Experimental results showed that addition of linear vector DNA to reactions with linear template DNA reduced the difference to non-statistical significance, though it did not rule out contributions from other factors, such as differences in periodicity of the DNA helix for linear vs. circular formats, which, in turn, could affect TetR binding. Depending upon the application, the use of linear template may require additional validation.

We further include representative data on potential issues with accurate dispensing using acoustic liquid handling (**Figure 4**). A solution of 1x phosphate buffered saline (PBS), pH 7.4 containing 0.25 mM tartrazine dye was used to evaluate two methods of programming an acoustic liquid handler to dispense volumes >1 µL. Following liquid dispensing, the destination plate was sealed and centrifuged at 1,500 x g for 1 min, and the absorbance at 425 nm measured with a plate reader. Representative results of nine experiments are shown and demonstrate more consistent dispensing across the series of eight destination wells when the 5 µL transfer is divided into separate 1 µL dispenses. Based on these observations, it is recommended that transfers >1 µL be broken down into multiple transfers of ≤1 µL. See the **Discussion** section for more details on troubleshooting this important aspect of the protocol.

FIGURE AND TABLE LEGENDS:

Figure 1: Single-day workflow for the evaluation of promoter parts in cell-free extract. (**A**) A reporter is PCR-amplified using primers containing genetic parts to be evaluated (2–5 h). (**B**) The cell-free reaction mix is prepared as detailed in the protocol and distributed into a 384-well plate with the PCR-amplified templates (30 min). (**C**) Acoustic liquid handling is used to distribute

additional components, which can include repressor proteins, effector molecules, and any other conditional effectors (10 min). (**D**) Reporter protein expression from each reaction is measured in a plate reader (2–16 h, depending on the CFPS recipe and construct).

Figure 2: Primer design for adding genetic parts to a reporter gene by PCR amplification. (**A**) The sfGFP reporter gene (green) will be amplified to add an RBS (red) and a T7 promoter (blue) by PCR. (**B**) The sfGFP (green) and an RBS (red) will be amplified to add a tetO sequence (gold) and a T7 promoter (blue) by PCR.

Figure 3: The effect of tetO position on the regulation of a T7-driven expression. Normalized maximum repression values for linear and circular template as a function of tetO position. Traces represent the mean and standard deviations for three replicates. This figure has been modified from McManus et al.[32] under a Creative Commons CC-BY license.

Figure 4: Using tartrazine dye to validate liquid dispensing with an acoustic liquid handler. Black bars indicate dispensing 5 µL of tartrazine solution from a single source well into each of the eight consecutive destination wells of a 384-well plate using a single programming command. Gray bars indicate dispensing 1 µL from a single source well into each of eight consecutive destination wells using a single programming command, and then repeat this step four times for a total of 5 µL dispensed in each destination well.

Table 1: Worksheet for the preparation of reagents for PCR reactions. Values in the rightmost column can be filled in by users depending on the intended number of reactions. [1]Variable primers contain a specific part to be added in the PCR reaction and can be the forward primer, reverse primer, or both. Fixed primers do not add a part and can be the forward primer or reverse primer but not both.

Table 2: Worksheet for the preparation of reagents for CFPS reactions. Values in the rightmost column can be filled in by users depending on the intended number of reactions.

DISCUSSION:

The protocols described here provide a cost-effective and rapid means to screen genetic parts via the expression of a reporter protein by CFPS. Well-characterized genetic parts are crucial to the design of predictable genetic circuits with useful function. This methodology increases throughput and decreases the time needed to screen new genetic parts by removing the requirement to work in living cells, while retaining functionality that mirrors the cellular environment by retaining the metabolic process of protein expression in the cell lysate. Our protocol can be performed in 1 day after receipt of primers (~2.5–6 h for reaction preparation, 2–16 h for CFPS reaction; **Figure 1**), compared to at least 3 days for traditional cloning (1 day each for construct assembly and transformation, sequence verification of clones, and culturing of cells for assessment). We further estimate that the cost per construct using linear DNA is roughly one-third of the traditional cloning ($78 vs. $237; **Supplementary Table 1**) methods. Commercial synthesis services currently take a minimum of 5 business days, though they would have similar

costs to our method if linear fragments are screened directly in CFPS ($78 vs. $91); we have not verified this approach. The cost to evaluate a part with CFPS is small compared to the generation of the template DNA ($0.05/reaction[22] vs. $78 per template), though it should be noted that the startup costs for bulk reagents and lysis equipment is at least several thousand dollars. The use of an acoustic liquid handler only marginally improves costs by enabling smaller volumes down to 0.5 µL[40]; the more significant advantage is the reduction of time to prepare reactions (~10 min vs. up to 1 h, depending on the number of reactions), especially when preparing a large number of reactions raises concerns of prepared reaction sitting for extended times before incubation.

While rapid and cost-effective, the limitations on when CFPS prototyping adequately predicts *in vivo* function remain to be seen. For example, any cross-reactivity with genomic DNA will not be detected due to removal of the host genome during the production of the CFPS system. Also, component concentrations can be 1–2 orders of magnitude lower in CFPS than in cells[51], which is likely to affect the behavior of some parts as a result of different macromolecular crowding conditions. Further, the ability of linear DNA to predict *in vivo* function may be limited, for example, when DNA secondary structure plays an important role. A final limitation is that constructs are not sequence-verified before testing for functions. There may be cases where the part characterized is not actually aligned with the intended theoretical sequence. All of these limitations can be mitigated by validating a subset of the parts screened by this method in the intended *in vivo* application.

We originally developed this methodology to investigate the effects of changing the operator position on hybrid T7-tetO promoters[32]. We have presented the protocols here in a more generic format, such that they can be applied to promoters, operators, ribosome binding sequences, insulators, and terminators. These genetic parts can be added to the 5′ or 3′ end of the reporter gene by PCR using primers for each design, obviating the need for synthesis or cloning of each variant to test. The resulting PCR products serve as template DNA for evaluation via the expression of a reporter protein. In our work, the affinity purification protocol provided here was used for TetR and GamS. The same procedure can be used for the expression and purification of other repressors, activators, polymerases, sigma factors, and other proteins cognate to a genetic part of interest, although modifications may be needed for the desired protein being expressed. Purification and titration of these proteins into CFPS reactions enables a more detailed characterization of a particular genetic part. Finally, numerous alternative CFPS protocols exist and each should be amenable to this methodology. Varying the concentrations of underlying constituent components of the CFPS is also possible. The use of liquid handling enhances the ability to test the myriad conditions by increasing throughput and decreasing the materials required[34,35].

One area that can require significant troubleshooting is optimization of the acoustic liquid handler. Acoustic liquid handler dispensing should be optimized for each component being transferred and it is strongly recommended to run controls to verify proper distribution and reproducibility before collecting data. The ideal source plate type and liquid class setting will depend on the specific liquid to be dispensed and its components. It is not recommended to use amine-coated plates to dispense DNA, as the amine coating may interact with the DNA. It should

also be noted that the ability to dispense higher concentrations of certain components may depend on the acoustic liquid handler model. A test liquid transfer may be conducted by dispensing onto a foil plate seal to visualize successful droplet formation; however, this test provides limited information and droplets from different settings may appear identical. The use of a water-soluble dye, such as tartrazine, may be used to more accurately verify the correct volume is dispensed with a given setting or workflow (see **Representative Results**). Optimal programming of liquid transfers can also influence the accuracy and consistency of data generated; for transfers >1 µL from one source well to one destination well, we have found that sequential transfers of ≤1 µL should be programmed to reduce systematic well-to-well variability (**Figure 4**). Lastly, theoretical and actual source well dead volumes can vary dramatically depending on the source plate type, liquid class setting, and components of the specific liquid; using the acoustic liquid handler survey function to assess the well volumes prior to running a program may help gauge how accurately the instrument is able to measure a particular liquid.

CFPS reaction performance can vary when comparing results between different users, batches of materials, plate readers, and laboratories[41]. For instances where such comparisons are required while prototyping genetic circuits, we recommend including internal control reactions with standard constitutive promoters in each reaction plate to help normalize results across experimental setups. The method of DNA preparation can also contribute majorly to CFPS activity; the inclusion of an ethanol precipitation step is recommended. In addition, the optimal reaction composition can vary by the batch of extract[34]. Optimal magnesium glutamate and potassium glutamate concentrations, in particular, have been shown to vary by batch[42] or with the promoter or reporter protein used[24]. Concentrations of these components should be optimized by screening across several concentrations of each component per genetic construct and per cell extract preparation to determine the optimal conditions for protein expression. Finally, best practices for consistent CFPS reaction performance include thorough mixing, careful pipetting, and consistency in the preparation of each reagent component.

Beyond characterization of individual parts, the same method can be used to screen combinations of parts that form complex circuits, such as logic circuits[16] or oscillators[52,53]. This method can also be applied to screening and optimizing biosensors for applications in epidemiological diagnostics[54–57] or hazard detection and quantification[3,58,59]. The application of AI-driven techniques such as active learning[34] can also be paired with the high-throughput nature of this method to drive rapid exploration of complex biological design spaces. Ultimately, we envision this approach supporting accelerated development times for new genetic designs in synthetic biology.

ACKNOWLEDGMENTS:

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DISCLOSURES:

RMM has a financial stake in Tierra Biosciences, a private company that makes use of cell-free technologies such as those described in this article for protein expression and screening.

The other authors have nothing to disclose.

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Figure 1

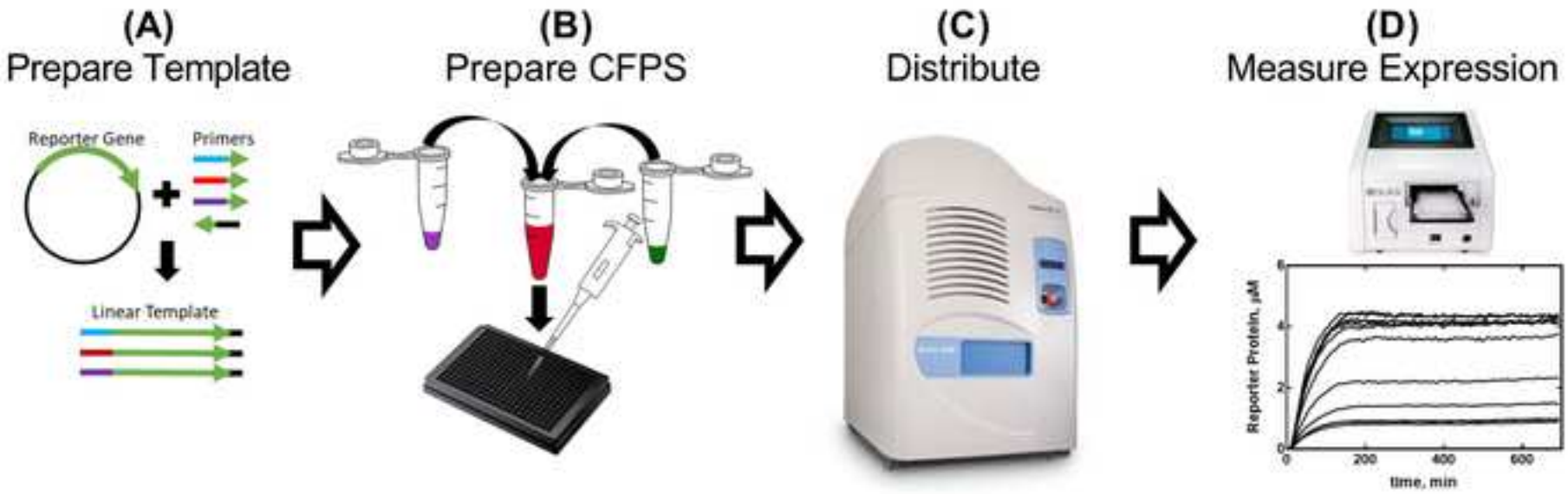

Figure 2

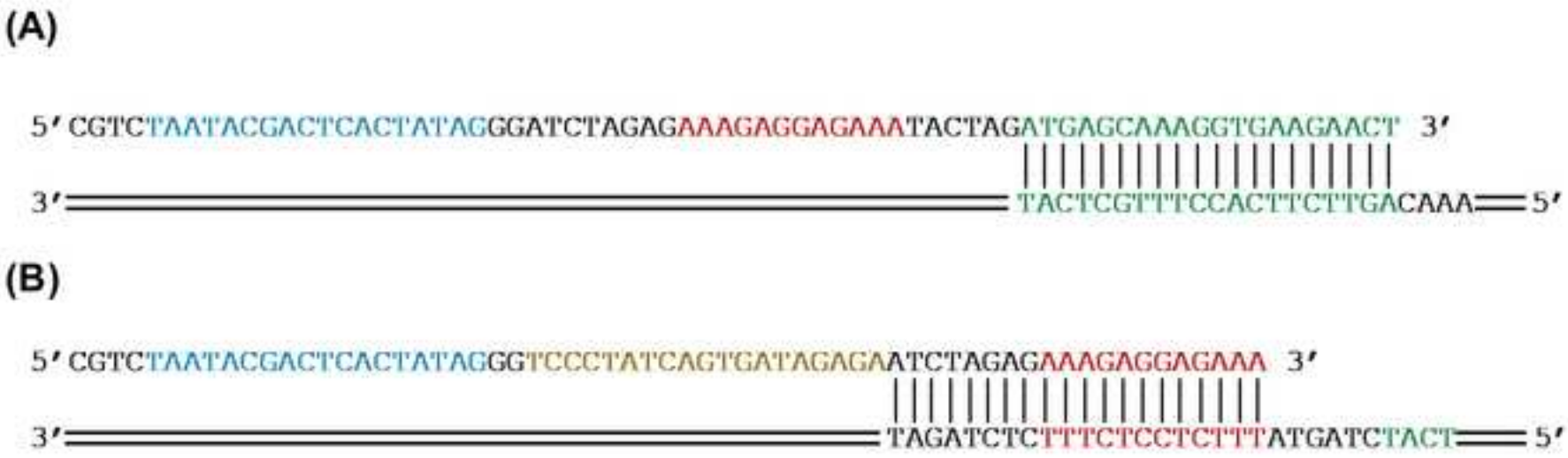

Figure 3

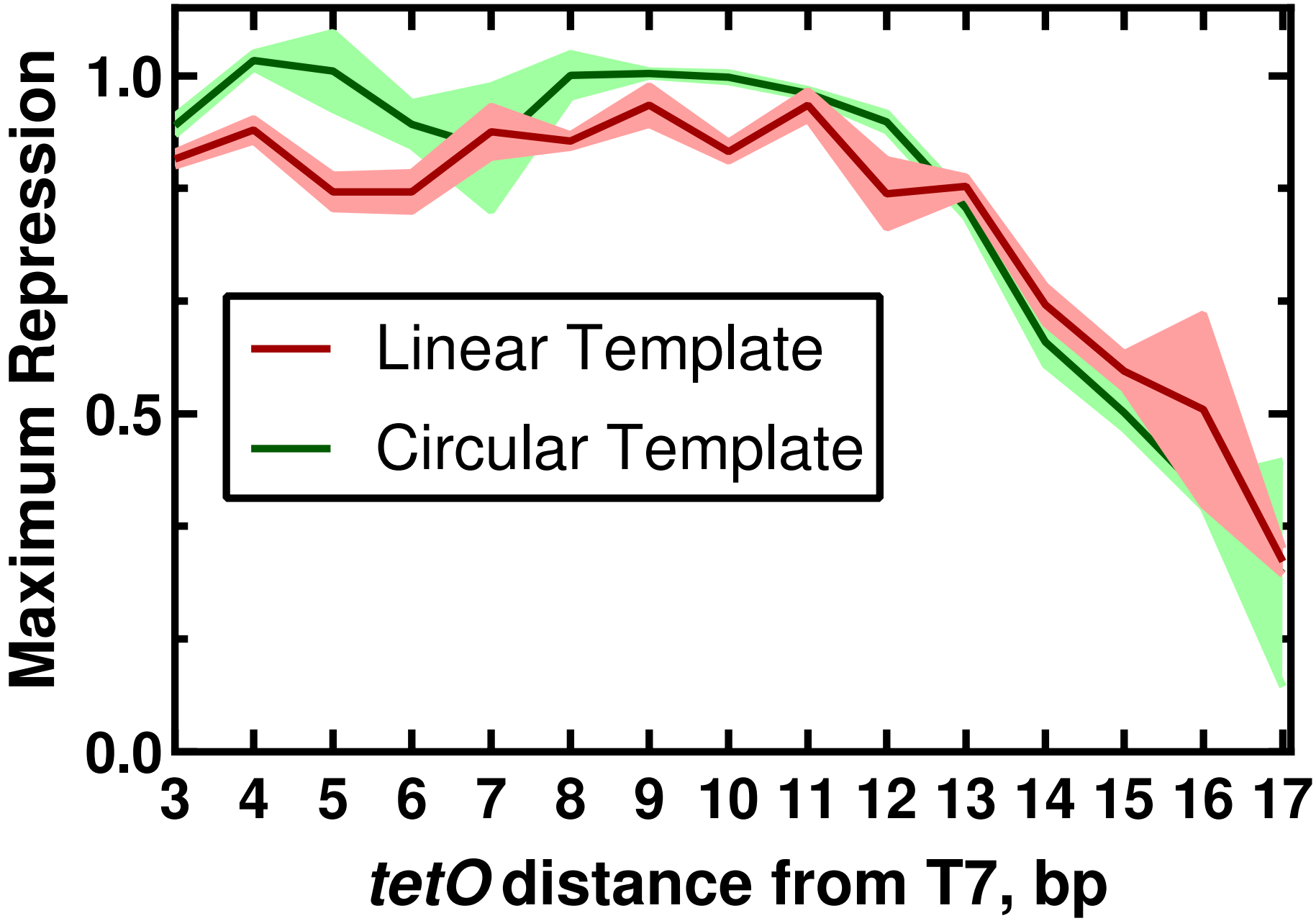

Figure 4

Click here to access/download;Figure;Fig 4 - echo.tif

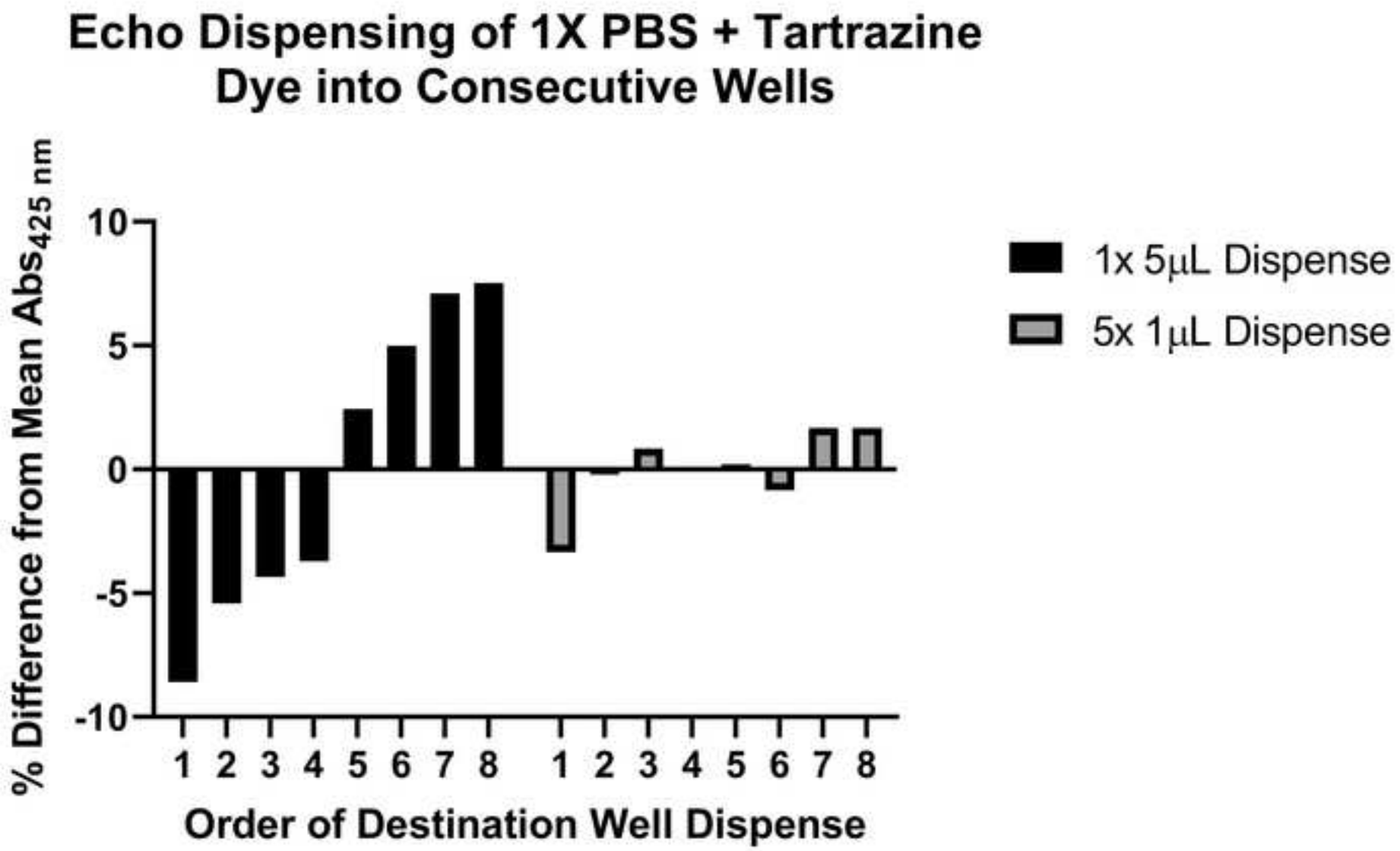

Table 1

Component Name	**Volume for 1 reaction (µL)**
Q5 PCR Premix	25
Water	4
Template (1–3 ng/µL)	1
(if fixed[1]) Forward Primer (5 µM)	0 or 10
(if fixed[1]) Reverse Primer (5 µM)	0 or 10
Master Mix Total:	30 or 40
(if variable[1]) Forward Primer (5 µM)	0 or 10
(if variable[1]) Reverse Primer (5 µM)	0 or 10

Volume for 110% of X number of reactions (µL)

Table 2

Click here to access/download;Table;Table 2 - CFPS reaction.xlsx

Component Name	**Volume for 1 reaction (µL)**	**Volume for 110% of X number of reactions (µL)**
Cell Extract	4.2	
Supplement Mix	3.3	
GamS Protein (207 µM)	0.15	
Template DNA (20 nM)	1	
T7 Polymerase (13 mg/mL)	0.12	
Water	0.73 (this number may vary)	
Master Mix Total:	9	
Repressor Protein:	1	

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
[Response: All spelling and grammar issues that we have found have been corrected.]

2. Please make the title concise and ensure that it directly reflects the protocol being presented.
[Response: We have changed the title with this comment in mind.]

3. Please define all abbreviations during the first-time use.
[Response: We have added missing definitions in a few cases and removed the use of two abbreviations that were not critical. We assume that extremely common abbreviations such as DNA and PCR are exempted, as are units like rpm.]

4. Please ensure the Introduction include all of the following with citation:
a) A clear statement of the overall goal of this method
b) The rationale behind the development and/or use of this technique
c) The advantages over alternative techniques with applicable references to previous studies
d) A description of the context of the technique in the wider body of literature
e) Information to help readers to determine whether the method is appropriate for their application
[Response: We have significantly revamped the introduction, and believe that all of these points are now well addressed.]

5. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points with one inch margin on all sides.
[Response: Fixed.]

6. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Cello, QIAquick PCR Purification Kit, GraphPad Prism, etc.
[Response: All commercial product references removed from manuscript text.]

7. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”
[Response: We have checked the protocol for imperative voice and edited accordingly.]

8. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections.
[Response: We have revisited the protocol section and edited areas where this applies.]

9. Please ensure that individual steps of the protocol should only contain 2-3 actions sentences per step.
[Response: We have edited the protocol with these instructions in mind.]

10. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?
[Response: We added greater detail to the protocols where it may cause confusion for experimenters unfamiliar with the methods related to cell-free work.]

11. 2.1: What and which region is being amplified in your experiment?
[Response: We have added a step to explain how to choose a core sequence for amplification.]

12. 2.2: What is the expected band size in your case?
[Response: The expected band size varies by the parts added and core sequence used. We have added a sentence to clarify this point.]

13. 3.1.1 how is this done?
[Response: There are many ways to do this step that will vary by lab. We have revised the text to be more clear about the process and indicate what approaches we use without being proscriptive.]

14. Only one note can follow one step. In the JoVE Protocol format, "Notes" should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step. Other details should be moved to the discussion section.
[Response: We have reviewed all notes with this guidance in mind and removed or edited many of them accordingly.]

15. There is a 10-page limit for the Protocol, but there is a 3-page limit for filmable content. Please highlight 3 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
[Response: Less than 3 pages of the protocol are highlighted accordingly.]

16. Please ensure the results are described in the context of the presented technique e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included.
[Response: We have significantly revised this section in accordance with this guidance.]

17. Figure 4 is not described in the results section.
[Response: Figure 4 is meant to facilitate troubleshooting and is described in that section. Nonetheless, we added a paragraph to the results section describing Figure 4 to comply with this comment.]

18. Each Figure Legend should include a title and a short description of the data presented in the Figure and relevant symbols. The Discussion of the Figures should be placed in the Representative Results. Details of the methodology should not be in the Figure Legends, but rather the Protocol.
[Response: We have revised the figure legends and moved inappropriate text to more appropriate sections.]

19. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."
[Response: We have added the requested .docx file.]

20. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
[Response: We have modified the discussion to better adhere to these guidelines.]

21. Please sort the materials table in alphabetical order.
[Response: Done.]

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
The manuscript "A Method for Cost-Effective and Rapid Characterization of Genetic Parts" makes a very useful contribution to the synthetic biologist's toolkit for prototyping DNA-encoded functions. The work provides a fitting example application—construction and optimization of an inducible promoter. The manuscript describes the procedures with a very good level of detail, enabling others to employ their approach. I recommend publication of this work with only minor revisions.
[Response: We thank the reviewer for helpful comments and catching several mistakes.]

Major Concerns:
My one area of concern that it could be clearer where the advantages of the method come from. As mentioned in the summary, the key advantages (cost-effectiveness and high throughput) of the method arise from using cell-free lysates, linear DNA templates, and acoustic liquid handling. These assertions could be justified more clearly in the manuscript. For example, the

manuscript states several times that the method is cost-effective, but doesn't do much to explain how itis cost-effective.

The only place where costs are described is in Supplemental Table 1. I value the effort that went into this table, as even a best attempt at such accounting is fraught with subjective decisions that are easy to criticize. Important elements that contribute to true costs (facilities, equipment/depreciation, overhead) are difficult to factor in. But the details given in this table don't quite produce a fair comparison. The heading "traditional cloning" seems to describe the process of producing the DNA template, while "Our method" and "Commercial Synthesis" add in the cost of CFPS Reaction Components, i.e., making the measurement.

Describing CFPS Reaction Components as $0.05 per reaction is misleading, even if it is an accurate reference to the numbers from Silverman, Karim, and Jewett (2019). A casual reader can be fooled into thinking this investment will be trivial, or that it scales linearly withthe number of reactions. Rather, a user following these recipes will need to purchase roughly two dozen reagents, some of which are expensive. The labor required to manipulate all these reagents and produce a high-quality cell-free extract is not trivial, and does not seem to show up in this accounting.

Conversely, it is not clear that the acoustic liquid handler makes a meaningful impact on cost. If CFPS Reaction Components contribute trivially to cost per reaction, it is not clear that a modest reduction in volumes (from 20 uL to 10 uL in 384-well plates?) really affects cost.

I do in fact believe the assertion the authors make, that their approach is more cost-efficient (and time efficient) than traditional cloning. But the reader can get confused about where the savings is coming from. It would be especially informative for the reader to be given a rough estimate of the breakpoint for when the advantages really kick in: how many reactions does oneneed before it becomes cost effective to buy all the cell-free reagents described in Silverman, Karim, and Jewett? Before it makes sense to put down the multichannel pipettor and buy an Echo liquid handler?

I am not requesting major additions to the manuscript, but would like to see a few more informative sentences in the discussion so the reader can understand where the key advantages come from.

[Response: We appreciate this comment and acknowledge that the advantages, especially for cost, were poorly discussed. We have addressed this point in several ways. First, we have somewhat downplayed the point until the Discussion section where it is more thoroughly considered. We have removed the CFPS costs from Table S1 to focus specifically on the cost of template preparation, which is the dominant cost per construct tested. We have included a note about the startup costs for CFPS (i.e. bulk reagents and sonicator) to avoid offering a misleading picture. We have also described how the advantage of the acoustic liquid handling is not in the cost (negligible savings per construct), but rather in the throughput enabled.]

Minor Concerns:
(Line numbers in the manuscript are indicated)

55 "Because this method retains a cell-like environment, the function of the genetic part will typically mimic its function in whole cells." This is not a generally accepted view, and there are many genetically-encoded functions that researchers have struggled to reconstitute in cell-free systems. Can you reference studies demonstrating this generality?
[Response: This is a fair criticism. We have revised the sentence to soften any claim of generality. We have also added an expanded discussion of this point to the introduction.]

123 H2O (needs subscript)
[Response: Corrected.]

225 (not clear what yellow highlighting is for)
[Response: The yellow highlighting indicates areas that we think would be most useful to film for the companion videography.]

459 Could plasmid supercoiling contribute to a performance difference between linear and plasmid templates? (Just curious, not a point that has to be addressed.)
[Response: We think it is possible, but have not seen compelling evidence so far. Indeed supercoiling was our theory until a colleague suggested non-specific binding of TetR to the vector backbone as an explanation of observed differences. To test that idea, we evaluated linear template plus linear vector. With linear template present, the IC50 was statistically different from linear vector alone but was not statistically different from circular template, which demonstrated that the presence of the vector DNA, and not supercoiling, explained at least most of the discrepancy between linear and circular templates. Thus, non-specific TetR binding to the vector is the most likely explanation in our view. The data for these experiments is available in our previous manuscript (10.1016/j.abb.2019.07.010) if you are interested in looking closer. In other work, we have observed more dramatic differences between linear and circular templates, but we have not explored the factors as thoroughly. We suspect that for some transcription factors or other genetic elements supercoiling is likely to play a significant role.]

496 "repeated a total of 5 times" means a total of 6 uL dispensed. Either "repeated 4 more times" or "performed a total of 5 times" would indicate 5 uL dispensed.
[Response: Corrected.]

Figure 3 has "Represion" on y-axis
[Response: Corrected.]

Reviewer #2:
Manuscript Summary:
McManus JB et al. provide a very well detailed and understandable protocol to characterize genetic parts using cell-free protein synthesis, linear DNA and automation with a liquid handler echo machine from Labcyte. Each steps are well explained with an excellent troubleshooting paragraph at the end of the paper. In addition, McManus JB et al. give a link to a paper with previous experiments using the same protocol giving a good example of the type of results to expect. Eventually, an interesting discussion is present at the end of the paper. The list of

material/ equipment, to produce homemade cell-free and purified proteins, is complete.
[Response: We thank the reviewer for identifying several useful points of clarification.]

Major Concerns:
None

Minor Concerns:
In the Introduction: you do not talk about the impact of the heterologous expression of protein on the cell physiology, which could in return affect the behavior of genetic parts. It would be interesting to add this limitation in the introduction somewhere with a reference
[Response: We agree that this is a relevant consideration. Rather than address this point explicitly, we have expanded our discussion of cell-free prototyping and the current state of its ability to predict function in cells. We cite three specific examples addressing this topic, including one on predicting burden of genetic circuits on cells using cell-free screening (10.1038/s41467-018-03970-x). While perhaps not exactly what the reviewer had in mind, we think that this change addresses the underlying intent.]

Line 55: the last sentence should be at the conditional. It is not clear to me that a characterization in cell-free is directly comparable with in vivo measurements.
[Response: This is fair criticism. We have modified the sentence accordingly. We have also added an expanded discussion of this point to the introduction.]

Line 126: (add to bring solution at ph 8.2) instead of just (ph 8.2)
[Response: The "(pH 8.2)" portion was redundant with the following sentence instructing adjustment to pH 8.2. We therefore removed the "(pH 8.2)" text and slightly rephrased the following sentence for clarity.]

Line 132: is there a difference between K12 Rosetta and BL21-Rosetta strain as named in Sun et al paper? Is it the same strain as BL21(DE3) Rosetta(described in line 270)?
[Response: We thank the reviewer for pointing out this discrepancy. While we have used several strains, we reviewed our records and confirmed that all work described here was done using the BL21(DE3) Rosetta2 strain (Novagen 70954). This strain differs from the one used in Sun et al. only by the presence of the λDE3 lysogen sequence, which enables induction of T7 polymerase. We do not use this feature in this work. We have updated the naming of strains in the main text and Table of Materials to make this more clear, and have corrected the error in the strain listed for use in protein purification. We also added a note to indicate that it is possible to use alternative strains and species.]

Line 144: At OD 2 to 4 you are in stationary phase. Why did you choose such high OD? Cell can be less rich in ribosomes than during exponential phase. In Sun et al, the OD was between 1.5 and 2. Do you obtain the same or better results at this OD?
[Response: In the media (2xYT) and growth conditions we use, stationary phase is not reached until around 6-8 depending on aeration. We have not explicitly tested the difference of harvesting at the ODs in Sun et al.; however, we note that other methods using sonication have shown success in this OD range, e.g. OD of 2.7-4 (doi: 10.3791/58882) or OD of 3 (doi: 10.1021/acssynbio.8b00430)]

Line 156: 755 g seems quite an odd value. Is it an optimal?
[Response: It is not optimized. We have used the 2000 g as recommended in Sun et al. as well as lower speeds, including 755 g. However, we have not done a careful comparison of these results. To avoid confusion, we have changed the text to 2000 g.]

Line 223: there are no numbers in the third column of table 1. What the point of it? Is it for people to write in it? (Same for table 2)
[Response: Yes, these tables are intended to serve as a worksheet for users. We have addressed this point by changing the Table caption to indicate the intent as a worksheet.]

Line 244: Why NdeI in particular? Is it like DpnI to digest only methylated dna?
[Response: This was a mistake; DpnI is the correct enzyme to use here. Thank you for pointing it out.]

Line 332: the company 5 prime described in sun et al. does not produce the RTS Amino Acid Sampler anymore. Can you provide another reference?
[Response: we have added the RTS Amino Acid Sampler from biotechrabbit (BR1401801) currently used by the Murray lab to the Table of Materials.]

Line 360: Is it possible to also distribute the cell-free mix with the Echo machine?
[Response: Yes, it is possible. However, we have experienced intermittent issues with dispensing the viscous cell-free mix and some of its subcomponents and usually perform this step by hand. We therefore hesitate to recommend it here. Anecdotally, other groups dispense the cell-free mix and do not report the same issues; however, based on our conversations, those groups do not verify dispensing accuracy of the Echo. We have added a note indicating that dispensing the cell-free mix is possible but caution should be used, and refer the reader to the later troubleshooting section.]

Line 389: You choose to do your reactions at 37°C when sun at al use 29°C. Why this difference? Is it due to a problem of evaporation?
[Response: We have used 29°C, 30°C, and 37°C for this protocol and with different cell-free systems across various projects. While there can be differences within each system, each temperature works well here. We have indicated this flexibility in the main text.]

Line 602: Could give the fluid class (BP2?) you used in the echo machine to distribute your samples?
[Response: We use 384PP_AQ_BP for most aqueous transfers. We do not include this in the manuscript due to JoVE's restriction on specifying commercial products.]

Line 612: The optimal concentration of other CFPS elements seems also to be batch dependent (https://doi.org/10.1021/acssynbio.8b00276, https://doi.org/10.1038/s41467-020-15798-5)
[Response: We agree that this is the case. We have added 10.1038/s41467-020-15798-5, as recommended; however, we don't think that 10.1021/acssynbio.8b00276 is quite relevant here as the authors explicitly state that "A single batch of extract was used for this study." That said, we do include both references elsewhere in the context of using robotics to increase throughput.]

Process/Reagent	Cost/ unit	Units/ construct	Cost/ construct	Notes
Traditional Cloning:				
60 bp Primers	$22.20	4	$88.80	Assuming 2 primers for back bone linearization, and 2 for part amplification. Prepared by IDT.
Q5 master mix	$1.72	2	$3.44	
Qiagen Gel Extraction Kit	$1.93	2	$3.86	
Gibson assembly	$20.00	1	$20.00	Based on NEB kit
Competent cells	$16.00	1	$16.00	Based on BL21(DE3) Rosetta 2 competent cells used in this study
Labor	$15.00	7	$105.00	Assuming a $15/hr (CA minimum wage) and 1 h PCR, 1 h gel purification, 1 h Gibson, 2 h transformation and plating, 1 h sequence verification and analysis, 1 h culturing.
Total Cost / Construct:			$237.10	
Our method:				
60 bp Primers	$22.20	2	$44.40	Assuming 2 primers for part amplification.
Q5 master mix	$1.72	1	$1.72	
Qiagen Gel Extraction Kit	$1.93	1	$1.93	
Labor	$15.00	2	$30.00	Assuming a $15/hr (CA minimum wage) and 1 h PCR plus 1 h gel purification
Total Cost / Construct:			$78.05	
Commercial Synthesis:				
Synthesis ($0.10/bp)	$91.40	1	$91.40	Assumes 714 bp for sfGFP plus 200 bp of regulatory sequences at $0.10/bp
Total Cost / Construct:			$91.40	

Figure 3 is reprinted https://doi.org/10.1016/j.abb.2019.07.010

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