CHAPTER TWO

Cell-Free Synthetic Biology for Pathway Prototyping

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Abstract

Engineering biological systems for the production of biofuels and bioproducts holds great potential to transform the bioeconomy, but often requires laborious, time-consuming design-build-test cycles. For decades cell-free systems have offered quick and facile approaches to study enzymes with hopes of informing cellular processes, mainly in the form of purified single-enzyme activity assays. Over the past 20 years, cell-free systems have grown to include multienzymatic systems, both purified and crude. By decoupling cellular growth objectives from enzyme pathway engineering objectives, cell-free systems provide a controllable environment to direct substrates toward a single, desired product. Cell-free approaches are being developed for prototyping and for biomanufacturing. In prototyping applications, the idea is to use

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cell-free systems to test and optimize biosynthetic pathways before implementation in live cells and scale-up. We present a detailed method for the generation of crude lysates for cell-free pathway prototyping, mix-and-match cell-free metabolic engineering using preenriched lysates, and cell-free protein synthesis driven cell-free metabolic engineering. The cell-free synthetic biology methods described herein are generalizable to any biosynthetic pathway of interest and provide a powerful approach to building pathways in crude lysates for the purpose of prototyping. The foundational principle of the presented approach is that we can construct discrete metabolic pathways through modular assembly of cell-free lysates containing enzyme components produced by overexpression in the lysate chassis strain or by cell-free protein synthesis (in vitro production). Overall, the modular and cell-free nature of our pathway prototyping framework is poised to facilitate multiplexed, automated study of biosynthetic pathways to inform systems-level cellular design.

1. INTRODUCTION

Increased demands for energy, climate change concerns, and reliance on petrochemicals as the source of 95% of today's chemicals and materials have intensified the need for sustainable, low-cost biofuels and bioproducts production (Sheldon, 2007; Werpy et al., 2004). Microbial cell factories offer one of the most attractive approaches for addressing this need (Curran & Alper, 2012). However, long research and development timelines (10–100s of person years) motivate the need for new methods to accelerate the design and optimization of biological systems (Bornscheuer et al., 2012; Curran & Alper, 2012; Erickson, Nelson, & Winters, 2012; Fritz, Timmerman, Daringer, Leonard, & Jewett, 2010; Nielsen et al., 2014; Rollié, Mangold, & Sundmacher, 2012).

1.1 The State of Metabolic Engineering

Often, biologically produced small molecules are insufficient for production at commercially relevant titers, rates, or yields because natural sources are difficult to optimize and to scale. Thus, engineers seek to design enzymatic reaction schemes in model microorganisms to meet manufacturing criteria. Success in these endeavors depends upon identifying sets of enzymes that can convert readily available molecules (e.g., glucose) to high-value products (e.g., medicines), with each enzyme performing one of a series of chemical modifications. For example, introducing heterologous pathways into model microbes (such as baker's yeast and *Escherichia coli*) and engineering them to maximize biochemical production has led to large-scale production of

1,3-propanediol, farnesene, and artemisinin with many more on their way to market (Hodgman & Jewett, 2012; Nielsen et al., 2014). Unfortunately, this is difficult because design-build-test (DBT) cycles—iterations of reengineering organisms to test new sets of enzymes—can be detrimentally slow due to the constraints of cell growth (Nielsen & Keasling, 2016). As a result, a typical project today might only explore dozens of variants of an enzymatic reaction pathway. This is often insufficient to identify a commercially relevant solution because selecting productive enzymes using existing single-enzyme kinetic data has limited applicability in multienzyme pathways and consequently requires more DBT iterations. While techniques continue to develop to multiplex DBT cycles for rationally engineering cells (Smanski et al., 2014), in vitro systems show promise in speeding up DBT cycles because they bypass many in vivo limitations by having direct access to the cellular contents (Carlson, Gan, Hodgman, & Jewett, 2012; Hodgman & Jewett, 2012; Moore et al., 2018; Siegal-Gaskins, Tuza, Kim, Noireaux, & Murray, 2014; Sun, Yeung, Hayes, Noireaux, & Murray, 2014). In concert with a trend toward automation, in vitro systems could transform the way we engineer metabolic pathways.

1.2 Emerging Cell-Free Biotechnology

Cell-free systems complement traditional cellular systems. By decoupling cellular growth objectives from engineering enzyme utilization objectives, cell-free systems provide controllable and open environment to direct substrates toward a single, desired product (Dudley, Karim, & Jewett, 2015). Thus, cell-free synthetic biology methods are being developed for both pathway prototyping and for biomanufacturing. In prototyping applications, the idea is to use cell-free systems to test and optimize biosynthetic pathways before implementation in live cells and scale-up. Already, cell-free biosynthetic pathway building methods are already being used for pathway operation and debugging (Dudley et al., 2015). However, using cell-free systems to study metabolism and enzymatic pathways are still in its early stages. Nevertheless, cell-free systems provide advantages in controlling enzymes and the reaction environment. In biomanufacturing applications, the idea is to use cell-free systems for making the product itself. There is also potential to make products unavailable to cells due to toxicity limitations and to focus substrates to products with yields unattainable in cells (Korman, Opgenorth, & Bowie, 2017; Korman et al., 2014). Cell-free systems might also offer exciting new directions in the synthesis of hybrid biochemicals

comprised of parts derived from organic syntheses and parts derived from biological syntheses (Swartz, 2012). The ability to not only use enzymes from multiple organisms but also unique metabolisms from across the phylogenic spectra is also on the horizon. As CFME emerges (Dudley et al., 2015; Guterl et al., 2012; Karim, Dudley, & Jewett, 2016; You & Zhang, 2013), two broad classes dominate in vitro small-molecule synthesis: purified enzymes and crude cell lysates (Fig. 1).

1.2.1 Purified Enzyme Systems

Purified enzyme approaches involve individual overexpression and purification of enzymes, which are then used as individual biocatalysts or recombined to assemble pathways of interest. The benefit of these systems is that the reaction network is explicitly defined, which gives exquisite control of reaction conditions and pathway fluxes. Indeed, the clarity of the biosynthetic pathway comes from eliminating unnecessary enzymes and cellular interferences (i.e., growth, other off-pathway metabolites). There are several examples of simple purified enzyme systems in industrial biocatalysis (Bruggink, Roos, & de Vroom, 1998; Jensen & Rugh, 1987). However, few industrial examples of synthetic enzymatic pathways exist, in part because of the high catalyst

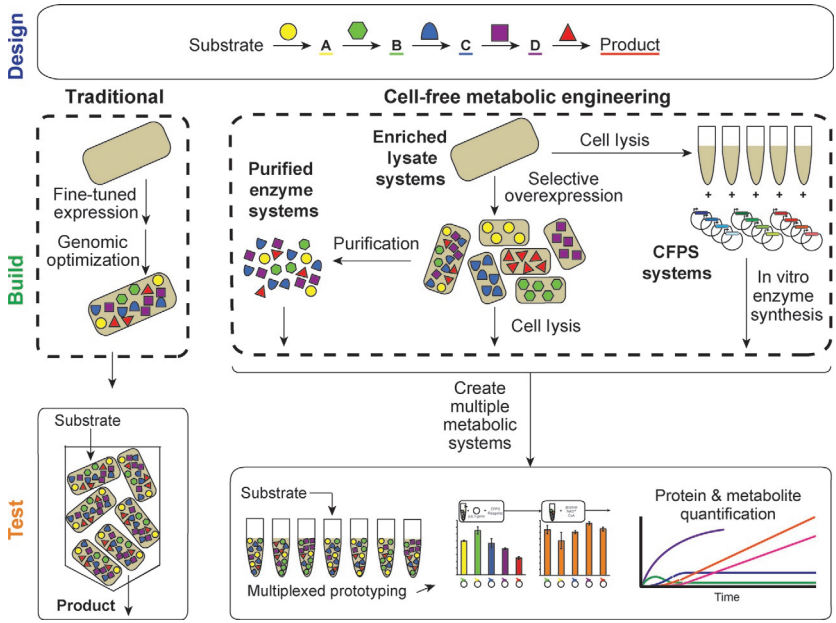

Fig. 1 Overview of cell-free synthetic biology methods for prototyping biosynthetic pathways.

and cofactor costs (as a result of enzyme purification and instability) and poor cofactor regeneration (Bujara & Panke, 2012). Despite these challenges, the majority of CFME research to date has utilized purified systems (Martin del Campo et al., 2013; Myung & Zhang, 2013; Toogood et al., 2015; Wang, Huang, Sathitsuksanoh, Zhu, & Zhang, 2011; Ye et al., 2009; Zhang, Evans, Mielenz, Hopkins, & Adams, 2007; Zhu, Kin Tam, Sun, You, & Percival Zhang, 2014). Recently, unique cofactor regeneration systems have been developed for purified systems that increase production capabilities and longevity of reactions (Opgenorth, Korman, & Bowie, 2014; Opgenorth, Korman, Iancu, & Bowie, 2017).

1.2.2 Crude Cell Lysate Systems

Crude extract-based systems rely on the ensemble of biocatalysts left after cell lysis. Upon centrifugation lipid membranes and genomic DNA are removed from cell lysates. Many groups have carried out extensive work in extract preparation and system optimization (Jewett, Calhoun, Voloshin, Wuu, & Swartz, 2008; Jewett & Swartz, 2004a, 2004b; Kwon & Jewett, 2015). There are several advantages to using crude lysates beyond their ease of preparation including lower system catalyst costs compared to purified counterparts, cofactor regeneration systems (Swartz, 2006, 2012), and the presence of native-like metabolism (Jewett et al., 2008; Jewett & Swartz, 2004a). Crude lysates thus allow for observations of metabolic interactions with biosynthetic pathways (Dudley, Anderson, & Jewett, 2016; Karim, Heggestad, Crowe, & Jewett, 2018; Kay & Jewett, 2015). There are a growing number of successes in using and characterizing crude lysate systems. A great example is the real-time monitoring and optimization of DHAP production (Bujara, Schumperli, Pellaux, Heinemann, & Panke, 2011; Hold, Billerbeck, & Panke, 2016). In addition, our group has shown that 2,3-butanediol (Kay & Jewett, 2015), mevalonate (Dudley et al., 2016), *n*-butanol (Karim et al., 2018; Karim & Jewett, 2016), and more complex products (Goering et al., 2016) can be constructed in crude lysates with high productivities (>g/L/h).

2. THE CELL-FREE METABOLIC ENGINEERING FRAMEWORK

The cell-free framework is a way to build pathways in the context of the DBT paradigm. The foundational principle of the cell-free metabolic engineering approach is that we can construct discrete metabolic pathways

through modular assembly of cell-free lysates containing enzyme components produced by overexpression in the lysate chassis strain or by cell-free protein synthesis (CFPS; in vitro production) (Fig. 2). In addition, the open reaction environment allows for the supplementation of components such as cofactors and intermediates at any time during a cell-free reaction, which can be maintained at precise concentrations. In the Design phase, desired small-molecule products are selected, enzymes, homologs, and their stoichiometries are chosen, and likely beneficial conditions (i.e., substrates, cofactors, buffers, pH, and temperature) are selected. In the Build phase, the heart of this framework consists of assembling planned pathways for making the desired molecules from lysates, which can occur through two routes. One pathway construction route we term mix-and-match cell-free metabolic engineering (CFME) involves preparing multiple cell extracts for a selected pathway. One enzyme in the candidate pathway will be preenriched in each extract by overexpression in the host strain prior to lysis. Then, enriched extracts can be mixed in multiple, different ratios to build complete biosynthetic pathways. Another route termed CFPS-driven metabolic

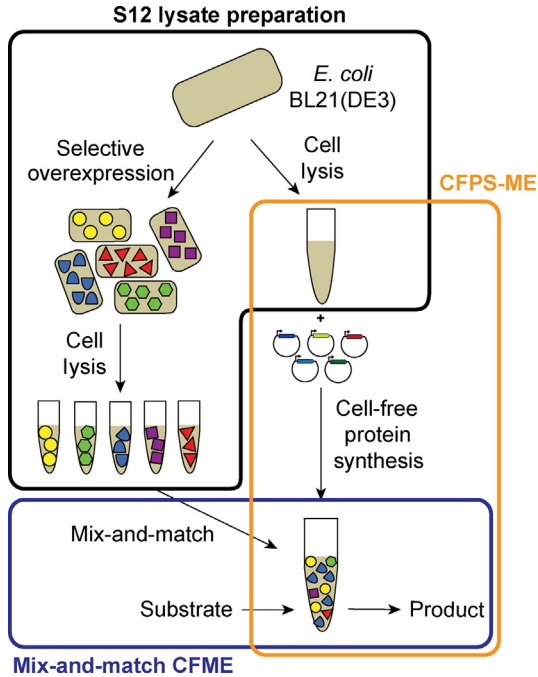

Fig. 2 Overview of methods for cell-free pathway prototyping. Lysate preparation, mix-and-match CFME, and CFPS-ME methods are outlined.

engineering (CFPS-ME) involves one-pot in vitro synthesis of biosynthetic enzymes and pathway operation. In this route, enzymes are made by CFPS in the extract postlysis by adding the DNA for each enzyme along with protein synthesis reagents followed by the addition of pathway operation reagents, faster than any previous approach (hours rather than days). Pathways can be built in 96- or 384-well plates, using liquid handling robotics, or in 1.5- or 2.0-mL microcentrifuge tubes (Karim et al., 2018). Significantly, this cell-free prototyping approach does not require the focus on flux balancing and delicate promoter tuning to maintain viability as is true for in vivo systems (Ajikumar et al., 2010; Alper & Stephanopoulos, 2007; Blazeck, Liu, Redden, & Alper, 2011; Du, Yuan, Si, Lian, & Zhao, 2012). Following the rapid and discrete construction of metabolic pathways, each pathway can be tested by using kinetically sampled batch reactions for fast DBT cycle times (Karim & Jewett, 2016).

3. S12 LYSATE PREPARATION FOR CELL-FREE METABOLIC ENGINEERING

The foundation of these cell-free prototyping strategies relies on crude lysates for pathway assembly. There are many ways to make *E. coli*-based crude lysates (Kigawa et al., 2004; Kim et al., 2006; Krinsky et al., 2016; Kwon & Jewett, 2015; Shrestha, Holland, & Bundy, 2012; Sun et al., 2013). In theory any of these methods would work to make lysates for pathway engineering, so our method focuses on one of the more high-throughput, high-yielding methods for crude lysate preparation (Kwon & Jewett, 2015). The following protocol is based on Kwon and Jewett's preparation with a few modifications for pathway prototyping strategies, also described in Karim and Jewett (2016).

3.1 Materials

3.1.1 Equipment

- 125 mL (small batch) and 4 L (large batch) baffled, Tunair shake flasks, or similar culture container; one per extract.
- 30°C and 37°C incubators with vigorous shaking at 250 rpm.
- Spectrophotometer, for cuvette-based OD_{600} measurements.
- Optically clear cuvettes for measuring optical density.
- Centrifuge capable of spinning 1-L centrifuge bottles at 5,000 × *g*, prechilled to 4°C.
- 1-L plastic centrifuge bottles and lids, prechilled to 4°C; one per extract.

- Table-top centrifuge capable of spinning 50-mL conical tubes at 12,000 × *g*, prechilled to 4°C.
- 50-mL conical tubes, prechilled to 4°C; one per extract.
- Weight scale to measure wet cell mass.
- Kim wipes.
- Bucket with ice.
- Beaker with ice water bath.
- Liquid nitrogen and dewar.
- −80°C freezer for storage.
- Q125 Sonicator (Qsonica, Newtown, CT), 3.175 mm diameter probe at frequency of 20 kHz.
- Heat block capable of heating to 90°C.
- XCell SureLock® Mini-Cell (Invitrogen) with power box.
- Gel Doc™ XR + Gel Documentation System (BioRad).

3.1.2 Media

- Luria–Bertani (LB) broth: 1.0% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl, autoclaved.
- LB agar: add 1.5% (w/v) agar to LB prepared as above. Aliquot 30 mL of LB agar per 150 mm Petri dish into a plastic conical tube. Add appropriate antibiotics.
- 2 × YTPG broth: 1.6% (w/v) tryptone, 1.0% (w/v) yeast extract, 0.5% (w/v) NaCl, 1.8% (w/v) glucose, 0.7% (w/v) K_2HPO_4, 0.3% (w/v) KH_2PO_4, pH adjusted to 7.2 with 5N KOH, autoclaved. Add 40% (w/v) glucose, separately autoclaved, to broth prior use. Use 1 L per extract.

3.1.3 Media Supplements

- Carbenicillin (Carb) (100 μg/mL): To make a 1000 × stock, mix 1 g in 10 mL nanopure water, sterile filtered.
- Kanamycin (Kan) (50 μg/mL): To make a 1000 × stock, mix 0.5 g in 10 mL nanopure water, sterile filtered.
- Isopropyl-β-D-thiogalactopyranoside (IPTG) (0.1 m*M*): To make a 1000 × stock, mix 0.238 g in nanopure water to 10 mL, sterile filtered.

3.1.4 Bacterial Strains and Plasmids (See *Table 1*)

- *E. coli* DH5α (NEB).
- *E. coli* BL21(DE3) (NEB).

Table 1 A List of Strains and Plasmids Used to Carry Out Cell-Free Pathway Prototyping

Strain or Plasmid	Description	References
E. coli strains		
NEB Turbo™	F′ proA + B + lacIq Δ lacZM15/ fhuA2 Δ(lac-proAB) glnV galK16 galE15 R(zgb-210::Tn10)TetS endA1 thi-1 Δ(hsdS-mcrB)5—used for cloning purposes only	New England Biolabs
BL21 (DE3)	fhuA2 [lon] ompT gal (λ DE3) [dcm] Δ hsdS λ DE3 = λ s*Bam*HIo Δ *Eco*RI-B int::(lacI::PlacUV5::T7 gene1) i21 Δ nin5—used for enzyme overexpression and for lysate production	New England Biolabs
Plasmids		
pETBCS-rbsU-gene	Plasmid used for protein production and extract preparation	Karim et al. (2018) and Karim and Jewett (2016)
pJL1-gene	Plasmid used for CFPS	Karim et al. (2018), Karim and Jewett (2016), and Addgene #69496

- pETBCS-rbsU vector, used in previous studies (Karim et al., 2018; Karim & Jewett, 2016).
- pJL1 vector, used in previous studies (Karim et al., 2018; Karim & Jewett, 2016) (Addgene #69496).

3.1.5 Buffers and Reagents

- S30 buffer: 10 m*M* Tris–acetate (pH 8.2), 14 m*M* magnesium acetate, and 60 m*M* potassium glutamate, prechilled to 4°C.
- QuickStart™ Bradford Protein Assay Kit (BioRad Laboratories, Inc.).
- NuPAGE® LDS Sample Buffer (Invitrogen).
- 1 *M* Dithiothreitol (DTT).
- 4%–12% Bis-Tris Nu-PAGE gel (Invitrogen).
- 20× NuPAGE MOPS SDS Running Buffer (Invitrogen).
- SeeBlue™ Plus2 Prestained Protein Standard (Thermo).
- SimplyBlue™ SafeStain (Thermo).

3.2 Procedure

3.2.1 Cell Preparation and Expression

Day 0 of procedure (about 16–18 h before starting Day 1):

1. Add 30 mL of LB media to a 125-mL baffled flask and sterilize. Add appropriate antibiotic if necessary following sterilization. Note: while you only need ~1 mL of culture per planned extract preparation for Day 1 of procedure, having additional liquid culture is suggested.
2. Inoculate media with desired *E. coli* strain from glycerol stock. Note: inoculating from a plate is also possible and should not make a substantial difference on lysate quality.
3. Incubate overnight at 37°C, shaking at 250 rpm.

Day 1 of procedure:

4. Add 1 L of 2 × YTPG media without antibiotic to an autoclaved 4-L Tunair flask. Note: we do not typically use antibiotics at this stage of the procedure. 200 mL of culture makes ~2 mL of extract.
5. Inoculate overnight culture (~1:100 dilution) into 2 × YTPG media so that the starting OD_{600} is within the range of 0.05–0.1. Note: the 1:100 dilution is not a strict guideline but undergoing several doublings before induction is ideal.
6. Incubate culture at 37°C, shaking at 250 rpm.
7. Check the OD_{600} about every 30 min. Note: there can often be a variable lag phase, so it is important to check the OD_{600} frequently. Use 2 × YTPG as a blank.
8. At OD_{600} of 0.5–0.8 (early exponential phase), induce recombinant protein overexpression by adding 0.1 m*M* (final concentration) IPTG. Note: IPTG concentrations can be tuned for optimal expression and other induction mechanisms can be used.
9. Incubate induced culture at 30°C, shaking at 250 rpm.
   - If enzyme overexpression is the objective, incubate in 30°C shaker for 4 h, or ~8 doublings, postinduction for protein expression. Note: this time it can be optimized to obtain the desired weight of cells and enzyme overexpression.
   - If cell-free protein synthesis is the objective, incubate in 30°C shaker to reach OD_{600} of 3. Check OD_{600} every ~30 min.
10. Following growth, immediately take the shake flask containing the culture out of the incubator and pour the contents into the prechilled 1-L centrifuge bottles on ice. Note: keeping cultures and subsequent handlings of the cultures on ice is imperative to maintain the quality of lysates produced.

11. Centrifuge samples in prechilled centrifuge bottles for 15 min at 5000 × *g* at 4°C.
12. Wipe the inside walls of the centrifuge bottle with large Kim wipes to remove as much of the leftover supernatant as possible before inverting the bottle back upright. Note: removing as much supernatant at each centrifugation steps reduces possible dilution of the extract.
13. Transfer cell pellet from centrifuge bottle to a prechilled 50-mL conical tube, using a spatula.
14. To obtain the remaining cells from the spatula and centrifuge bottle, add 2 mL of S30 buffer in the bottle and use the spatula to mix the cells into solution. Pipette the cell/buffer mixture into the 50-mL conical tube. Proceed with your remaining cell samples.
15. Place the 50-mL conical tube containing your cell sample submerged in ice.
16. Resuspend in 25 mL S30 buffer. Alternate between vortexing using a medium-high setting for 15 s and resting on ice for 15 s. Note: this can take 5–15 min or longer.
17. Once resuspended, centrifuge samples in 50-mL conical tubes at 5,000 × *g* for 10 min at 4°C to pellet cells.
18. Pour off supernatant into waste. Again, wipe the inside walls of the centrifuge bottle with Kim wipes to remove as much of the leftover supernatant as possible before inverting the bottle back upright.
19. Repeat steps 15–18.
20. Repeat step 16.
21. Once resuspended, centrifuge samples in 50-mL conical tubes at 7000 × *g* for 10 min at 4°C to pellet cells. Note: the change in centrifugation speed is to keep the cells somewhat tight to remove residual liquid but somewhat loose before freeze–thaw to increase ease of resuspension.
22. Completely remove supernatant by pouring into waste. Before inverting your tubes back upright, clean the residual supernatant on inside of tubes as much as possible using a Kim wipe.
23. Use a clean pipette tip to split the cell pellet in half to increase the surface area of the pellet. Note: this will increase the speed of pellet resuspension when making extract.
24. Measure pellet mass on weight scale and record weight on tube.
25. Flash freeze the pellets in liquid nitrogen and store at −80°C. Note: flash freezing in liquid nitrogen helps maintain the quality of cells before extract preparation compared to just placing pellets in −80°C storage.

3.2.2 Extract Preparation

1. Take out cell pellets from −80°C freezer. Let pellets thaw on ice (∼1–2 h).
2. Resuspend pellets in 1 mL S30 buffer per g of weighed pellet via vortex. It is important that the cells are kept on ice. Vortex as necessary, but do not hold off ice for more than 15 s at a time. Note: this can take 15 min or more and the resuspension volume may be optimized for other strains.
3. Let samples rest on ice for suspension to settle.
4. Prepare a glass beaker with an ice water bath (90% ice, 10% water).
5. Transfer 1.4 mL of cell suspension into 1.5-mL microcentrifuge tubes. Note: avoid transferring bubbles to 1.5-mL microcentrifuge tubes.
6. Place microcentrifuge tube containing cell suspension in ice bath.
7. Turn on the Q125 sonicator with 3.175 mm diameter probe at frequency of 20 kHz. Note that this protocol is optimized for the Q125 sonicator and might need to be adjusted for other sonicator models and probe diameters.
8. Place the sonicator tip into the cell suspension just below center of the microcentrifuge tube. Note: tip should not rest against the sides of the tube. Moving tip around the cell suspension can improve energy transfer.
9. Set sonicator to 50% amplitude with pulsing at 10 s on, 10 s off. Note: this amplitude and pulsing time may be optimized for other strains.
10. Sonicate the 1.4-mL cell suspension with 820 J of sonication energy according to step 11 settings. Note: for a 1-mL suspension, the energy should be 530 J. For volumes between 1 and 1.4 mL, use linear interpolation to calculate sonication energy required. Refer to Kwon and Jewett for more information on sonication energies (Kwon & Jewett, 2015).
11. Different bacterial strains may require a preincubation step. Refer to Kwon and Jewett for more information on preincubation (Kwon & Jewett, 2015), noting that experiments have shown that variability at this step can lead to significant differences in extract performance. Note: for our previous pathway prototyping studies, preincubation was not needed.
12. Clean sonicator according to step 9 before moving to next sample. Repeat steps 9–13 for each sample.
13. Centrifuge samples at 12,000 × *g* for 10 min at 4°C.
14. Aliquot ∼500–820 μL (if started with 1.4 mL extract mixture), top layer only, into a new tube. All samples from the original cell suspension should be pooled into the same new tube. Note: there may be three layers. Collect top layer only. In addition, the pellet after the first centrifugation tends to be loose. Therefore, immediately transfer

the supernatant of the first centrifugation (typically with a pipette) into another tube to avoid carrying over layers. Experiments have shown that multiple centrifugations yield more active extracts as compared to extracts performed with one centrifugation.

15. Pipet mix thoroughly but gently. Aliquot 100–200 μL each (makes ~20 aliquots) on ice in new 1.5-mL or 0.6-mL microcentrifuge tubes.
16. Flash freeze on liquid nitrogen and store at −80°C.

3.2.3 Extract Quantification of Total Protein by Bradford Assay

1. Remove the 1 × dye reagent (QuickStart™ Bradford Protein Assay Kit) and equilibrate to room temperature before use.
2. Refer to Table 2 for preparing the protein standards (use nanopure H_2O to dilute) Note: we use the bovine serum albumin standard that comes with the QuickStart™ Bradford Protein Assay Kit.

Table 2 Bradford Assay Sample Setup

Tube #	Source Volume (μL)	Source	Water Volume (μL)	[Protein] (μg/mL)
Standards				
1	10	2 mg/mL BSA stock	790	25
2	10	2 mg/mL BSA stock	990	20
3	6	2 mg/mL BSA stock	794	15
4	500	Tube 2	500	10
5	500	Tube 4	500	5
6	500	Tube 5	500	2.5
7	500	Tube 6	500	1.25
8	—	—	500	0
Extracts				
Dilute extract	10	Extract to measure	990	
E4x	6	Dilute extract	594	
E7x	8	Dilute extract	552	
E10x	16	Dilute extract	624	

The following is the experimental setup of samples for extract quantification.
This is adapted from the QuickStart™ Bradford Protein Assay Kit (BioRad Laboratories, Inc.) instruction manual.

3. Dilute extract samples (assuming extract concentration is ~40 mg/mL) by adding 10 μL extract to 990 μL water. This will be the "dilute extract" used to make further dilutions (See Table 2).
   - Make a 10,000× dilution by adding 6 μL "dilute extract" into 594 μL water.
   - Make a 7000× dilution by adding 8 μL "dilute extract" into 552 μL water.
   - Make a 4000× dilution by adding 16 μL "dilute extract" into 624 μL water.
4. Pipette 140 μL each standard and extract dilutions into separate microplate wells (refer to Fig. 3).
5. Use a multichannel pipette to add 140 μL 1× dye reagent to each well and carefully mix. Note: avoid bubbles as they can drastically alter Bradford assay readings.
6. Incubate at room temperature for ~5 min. Samples should not be incubated longer than 1 h at room temperature.
7. Measure the absorbance of the standards and extract samples at 595 nm on a plate reader. Note: the linear range of these assays for BSA is 1.25–10 μg/mL.
8. Create a standard curve of the BSA standards measured and use this curve to calculate the protein content of each extraction dilution. Multiple each dilution to get a 1× concentration of the measurement. Then, average the values together to get an extract total protein

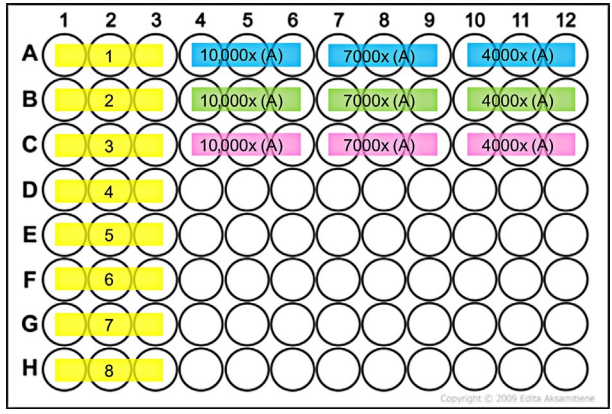

Fig. 3 Layout of Bradford assay plate. *Yellow* (columns 1–3) are the standards. *Blue*, *green*, and *purple* (rows 1, 2, and 3; columns 4–12) are three separate extracts with triplicates for each of the three dilutions: 10,000×, 7000×, and 4000×.

concentration. Note: total extract concentrations are typically between 35 and 45 mg total *E. coli* protein/mL. Refer to QuickStart™ Bradford Protein Assay Kit instructions.

3.2.4 Overexpressed Protein Quantification by Densitometry

1. Add ~2 μL of extract to 3 μL NuPAGE® LDS Sample Buffer (4×), 0.6 μL 1 *M* DTT, and water to make a total of 12 μL sample per extract being tested. Note: it is important for overexpressed protein quantification that a control sample of *E. coli* extract with no protein overexpressed is also made.
2. Heat each sample at 90°C for 10 min in a heat block.
3. Load 10 μL of each protein samples on a 4%–12% Bis-Tris Nu-PAGE gel with 1× MOPS buffer and protein standards (SeeBlue plus2 ladder). Note: other protein ladders can be used in place of the SeeBlue plus2 ladder.
4. Run gel at a constant 120 V for 105 min.
5. After the run, open gel container, and place gel in staining container.
6. Rinse 2× and shake in nanopure water for 5 min, then drain.
7. Stain with ~100 mL SimplyBlue™ SafeStain for 1 h on shaker. Note: any Coomassie stain can be used in place of SimplyBlue™ SafeStain.
8. Rinse 2× and shake in water for 2 h or overnight.
9. Image on white background with Gel Doc™ XR + Gel Documentation System using a Coomassie stain filter.
10. Once imaged the gel can be used to loosely quantify what percentage of protein in the extract is the overexpressed protein of interest using ImageJ software and documentation (http://rsb.info.nih.gov/ij/index.html). See Fig. 4 for details.

4. MIX-AND-MATCH CELL-FREE METABOLIC ENGINEERING

One approach to building pathways is to construct them by mixing lysates together that separately contain heterologous enzymes catalyzing the chemical reactions to get to a product of interest. Rather than needing to engineer one organism to contain all enzymes of a pathway, we can overexpress single enzymes in chassis strains which can then be lysed and mixed to assemble the pathway. Provided are methods for biosynthetic reaction assembly.

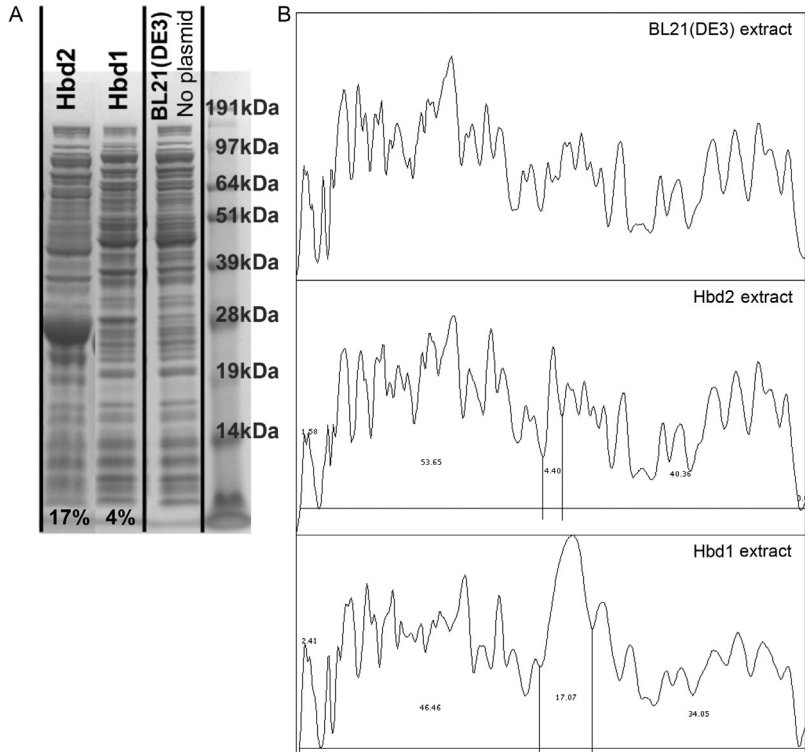

Fig. 4 An example of semiquantification overexpressed proteins in *E. coli* extracts. (A) An SDS-PAGE gel is shown for three extracts: BL21 (DE3) containing no plasmid, containing a plasmid expressing Hbd1, and containing a plasmid expressing Hbd2. The percent of overexpressed protein relative to total protein content is listed at the bottom of each lane. (B) ImageJ analysis described is demonstrated here. This analysis is performed on the SDS-PAGE gel in A.

4.1 Materials

4.1.1 Equipment

- 1.5-mL Eppendorf tubes
- 37°C incubator

4.1.2 Buffers and Reagents

- *E. coli* extracts individually expressing enzymes to assemble a biosynthetic pathway.
- 15 × Salt Solution: magnesium glutamate (150 m*M*), ammonium glutamate (150 m*M*), and potassium glutamate (2010 m*M*).
- Glucose (2.2 *M*).

- Dipotassium phosphate (1 *M*, pH 7.2).
- Bis-Tris (2 *M*).
- NAD (100 m*M*).
- ATP (100 m*M*).
- CoA (50 m*M*).
- DNAse/RNAse-free water.
- 10% (w/v) trichloroacetic acid (TCA), sterile filtered.

4.2 Procedure

4.2.1 Mix-and-Match Biosynthesis Reactions

1. Let reagents thaw on ice. Do not hand-thaw lysates.
2. For a first test of a pathway, label 21- to 1.5-mL Eppendorf tubes as "reagent mix," "extract mix," "reaction mix," and 0, 3, 6, 9, 18, and 24 h time points in triplicate. It is important to do a time course for new pathways.
3. Keep all tubes on ice.
4. Assemble the "reagent mix" by combining magnesium glutamate (8 m*M*), ammonium glutamate (10 m*M*), potassium glutamate (134 m*M*), glucose (200 m*M*), dipotassium phosphate (10 m*M*, pH 7.2), Bis-Tris (100 m*M*), NAD (1 m*M*), ATP (1 m*M*), and CoA (1 m*M*). It is helpful to make this mix at 1.2× the volume needed for all reaction tubes (18 tubes, 25 μL in this example). Mix thoroughly by vortex and keep tube on ice.
5. Assemble the "extract mix" by combining each lysate preenriched with heterologous enzymes for the given pathway (in a five-enzyme pathway this would involve mixing five lysates). Start with each lysate at a final concentration of 2 mg/mL based on lysate quantification results. It is helpful to have this mix at 1.2× the volume needed for all reaction tubes. Gently pipette mix and keep on ice.
6. To create the "reaction mix" combine the "extract mix" and "reagent mix" the volume in here should be at 1.1× the volume needed to pipette each reaction tube at 25 μL each. Keep on ice.
7. Pipette mix and transfer 25 μL of "reaction mix" into each labeled reaction tube.
8. Immediately pull the 0 h time point (all replicates) and quench those reactions with 10% (w/v) trichloroacetic acid in a 1:1 ratio.
9. Incubate all other time points at 37°C.
10. Pull each reaction with replicates at time points. Terminate reactions by adding 10% (w/v) trichloroacetic acid in a 1:1 ratio.

11. Precipitate proteins by pelleting through centrifugation at 15,000 × *g* for 10 min.
12. The supernatant was stored at −80°C until analysis.

4.2.2 Biosynthesis Analysis

Metabolites can be quantified with current chromatography and mass spectroscopy techniques. In addition, chemical and enzymatic plate-based assays can be used when available (Dudley et al., 2016; Karim et al., 2018; Karim & Jewett, 2016; Kay & Jewett, 2015).

5. CELL-FREE PROTEIN SYNTHESIS-DRIVEN METABOLIC ENGINEERING

Using cell-free protein synthesis to enrich lysates with different enzymes for combinatorial assembly of different pathways enables parallelized pathway construction of combinatorial designs to accelerate DBT cycles.

5.1 Materials

5.1.1 Equipment

- 1.5-mL Eppendorf tubes.
- 37°C incubator.
- Microbeta scintillation detection instrument, PerkinElmer.
- Typhoon 7000 Imager (GE Healthcare Life Sciences, Pittsburgh, PA).
- Waterman chromatography paper.
- 12-in square cellophane sheets.
- Autoradiography cassettes.

5.1.2 Buffers and Reagents

- *E. coli* BL21 Star (DE3) extracts with or without individually expressing enzymes as part of a biosynthetic pathway.
- DNA encoding each enzyme in the biosynthetic pathway.
- 15 × Salt Solution: magnesium glutamate (150 m*M*), ammonium glutamate (150 m*M*), and potassium glutamate (2010 m*M*).
- 15 × Nucleotide master mix: ATP (18 m*M*), GTP (12.75 m*M*), UTP (12.75 m*M*), CTP (12.75 m*M*), folinic acid (0.51 mg/mL), and 2.559 mg/mL tRNA.
- Amino acid solution containing all 20 amino acids at 50 m*M*.
- Phosphoenolpyruvate (PEP) at 1 *M*.

- Putrescine (250 m*M*).
- Spermidine (250 m*M*).
- HEPES Buffer (1 *M*, pH 7.2).
- Glucose (2.2 *M*)
- Dipotassium phosphate (1 *M*, pH 7.2)
- NAD (100 m*M*)
- ATP (100 m*M*)
- CoA (50 m*M*)
- DNAse/RNAse-free water
- ^{14}C-leucine (10 μ*M*)
- 0.1 *M* sodium hydroxide.
- 5% (w/v) TCA.
- 10% (w/v) TCA, sterile filtered.
- Cytoscint™-ES liquid scintillation cocktail.
- Split-GFP Buffer: 50 m*M* Tris pH 7.4, 0.1 *M* NaCl, 10% glycerol (TNG buffer) (Cabantous, Terwilliger, & Waldo, 2005).

5.2 Procedure

CFPS reactions were performed to express enzymes involved in *n*-butanol production prior to starting the CFME portion of the reactions using a modified PANOx-SP system (Jewett & Swartz, 2004a).

5.2.1 CFPS-ME Reactions

1. Let reagents thaw on ice. Do not hand-thaw lysates.
2. For a first test of a pathway, label 21 1.5-mL Eppendorf tubes as "reagent mix," "extract mix," "reaction mix," and 0, 3, 6, 9, 18, and 24 h time points in triplicate. It is important to do a time course for new pathways.
3. Keep all tubes on ice.
4. Assemble the "CFPS Master Mix" by combining 15× Salt Solution (magnesium glutamate (8 m*M*), ammonium glutamate (10 m*M*), potassium glutamate (134 m*M*)), 15× nucleotide mix (ATP (1.2 m*M*); GTP, UTP, and CTP (0.85 m*M* each); folinic acid (34.0 μg/mL); *E. coli* tRNA mixture (170.0 μg/mL)), 20 standard amino acids (2 m*M* each), NAD (0.33 mm), CoA (0.27 m*M*), spermidine (1.5 m*M*), putrescine (1 m*M*), and PEP (33 m*M*). It is helpful to make this mix at 1.2× the volume needed for all reaction tubes (18 tubes, 22.5 μL in this example). Mix thoroughly by vortex and keep tube on ice.

5. Assemble the "extract mix" by using plain *E. coli* extract with no enzyme enrichment or by combining each lysate preenriched with heterologous enzymes for the given pathway (in a five-enzyme pathway this would involve mixing five lysates). Start with each lysate at a final concentration of 2 mg/mL based on lysate quantification results. The total lysate concentration should be 10 mg/mL. It is helpful to have this mix at 1.2× the volume needed for all reaction tubes. Gently pipette mix and keep on ice.
6. To create the "reaction mix" combine the "extract mix," "reagent mix," and plasmid DNA encoding each enzyme (~13.3 ng/mL each). The volume in here should be at 1.1× the volume needed to pipette each reaction tube at 22.5 μL each. Keep on ice.
7. Pipette mix and transfer 22.5 μL of "reaction mix" into each labeled reaction tube. Note: it is important that the reaction components are evenly mixed. Cell-free protein synthesis has been plagued by variable results. One cause for this variability in our laboratory has been the lack of complete mixing of the reaction components. Specifically, this can be a problem when setting up numerous reactions from the same premix. Air bubbles, especially in the extract can also be an issue. When reactions are well controlled, variability is low.
8. Incubate all reaction tubes at 30°C.
9. Assemble the "CFME Master Mix" by combining glucose (200 m*M*), NAD (0.67 m*M*), and CoA (0.73 m*M*). Mix thoroughly by vortex and keep tube on ice.
10. After 3 h, spike in 2.5 μL "CFME Master Mix" to each reaction tube to initiate biosynthetic activity.
11. Immediately pull the "0 h" time point (all replicates) and quench those reactions with 10% (w/v) TCA in a 1:1 ratio.
12. Incubate all other time points at 30°C.
13. Pull each reaction with replicates at time points. Terminate reactions by adding 10% (w/v) TCA in a 1:1 ratio.

5.2.2 Quantification of Protein Produced In Vitro via Radioactive Incorporation

1. Using the procedure for CFPS-ME Reactions in Section 5.2.1, the "CFPS Master Mix" can be made with radioactive ^{14}C-leucine (10 μ*M*) at step 4.
2. Samples are quenched with 100 μL of 0.1 *M* sodium hydroxide and incubated at 37°C for 20 min.

3. Quenched samples are then split in half ("washed" vs "unwashed") and pipetted onto 0.25-in by 1-in Waterman paper strips. The strips are then dried.
4. The "washed" half of samples are then washed three times with 5% (w/v) TCA to precipitate radioactive protein samples.
5. The "washed" half of samples are then washed with molecular biology grade ethanol.
6. Radioactivity of TCA-precipitated samples and "unwashed" were measured by liquid scintillation counting to then quantify the protein produced as previously reported (MicroBeta2; PerkinElmer)(Jewett et al., 2008; Jewett & Swartz, 2004a).
7. These reactions were also run on a Coomassie-stained SDS-PAGE gel as done in Section 3.2.4 steps 1–11.
8. The gels can then be dried between 2- and 12-in square cellophane sheets overnight.
9. The dried gel can now be exposed by autoradiography for 3 days.
10. Autoradiographs are imaged with a Typhoon 7000 Imager (GE Healthcare Life Sciences, Pittsburgh, PA).
11. Multiple proteins produced in vitro were further quantified by gel image intensity comparisons using ImageJ (NIH) similar to Section 3.2.4 (Fig. 5A).

5.2.3 Quantification of Protein Produced In Vitro via Split-GFP Construct

If radioactive ^{14}C-leucine is unavailable, other quantification methods can be used. Here, we present a method for quantification by split-GFP fluorescence. By adding a 20-amino acid "GFP11" tag onto the end of each protein and expressing the corresponding "GFP1–10" protein, the association of the two will elicit a quantifiable fluorescent signal.

1. DNA constructs encoding enzymes to prototype can be designed to include the following encoded amino acid sequence directly at the end of the coding sequence before the stop codon "DGGSGGGSTSR DHMVLHEYV."
2. Also, a DNA construct pJL1-split-GFP encoding the other portion of GFP can be made "MGGTSMSKGEELFTGVVPILVELDGDVNGH KFSVRGEGEGDATIGKLTLKFICTTGKLPVPWPTLVTTLTYGV QCFSRYPDHMKRHDFFKSAMPEGYVQERTISFKDDGKYKTR AVVKFEGDTLVNRIELKGTDFKEDGNILGHKLEYNFNSHNVY ITADKQKNGIKANFTVRHNVEDGSVQLADHYQQNTPIGDGP VLLPDNHYLSTQTVLSKDPNEK" (Cabantous et al., 2005).

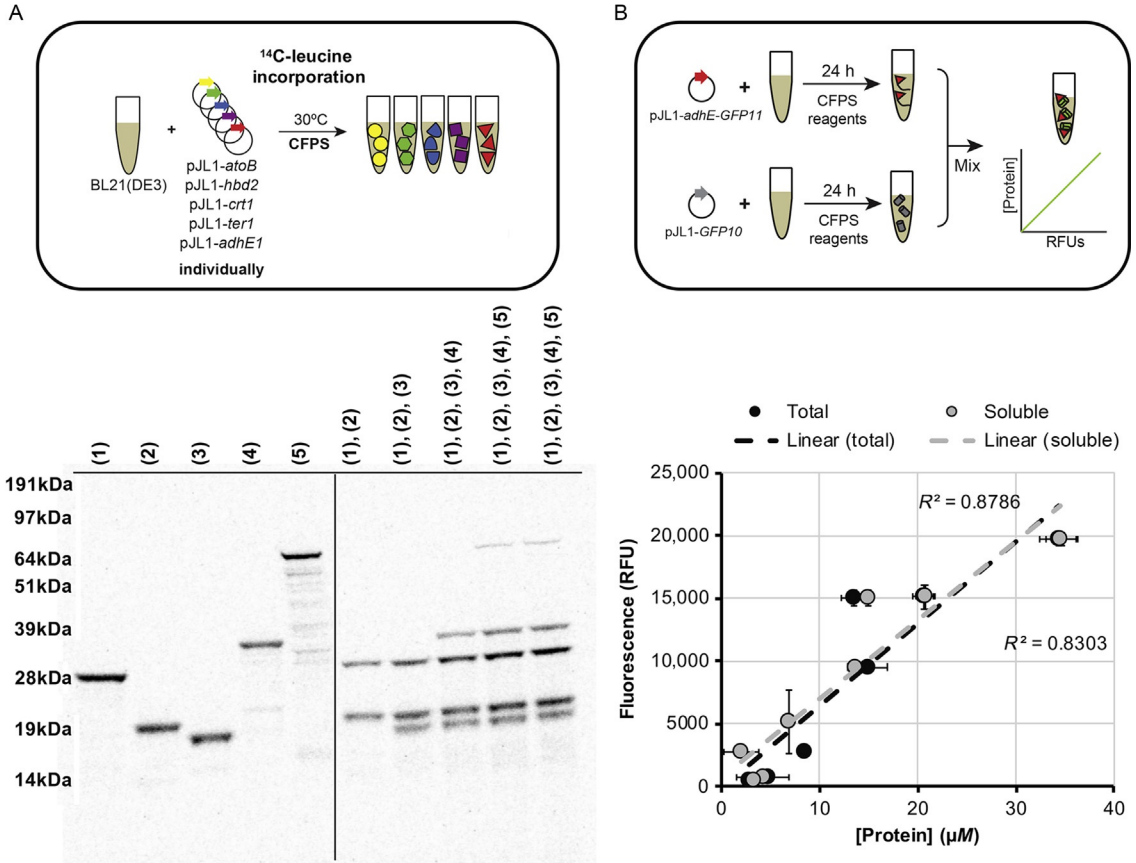

Fig. 5 Quantification of cell-free protein synthesis examples. (A) Data are representative of quantification by radioactivity. SDS-PAGE autoradiogram shows each protein produced by cell-free protein synthesis. Each column is a separate cell-free reaction producing one of five proteins or a combination of the five proteins. (B) Data are representative of quantification by splitGFP reporter. Each *circle* represents a concentration of protein measured by radioactive ^{14}C-leucine incorporation and the corresponding fluorescent readout.

3. Perform CFPS reactions of pJL1-gene-gfp according to Section 5.2.1, steps 1–9 (CFPS RXN 1).
4. Separately, perform CFPS reaction of pJL1-split-GFP (CFPS RXN 2).
5. Mix 10 μL of CFPS RXN 1, 5 μL of CFPS RXN 2, and 5 μL Split-GFP Buffer.
6. Measure fluorescence over a 20-h period (Fig. 5B).

5.2.4 Metabolite Quantification

Metabolites can be quantified with current chromatography and mass spectroscopy techniques (Goering et al., 2016; Karim et al., 2018). In addition, chemical and enzymatic plate-based assays can be used when available through commercially available kits or documented in the literature.

6. SUMMARY AND CONCLUSIONS

Cell-free synthetic biology provides powerful tools to prototype biosynthetic pathways, providing an unprecedented capability to test hundreds to thousands of pathways by avoiding inherent limitations of cell growth. The methods provided here describe multiple ways of constructing cell-free enzymatic pathways to iterate through DBT cycles at speeds 10 × faster than traditional approaches. Coupling cell-free protein synthesis, in particular, to the construction of a metabolic pathway in tandem with high-end metabolomics will offer a high degree of flexibility to model the kinetics and stability of individual enzymes, measure metabolite fluxes in multistep pathways, and experimentally isolate many other parameters confounded in living organisms. Overall, the modular and cell-free nature of our framework is poised to facilitate multiplexed, automated study of biosynthetic pathways to inform systems-level cellular design.

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