

1 **TITLE:**

2 A Method for Cost-Effective and Rapid Characterization of Genetic Parts

3

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28 **KEYWORDS:**

29 Synthetic Biology, TXTL, CFPS, Cell-free protein synthesis, T7 RNA Polymerase, genetic parts,
30 genetic circuits

31

32 **SUMMARY:**

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

37

38 **ABSTRACT:**

39 Characterizing and cataloging genetic parts are critical to the design of useful genetic circuits.
40 Having well-characterized parts allows for the fine-tuning of genetic circuits, such that their
41 function results in predictable outcomes. With the growth of synthetic biology as a field, there
42 has been an explosion of genetic circuits that have been implemented in microbes to execute
43 functions pertaining to sensing, metabolic alteration, and cellular computing. Here, we show a
44 cost-effective and rapid method for characterizing genetic parts. Our method utilizes cell-free

45 lysate, prepared in-house, as a medium to evaluate parts via the expression of a reporter
46 protein. Template DNA is prepared by PCR-amplification using inexpensive primers to add
47 variant parts to the reporter gene, and the template is added to the reaction as linear DNA
48 without cloning. Parts that can be added in this way include promoters, operators, ribosome
49 binding sites, insulators, and terminators. This approach, combined with the incorporation of an
50 acoustic liquid handler and 384-well plates, allows the user to carry out high-throughput
51 evaluations of genetic parts in a single day. By comparison, cell-based screening approaches
52 require time-consuming cloning and have longer testing times due to overnight culture and
53 culture density normalization steps. Further, working in cell-free lysate allows the user to exact
54 tighter control over the expression conditions through the addition of exogenous components,
55 or by titrating DNA concentrations rather than relying on limited plasmid copy numbers.
56 Because this method retains a cell-like environment, the function of the genetic part will
57 typically mimic its function in whole cells.

58

59 INTRODUCTION:

60 A core effort of synthetic biology is the development of genetic tool kits containing well-
61 characterized parts, which can be used to construct genetic circuits¹ that carry out useful
62 functions when deployed in microbes or cell-free lysate. Areas in which such genetic circuits
63 have found purchase are sensing²⁻⁵, human performance^{6,7}, biofuels^{8,9}, materials
64 production^{10,11}, and cellular computing¹². Registries of standardized genetic parts have been
65 established¹³ to catalog new and existing parts into categories like promoters, operators, coding
66 sequences, and terminators, to name just a few. Efforts such as the iGEM competition¹⁴ have
67 been instrumental in characterizing and cataloging these genetic parts. Methods, such as UNS¹⁵
68 and 3G¹⁶, have been developed to facilitate the rapid assembly of these parts into useful
69 genetic circuits. Software, such as Cello¹⁷, have even been developed to automate the
70 composition of well-characterized parts into circuits that achieve a desired function. However,
71 the assembly of useful genetic circuits with predictable function rests on the presumption that
72 the genetic tool kits contain well-characterized genetic parts. Due to the necessity of these tool
73 kits toward the advancement of synthetic biology, numerous undertakings to better catalog
74 circuits and parts with appropriate characterization data have been described¹⁸⁻²².

75

76 One category of components useful to the implementation of genetic circuits are orthogonal
77 parts, such as T7 RNA polymerase (T7 RNAP) and its cognate T7 promoter. The genetic systems
78 of *Escherichia coli* are well-developed and many genetic circuits have been deployed and
79 characterized in this organism. T7 RNAP is particularly well-suited as an orthogonal actuator for
80 genetic circuits in *E. coli*, owing to its ability to partially insulate circuit function from the host
81 metabolism²³. However, the major drawback of T7 RNAP is the lack of direct regulatory
82 mechanisms. Until recently, regulated T7 promoters were limited to a small set that had been
83 engineered by insertion of bacterial operator sequences^{24,25}. In order to fill this gap, we wanted
84 to develop a method for the rapid characterization of libraries of regulated T7 promoters. We
85 originally developed the method, presented here, for rapidly characterizing spatial
86 combinations of the T7 promoter and bacterial operator sequences along with their cognate
87 repressor proteins. We validated our methodology using the tetracycline regulatory system
88 (*tet*), by investigating the effects of proximity of the tetracycline operator (*tetO*) sequence to

89 the T7 promoter on the regulation of T7 RNAP-driven expression. Our results revealed
90 important insights into the kinetics of T7-driven expression and into the future design of
91 engineered T7-based transcription factors²⁶. We believe that this methodology can be applied
92 to the characterization of other types of genetic parts as well. In the meantime, others have
93 expanded the range of such regulated T7 promoters considerably²⁷.

94
95 Here, we present methodology that uses PCR to amplify template DNA via primers that add
96 variant genetic parts to a reporter gene. These genetic parts are evaluated using cell-free
97 lysate, prepared in-house, as a cost-effective medium to measure the expression of the
98 reporter protein by cell-free protein synthesis (CFPS). Several studies have demonstrated the
99 utility of CFPS for prototyping genetic components²⁸⁻³². Note that while we prepare our cell-
100 free lysates here based on Sun et al.³³, numerous other commercial kits and protocols^{34,35}
101 should work similarly. We have included the use of an acoustic liquid handler and 384-well
102 plates to increase throughput and decrease the volume of materials required. Previous work
103 has demonstrated successful use of acoustic liquid handling at significantly lower volumes^{36,37}
104 with variability comparable to manual pipetting at larger volumes³⁸. CFPS removes the
105 requirement of working in whole cells and reduces the amount of time for screening many
106 genetic parts to a single day, while still maintaining a cell-like environment. A second
107 advantage of CFPS is that the user can exact tighter control over the expression conditions
108 through the addition of exogenous components via the acoustic liquid handler. The generation
109 of variants by changing PCR primers and then using linear DNA in the CFPS reactions
110 dramatically cuts the cost and time of variant preparation compared to cloning or synthesis.
111 Though we had initially developed this method for the characterization of engineered T7
112 promoters regulated by transcription factors²⁶, other parts that can be changed by PCR-
113 amplification include promoters, operators, RBS sequences, insulators, and terminators. We
114 hope that through this methodology, the synthetic biology community can grow the number of
115 characterized parts for the assembly of predictable genetic circuits with useful function.

116

117 **PROTOCOL:**

118 1. Preparation of Cell Extract

119

120 1.1. Preparation of Media

121

122 1.1.1. For 2xYT media: Add 16 g of tryptone, 10 g yeast extract, 5 g NaCl to 900
123 mL of deionized water. Adjust pH to 7.0 with 5 M NaOH and adjust
124 volume of solution to 1 L using deionized H₂O. Alternatively, purchase
125 2xYT media.

126

127 1.1.2. For S30B buffer: Prepare a solution of 14 mM Mg-glutamate, 60 mM K-
128 glutamate, and 5 mM Tris (pH 8.2) in 2 L of deionized water. Use 2 M Tris
129 to get pH to 8.2. Store at 4 °C. Complete solution by adding DTT to 1 mM
130 final concentration just before use.

131

132 1.2. Preparation of Cells

- 133
- 134 1.2.1. Streak *E. coli* K12 Rosetta cells onto a plate of LB agar and incubate at
- 135 37°C for 10 to 14 h.
- 136
- 137 1.2.2. From a single colony, inoculate one 10 mL culture tube containing 3 mL of
- 138 2xYT medium with *E. coli* K12 Rosetta cells. Incubate this tube at 37°C,
- 139 shaking at 250 rpm, for 8 h.
- 140
- 141 1.2.3. From the 3 mL culture, inoculate 50 µL into a 500 mL flask containing 50
- 142 mL 2xYT medium. Incubate this flask at 37°C, shaking at 250 rpm, for 8 h.
- 143
- 144 1.2.4. From the 50 mL culture, inoculate 7.5 mL into four 4 L baffled flasks
- 145 containing 0.75 L 2xYT medium. Incubate these flasks at 37°C, shaking at
- 146 220 rpm, until they have reached an optical density at 600 nm of 2 to 4,
- 147 approximately 3-4 h.
- 148
- 149 1.2.5. Harvest the cells from each flask by centrifugation, in 1 L containers, at
- 150 5000 x g for 12 min. Discard the supernatant.
- 151
- 152 1.2.6. Wash each cell pellet with 150 mL of ice cold S30B buffer by completely
- 153 resuspending them, then collect the cells again by centrifugation at 5,000
- 154 x g for 12 min. Discard the supernatant.
- 155
- 156 1.2.7. Wash each cell pellet again in 40 mL of ice cold S30B buffer by completely
- 157 resuspending them. Transfer the cells to a 50 mL conical tube and collect
- 158 the cells again by centrifugation at 775 x g for 8 min. Discard the
- 159 supernatant.
- 160
- 161 1.2.8. Weigh the cell pellets. Flash freeze the cell pellets in liquid nitrogen. Store
- 162 the cell pellets at -80°C.
- 163

164 1.3. Cell Lysis

- 165
- 166 1.3.1. Thaw cell pellets on ice.
- 167
- 168 1.3.2. Resuspend each cell pellet in 1.4 mL of S30B buffer per 1 g of cell pellet.
- 169
- 170 1.3.3. Lyse the cells by French pressure cell at 640 psi at 4°C. Collect the lysate
- 171 on ice and add 3 µL of 1 M DTT per 1 mL of lysate immediately after lysis.
- 172
- 173 NOTE: It is best to tap the French press release valve with a small metal
- 174 rod in order to maintain even pressure and avoid sudden drops in
- 175 pressure.
- 176

- 177 1.3.4. Clear the lysate by centrifugation at 30,000 x g for 30 min at 4°C and
178 discard the pellet.
179
- 180 1.3.5. Centrifuge the supernatant a second time at 30,000 x g for 30 min at 4°C
181 and discard the pellet.
182
- 183 1.3.6. Incubate the supernatant in a 37°C water bath for 1 h.
184
- 185 1.3.7. Clear the supernatant by centrifugation at 15,000 x g for 15 min at 4°C
186 and discard the pellet.
187
- 188 1.3.8. Centrifuge the supernatant a second time at 15,000 x g for 15 min at 4°C
189 and discard the pellet.
190
- 191 1.3.9. Distribute the supernatant in 100 µL aliquots into 1.5 mL microcentrifuge
192 tubes and flash freeze them in liquid nitrogen. Store the supernatant at -
193 80°C.
194

195 2. Linear Template Preparation

196 2.1. Primer Design

- 197
- 198
- 199 2.1.1. For the forward primers, choose a minimum of 20 bp on the 3' end of
200 the primer to match the 5' end of the coding strand of the reporter gene.
201 Design the remainder of the 5' end of the primer to add the genetic
202 parts of interest to the reporter gene via PCR-amplification (Fig. 1A and
203 Fig. 2).
204
- 205 2.1.2. For the reverse primer, choose a sequence on the 5' end of the non-
206 coding strand of the reporter gene, directly downstream from the
207 terminator. Alternatively, for parts at the end of the gene, such as C-
208 terminal tags, design primers to introduce the part of interest. Note that
209 inclusion of a terminator may not be required for expression from linear
210 DNA in cell-free systems. Be sure that the reverse primer's annealing
211 temperature is within 5°C of the annealing temperature of the entire
212 forward primer.
213

214 NOTE: DNA providers typically synthesize primers with a standard length
215 of up to ~60 bases. While longer primers can be synthesized and
216 implemented, costs often increase dramatically. Alternatively, multiple
217 overlapping primers can be designed to the 5' or 3' ends of the gene,
218 and longer sequences or multiple parts can be added to the reporter
219 through multiple rounds of PCR.
220

221 NOTE: The template containing the reporter gene to be amplified need
222 not be a plasmid. Genomic DNA or linear blocks ordered from
223 commercial vendors may also be suitable as template DNA.

224

225 2.2. Linear Template Amplification

226

227 2.2.1. Determine the number of PCR reactions to perform and calculate the
228 amount of each component required using Table 1.

229

230 2.2.2. Prepare the master mix and store it on ice. Aliquot 40 μ L of the master
231 mix into the determined number of PCR tubes and add 10 μ L of Forward
232 Primer (5 μ M) to each appropriately-labeled, corresponding PCR tube.

233

234 2.2.3. Place PCR tubes into the thermocycler and run the following PCR
235 program:

236

237	98°C - 3 min	} 30 cycles
238	98°C - 15 s	
239	XX°C - 20 s	
240	72°C - YY min	
241	72°C - 10 min	
242	4°C - hold	

243

244 Where XX represents the temperature for the lower annealing
245 temperature primer. YY represents the extension time calculated for
246 the length of the amplicon (30 s – 1 min / kb for Q5 polymerase). These
247 conditions may need to be optimized for different primers and/or
248 templates (see 2.3 and Discussion).

249

250 2.2.4. Add 1 μ L of NdeI restriction enzyme to digest the original template.
251 Incubate the reaction at 37°C for 1 h. This step is not necessary if a
252 synthetic block was used as the original template.

253

254 2.2.5. Analyze 5 μ L of each PCR product by gel electrophoresis. Separate the
255 product using a 1% agarose gel at 180 V for 20 min.

256

257 2.3. Purify the linear template according to the QIAquick PCR Purification Kit, or by your
258 preferred PCR cleanup method. If multiple bands were present by gel
259 electrophoresis analysis, see the Discussion section for troubleshooting OR purify
260 the correct molecular weight bands by QIAquick Gel Extraction Kit, or by your
261 preferred extraction method.

262

263 2.4. Quantify each DNA template using a NanoDrop spectrophotometer, or your
264 preferred spectrophotometric instrument. Be sure to assess DNA template quality

265 by assuring that the 260 nm / 280 nm ratio is approximately 1.8. Further, a portion
266 of the samples can be separated using a 1% agarose gel at 180 V for 20 min, a
267 second time, to check that any unwanted bands were removed during template
268 purification. DNA samples may be stored at -20 °C.
269

270 3. Purified Protein Preparation

271

272 3.1. Protein Expression

273

274 3.1.1. For each protein to be expressed, assemble the *E. coli*-codon optimized
275 gene into a pET-22b expression vector and transform the expression
276 plasmid into BL21(DE3) Rosetta expression cells.
277

278

279 3.1.2. For each protein, inoculate, from a single colony, a 10 mL culture tube
280 containing 3 mL of LB medium. Incubate these tubes at 37°C, shaking at
281 250 rpm, overnight.

282

283 3.1.3. Inoculate a 2 L flask containing 750 mL of LB medium with 1 mL of
284 overnight culture. Incubate these flasks at 37°C, shaking at 250 rpm, until
285 they reach an OD₆₀₀ of 0.6–1.0.

286

287 3.1.4. Induce protein expression by adding 0.75 mL of 1 M IPTG in water to each
288 flask, and continue to incubate these flasks at 37°C, shaking at 250 rpm,
289 for 4 h.

290

291 3.1.5. Harvest the cells from each flask, using a 1 L centrifuge bottle, by
292 centrifugation at 5000 x g for 12 min. Discard the supernatant.

293

294 3.1.6. Transfer the pellets to a 50 mL conical tube and weight each pellet.
295 Expect 2-5 g per 0.75 mL. Flash-freeze the cells in liquid nitrogen and
296 store them at -80 °C or proceed to 4.2.1.

297

298 3.2. Protein Purification by Nickel Affinity Column Chromatography

299

300 3.2.1. Thaw the cell pellet in room temperature water and resuspend,
301 homogenously, in lysis buffer, using 5 mL lysis buffer per 1 g cell pellet.

302

303 3.2.2. Lyse the cells by sonication. Separate the cell homogenate so that there
304 is no more than 30 mL per 50 mL conical tube and place each tube on ice.
305 Lyse the cells using a QSonica Ultrasonic Processor sonicator with a 0.16
306 cm diameter probe. Lyse the cells in 15 s rounds with 30 s breaks, 10
307 times.

308

NOTE: Avoid foaming, as this denatures protein. Formation of foam can

- 309 be avoided by keeping the tip at least 2/3 submerged in the lysate while
310 it is operational.
311
312 3.2.3. Clear the lysate by centrifugation at 15,000 x g for 30 min at 4 °C.
313
314 3.2.4. Incubate each 5 mL of supernatant with 1 mL of Ni-NTA resin at 4 °C, on a
315 Thermo tube rotator (or equivalent) at 10 rpm for 1 h. Separate the cell
316 lysate / Ni-NTA slurry so that there is no more than 36 mL per 50 mL
317 conical tube.
318
319 3.2.5. Load the resin into a 5 cm diameter column. Wash the resin with 10 resin
320 bed volumes of wash buffer.
321
322 3.2.6. Collect the protein with three resin bed volumes of elution buffer and
323 concentrate the volume to 1.5 mL using a centrifugal concentrator with
324 the appropriate molecular weight cut-off membrane for each protein.
325
326 3.2.7. Dialyze the protein against 2 L of dialysis buffer at 4 °C for 1 h. Dialyze
327 the protein again against 2 L of dialysis buffer overnight at 4 °C.
328
329 3.2.8. Quantify the protein using its molar extinction coefficient and absorbance
330 at 280 nm. Analyze the protein for purity by separating it using SDS-PAGE
331 electrophoresis. Store the protein at -80 °C.
332

333 4. Cell-Free Protein Synthesis

334 4.1. Preparation of CFPS Reaction Mixture

- 335
336
337 4.1.1. Prepare Supplement Mix by following the Amino Acid Solution
338 Preparation, Energy Solution Preparation, and Buffer Preparation steps in
339 Sun et al.³³ These three solutions can be combined ahead of time,
340 aliquoted, and stored at -80 °C. Final concentrations should match those
341 described in Sun et al.³³ in the Experimental Execution of a TX-TL Reaction
342 section.
343
344 4.1.2. Prepare GamS (protects linear DNA from degradation; see note in next
345 section), T7 polymerase, and repressor proteins using steps above (see 3.
346 Purified Protein Preparation), or obtain from a commercial vendor.
347
348 4.1.3. Determine the number of CFPS reactions to be performed and calculate
349 the amount of each component required using Table 2.
350

351 NOTE: The table above is a for a standard CFPS reaction. The volume of the
352 template, polymerase, and repressor protein can be varied or other

353 components can be added (or subtracted) by adjusting the amount of water
354 such that the final volume of each reaction mixture is always 10 μ L.
355 Additionally, other components can be optionally dispensed by acoustic liquid
356 handling instead of being present in the master mix (see Troubleshooting).

357
358 NOTE: While GamS protein is used here to limit degradation of linear DNA by
359 blocking the RecBCD complex^{39,40}, other approaches are available⁴¹⁻⁴³ and
360 some CFPS recipes based on purified components do not require any
361 additions⁴⁴.

362
363 4.1.4. Thaw all components on ice and prepare a master mix by mixing each
364 component as calculated above. Mix all the components thoroughly, by
365 pipette. Keep the master mix on ice.

366
367 4.1.5. Chill a 384-well plate on ice and distribute the master mix in 9 μ L aliquots
368 into each well using an electronic repeater pipette.

369
370 4.2. Distribution of Additional Components by Acoustic Liquid Handling

371
372 4.2.1. Calculate the amount of repressor protein (and optional other
373 components) required for all the CFPS reactions.

374
375 4.2.2. Thaw the repressor protein on ice and distribute it into a Labcyte Echo
376 source plate or other appropriate plate. Be sure to consider the
377 appropriate amount of dead volume required for the type of source plate
378 used.

379
380 4.2.3. Distribute the repressor protein in 1 μ L volumes into the appropriate
381 wells via the Echo acoustic liquid handler or similar instrument. More
382 information on distribution troubleshooting can be found in the
383 Discussion sections.

384
385 4.3. Standard Curves

386
387 4.3.1. Include a serial dilution of purified reporter protein (see Section 3 for
388 protein purification)³⁸ or appropriate chemical standard⁴⁵ on the plate to
389 enable comparison of results with other studies and other labs. In our
390 previous publication²⁶, a standard curve for sfGFP was generated using
391 purified protein with concentrations ranging from 0 μ M to 10 μ M.

392
393 4.4. Running CFPS Reactions

394
395 4.4.1. Pre-warm the plate reader (Biotek H10 used in our previous
396 publication²⁶) to 37°C. Ensure that the settings match those for the

397 reporter protein being measured (Ex: GFP, fluorescence, 480 nm ex / 528
398 nm em). No shaking steps are required. It may be helpful to run a test
399 reaction first to set the appropriate gain or sensitivity setting that will
400 capture the change in fluorescence without signal overflow.

401
402 NOTE: Read intervals of 10 minutes are sufficient to achieve good
403 resolution on sfGFP expression curves using the Biotek H10 plate reader.
404 However, this may change depending on the reporter protein and
405 particular CFPS recipe.

406
407 4.4.2. Seal the 384-well plate with an impermeable plastic sealable lid to
408 prevent evaporation. The instrument should be set to a 1 °C vertical
409 temperature gradient. This ensures that the condensation does not form
410 on the seal. Place the 384-well plate on the plate holder and begin
411 reading.

412 413 5. Data Interpretation

414
415 5.1. At the completion of the run, export the data as a .csv file.

416
417 5.2. Data should be converted to standardized units if applicable (see Section 4.3).

418
419 5.3. For characterization of repressible promoters, a dose response curve of reporter
420 expression against a titration of repressor concentration is informative. To interpret
421 these data, plot the maximum output values from each reaction against the
422 repressor protein concentration, then fit each dose response profile to a four-
423 parameter logistic curve using GraphPad Prism or the software of your choice.

424
425 NOTE: The maximum output values used here are derived from the sigmoidal fit of
426 each protein expression curve.

427
428 5.4. Calculate the maximum repression values for each data set by subtracting the top
429 and bottom values, determined by the four-parameter logistic curve fit. The
430 maximum repression values and EC₅₀ values, determined by the four-parameter
431 logistic fit, can be used to identify changes in the operator- promoter relationship.

432 433 **REPRESENTATIVE RESULTS:**

434 435 ***TetO Represses T7 RNAP within the First 13 Bases Downstream from the T7 Promoter***

436
437 To demonstrate the utility of our methods, we present results that describe the effects of
438 proximity of the *tetO* sequence to the T7 promoter on the regulation of T7 RNAP-driven
439 expression. The full results and their implications can be found in the work of McManus et al.²⁶.

440

441 Fifteen linear templates, varying only in the distance of the T7 promoter relative to the *tetO*
442 sequence, were prepared by PCR-amplifying the sfGFP reporter as described in Linear Template
443 Preparation (see Section 2). Amplicons were analyzed by gel electrophoresis and added to CFPS
444 reactions, then distributed into a 384-well plate. sfGFP expression was measured from each
445 template with a titration of 12 different concentrations of the TetR protein, in triplicate, using
446 an Echo acoustic liquid handler. At 36 CFPS reactions per template and 15 templates, a total of
447 540 reactions for the entire set of T7- *tetO* combinations were performed. The entire
448 evaluation was carried out on two plates in two Biotek H10 plate readers.

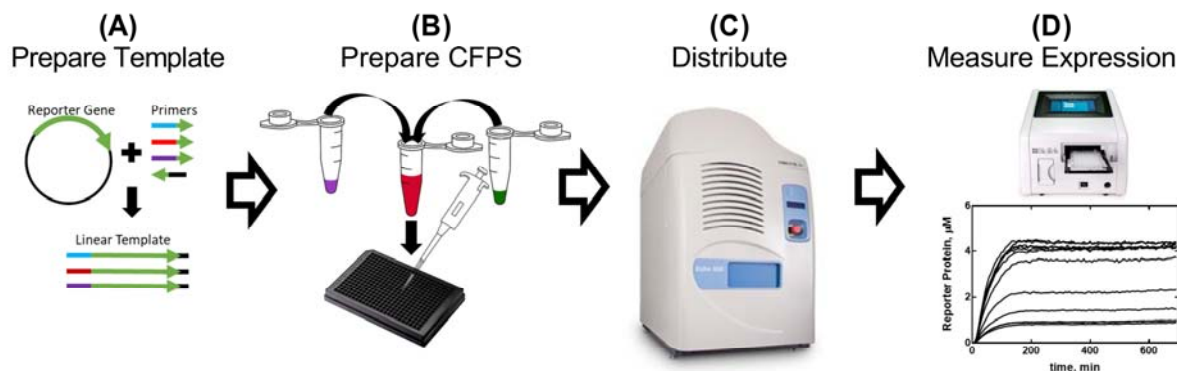
449
450 For data analysis, the sfGFP expression curves were first converted to units of μM sfGFP via
451 standard curve, then fit to a sigmoidal regression. The maximum expression values as
452 determined by the sigmoid fit were plotted against TetR concentration, and these dose-
453 response profiles were fit to a four-parameter logistic to yield EC_{50} values and maximum
454 repression values as described in the Protocol. We found that expression values varied from
455 template to template. This may be in part due to impurities carried over during template
456 purification. However, as shown in our previous publication²⁶, we were able to normalize these
457 expression values and derive meaningful conclusions by comparing these normalized values.
458 Analysis of these values shows that the T7 RNAP downregulates T7-driven expression equally
459 up through 13 bp downstream from the start of the T7 transcript (Fig. 3). This has implications
460 for the future design of regulatable T7-driven gene circuits.

461
462 We also performed the same experiment using circular plasmid DNA as template, rather than
463 PCR-amplified template. The purpose of this experiment was to determine the differences, if
464 any, between circular and linear template. Our results, detailed in our previous manuscript²⁶,
465 indicated that while the pattern of regulation, presented in the previous section, remains
466 unchanged (Fig. 3), the EC_{50} values show a significant difference. We hypothesized that non-
467 specific binding of TetR to the vector DNA could explain the observed difference. Experimental
468 results showed that addition of linear vector DNA to reactions with linear template DNA
469 reduced the difference to non-statistical significance, though did not rule out contributions
470 from other factors, such as differences in periodicity of the DNA helix for linear vs. circular
471 formats, which, in turn, could affect TetR binding. Depending upon the application, the use of
472 linear template may require additional validation.

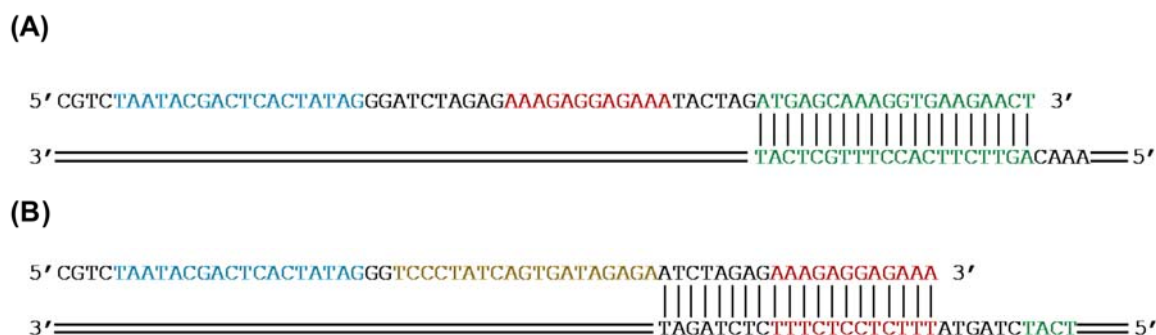
473

474 **FIGURE AND TABLE LEGENDS:**

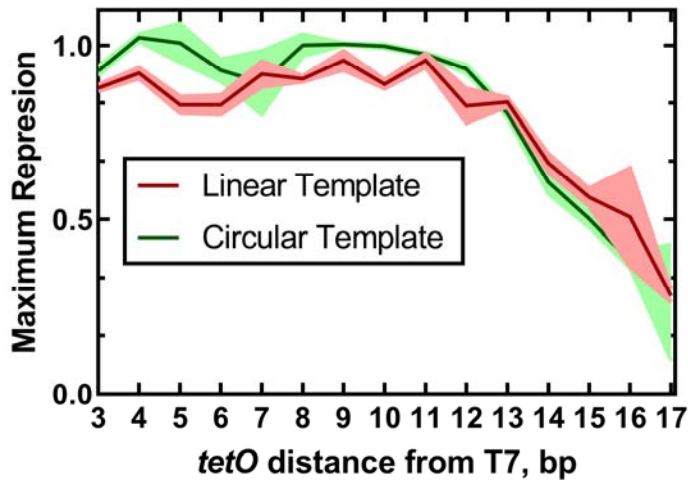
475



476
477 **Figure 1. Single-day Workflow for the Evaluation of Promoter Parts in Cell-Free Extract.** (A) A
478 reporter gene is PCR-amplified using primers containing genetic parts to be evaluated in order
479 to prepare template for CFPS reactions (2–5 h). (B) The cell-free reaction mix is prepared as
480 detailed in the Protocol and distributed into a 384 well plate (30 min). The template can be
481 added into the cell-free master mix or as part of the (C) distribution of additional components,
482 which can include repressor proteins, effector molecules, and any other conditional effectors (10
483 min). (D) Reporter protein expression from each reaction is measured in a plate reader (2 – 16
484 h, depending on CFPS recipe and construct).
485

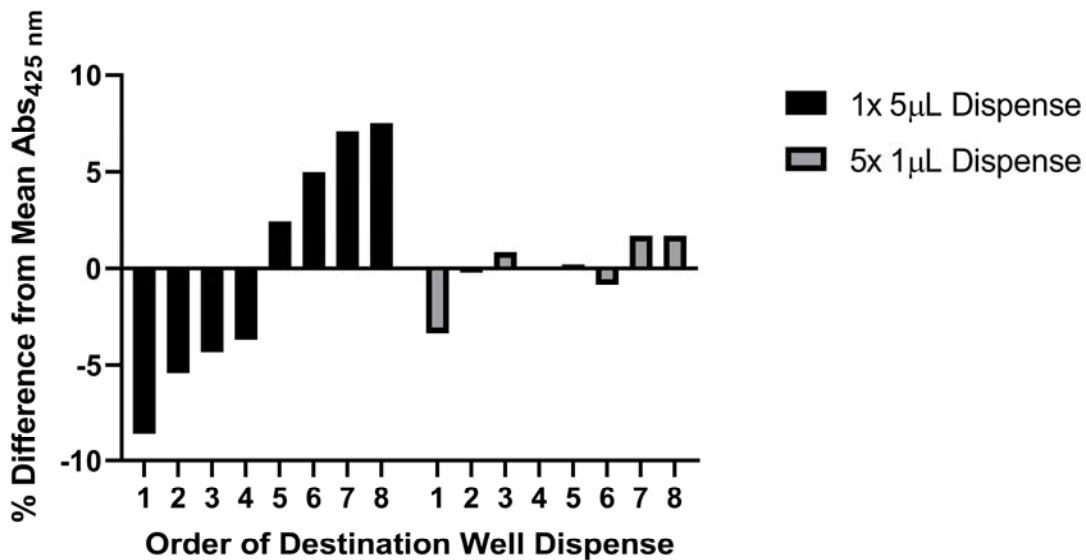


486
487 **Figure 2. Primer Design for Adding Genetic Parts to a Reporter Gene by PCR-Amplification.**
488 Design the 3' end of the primer (top) such that it will hybridize to at least 20 bp of the 3' end of
489 the non-coding strand of the reporter gene (bottom). (A) The sfGFP reporter gene (green) will
490 be amplified to add an RBS (red) and a T7 promoter (blue) by PCR. (B) The sfGFP (green) AND an
491 RBS (red) will be amplified to add a tetO sequence (gold) and a T7 promoter (blue) by PCR.
492



493
494 **Figure 3. The Effect of tetO Position on the Regulation of T7-Driven Expression.** Maximum,
495 normalized repression values were plotted against tetO position to reveal the effects of tetO
496 position on the regulation of T7-driven expression. The traces represent the mean and standard
497 deviations for three replicates. This figure has been modified from McManus et al.²⁶ under a
498 Creative Commons CC-BY license.
499

Echo Dispensing of 1X PBS + Tartrazine Dye into Consecutive Wells



500
501 **Figure 4. Using Tartrazine Dye to Validate Liquid Dispensing with an Acoustic Liquid Handler.**
502 A solution of 1x phosphate buffered saline (PBS), pH 7.4 containing 0.25 mM tartrazine dye was
503 used to evaluate two methods of programming an Echo acoustic liquid handler to dispense
504 volumes > 1 µl. In one method (results shown in black bars), 5 µl of the tartrazine solution were
505 dispensed from a single source well into each of eight consecutive destination wells of a 384-

506 well plate using a single programming command. In a second method (results shown in gray
 507 bars), 1 μ l was dispensed from a single source well into each of eight consecutive destination
 508 wells using a single programming command, and then this step repeated a total of five times.
 509 The destination plate was sealed and centrifuged at 1,500 x g for 1 min, and the absorbance at
 510 425 nm measured with a plate reader. Representative results of nine experiments are shown
 511 and demonstrate more consistent dispensing across the series of eight destination wells when
 512 the 5 μ l transfer is divided into separate 1 μ l dispenses. While the theoretical final volume in
 513 each destination well is the same for both methods (5 μ l), the second method utilizes five
 514 separate programming commands resulting in the Echo resurveying the source well between
 515 each command. Based on these observations and personal communications with Labcyte
 516 engineers, it is recommended that transfers > 1 μ l be broken down into multiple transfers of \leq 1
 517 μ l to improve accuracy.
 518

Component Name	Volume for 1 reaction (μ L)	Volume for 110% of X number of reactions (μ L)
Q5 PCR Premix	25	
Water	4	
Reverse Primer (5 μ M)	10	
Template (1–3 ng/ μ L)	1	
Master Mix Total:	40	
Forward Primer (5 μ M)	10	

519 **Table 1. Reagents for PCR reactions.**

520

Component Name	Volume for 1 reaction (μ L)	Volume for 110% of X number of reactions (μ L)
Cell Extract	4.2	
Supplement Mix	3.3	
GamS Protein (207 μ M)	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	

521 **Table 2. Reagents for CFPS reactions.**

522

523 DISCUSSION:

524

525 The protocols described here provide a cost-effective and rapid means to screen genetic parts
 526 via the expression of a reporter protein by CFPS. Well-characterized genetic parts are crucial to

527 the design of predictable genetic circuits with useful function. This methodology increases
528 throughput and decreases the time needed to screen new genetic parts by removing the
529 requirement to work in living cells, while retaining functionality that mirrors the cellular
530 environment by retaining the metabolic process of protein expression in the cell lysate. Our
531 protocol can be performed in one day after receipt of primers, compared to at least three for
532 traditional cloning (one day each for construct assembly and transformation, sequence
533 verification of clones, and culturing of cells for assessment). We further estimate the cost per
534 construct to be roughly one third compared to traditional cloning (Supplementary Table 1).
535 Commercial synthesis services take a minimum of 5 business days, though may have similar
536 costs to our methods if linear fragments are screened directly in CFPS; we have not verified this
537 approach.

538

539 ***Customizing the Methodology***

540

541 We originally developed this methodology to investigate the effects of operator proximity on
542 the T7 promoter²⁶. We have attempted to present the protocols here in a more generic
543 format, such that they can be applied to promoters, operators, ribosome binding sequences,
544 insulators, and terminators. These genetic parts can be added to the 5' or 3' end of the
545 reporter gene by PCR using primers for each design, obviating the need for synthesis or cloning
546 of each variant to test. The resulting PCR products serve as template DNA for evaluation via the
547 expression of a reporter protein. In our work, the affinity purification protocol provided here
548 was used for TetR and GamS. The same procedure can be used for the expression and
549 purification of other repressors, activators, polymerases, sigma factors, and other proteins
550 cognate to a genetic part of interest. Purification and titration of these proteins into CFPS
551 reactions enables a more detailed characterization of a particular genetic part. The procedure
552 given here for purifying protein may need to be altered to the desired protein being expressed.
553 Finally, numerous alternative CFPS protocols exist and each should be amenable to this
554 methodology. Varying the concentrations of underlying constituent components of the CFPS is
555 also possible. The use of acoustic liquid handling enhances the ability to test the myriad
556 conditions by increasing throughput and decreasing materials required.

557

558 ***Future Directions***

559

560 Beyond characterization of individual parts, the same method can be used to screen
561 combinations of parts that form complex circuits, such as logic circuits¹⁷ or oscillators^{46,47}.
562 Alternatively, the method can be used for screening and optimization of biosensors for
563 applications in epidemiological diagnostics⁴⁸⁻⁵¹ or hazard detection and quantification^{3,4,52}.
564 Similar to our TetR-responsive T7 promoters study, these detection circuits are frequently
565 designed around the interaction between a repressor protein and its cognate effector in order
566 to regulate the expression of genes via an operator sequence, making our method highly
567 appropriate; however, other biosensor mechanisms, such as riboswitches, can also be screened
568 with our approach. Further, the *E. coli* cell-free lysate can also be replaced with lysate from the
569 organism in which a biosensor is to be deployed in order to better mimic the function of that
570 sensor in its host organism. Several publications have described the preparation of different

571 cell lysates for protein expression including those from *Bacillus subtilis*⁵³, *Pseudomonas*
572 *putida*⁵⁴, and *Vibrio natrigens*⁵⁵, to name a few. Their methodology can be used in place of the
573 Preparation of Cell Extract (1) protocol, if desired.

574

575 **Troubleshooting**

576

577 Finally, it is worth noting that different applications may require different levels of optimization
578 before the collection of useful experimental data can begin. Four key areas are: (1) in the PCR-
579 amplification of template, (2) in the distribution of components by the acoustic liquid handler,
580 (3) protein expression and purification, and (4) CFPS performance.

581

582 Depending on the length and identity of the primers used to amplify linear template, the
583 conditions of the PCR reaction may have to be optimized to increase the amount of template
584 synthesized. Some problems users may encounter are: (a) weak or no amplification of the
585 desired product, (b) primer-dimer formation, and (c) products resulting from amplification due
586 to non-specific binding. It is helpful to check the primers, before ordering them, to ensure that
587 they do not form primer-dimers. There are several web-based tools (ex: idtDNA) that can be
588 accessed to analyze primer pairs for primer-dimer formation. If little or no product is produced,
589 applying a temperature gradient at the annealing step may be useful in order to optimize this
590 step. If the user is still unable to generate product in this way, consider redesigning the primers
591 that hybridize with a new sequence on or near the reporter gene. Finally, the synthesis of
592 products due to non-specific amplification can be reduced or eliminated by thermocycling
593 methods like touchdown PCR⁵⁶. Again, redesigning primers may also eliminate the problem of
594 non-specific amplification.

595

596 Acoustic liquid handler dispensing should be optimized for each component being transferred
597 and it is strongly recommended to run controls to verify proper distribution and reproducibility
598 before collecting data. The ideal source plate type and liquid class setting will depend on the
599 specific liquid to be dispensed and its components. It is not recommended to use Echo
600 Qualified 384-Well Polypropylene 2.0 Plus Microplates to dispense DNA, as the amine coating
601 may interact with the DNA. It should also be noted that the ability to dispense higher
602 concentrations of certain components may depend on the acoustic liquid handler model. For
603 example, the Echo 550 model is able to dispense liquids containing high concentrations of salts
604 and DMSO, whereas these components must be at lower concentrations for successful transfer
605 with the Echo 525. A test liquid transfer may be conducted by dispensing onto a foil plate seal
606 to visualize successful droplet formation; however, this test provides limited information and
607 droplets from different settings may appear identical. The use of a water-soluble dye, such as
608 tartrazine, may be used to more accurately verify the correct volume is dispensed with a given
609 setting or workflow (Fig. 4). Optimal Echo programming of liquid transfers can also influence
610 the accuracy and consistency of data generated; for transfers > 1 μ l from one source well to
611 one destination well, sequential transfers of \leq 1 μ l should be programmed to reduce systematic
612 well-to-well variability (Fig. 4). Lastly, theoretical and actual source well dead volumes can vary
613 dramatically depending on source plate type, liquid class setting, and components of the
614 specific liquid; using the Echo to survey the well volumes prior to running a program may help

615 to gauge how accurately the Echo is able to measure a particular liquid.

616

617 CFPS reaction performance can vary when comparing results between different users, plate
618 readers, and laboratories³⁸. For instances where such comparisons are required while
619 prototyping genetic circuits, we recommend including internal control reactions with standard
620 constitutive promoters (such as pT7) in each reaction plate to help normalize results across
621 experimental set-ups. The method of DNA preparation can also contribute majorly to CFPS
622 activity; inclusion of an ethanol precipitation step is recommended. In addition, it is useful to
623 validate certain aspects of the reaction preparation. For instance, it has been noted that
624 optimal magnesium and potassium glutamate concentrations can vary depending on the
625 promoter or reporter protein being expressed^{29,57} or per batch of cell extract prepared³³.
626 Concentrations of these components should be optimized by screening across several
627 concentrations of each component per genetic construct and per cell extract preparation to
628 determine the optimal conditions for protein expression. Finally, best practices for consistent
629 CFPS reaction performance include thorough mixing, careful pipetting, and consistency in the
630 preparation of each reagent component.

631

632 Though this section does touch on some more common issues that may arise during screening,
633 it is by no means an exhaustive list. Once the proper controls have been run to ensure the
634 collection of usable and reproducible data, the method developed here is an ideal way to
635 rapidly characterize genetic parts with a high degree of resolution.

636

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638

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644

645 **DISCLOSURES:**

646

647 RMM has a financial stake in Tierra Biosciences, a private company that makes use of cell-free
648 technologies such as those described in this article for protein expression and screening.
649 The other authors have nothing to disclose.

650

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