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**TITLE:****Cell-Free Protein Synthesis from Exonuclease-Deficient Cellular Extracts Utilizing Linear DNA Templates****AUTHORS AND AFFILIATIONS:**

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**SUMMARY:**

Presented here is a protocol for the preparation and buffer calibration of cell extracts from exonuclease V knockout strains of *Escherichia coli* BL21 Rosetta2 ( $\Delta recBCD$  and  $\Delta recB$ ). This is a fast, easy, and direct approach for expression in cell-free protein synthesis systems using linear DNA templates.

**ABSTRACT:**

Cell-free protein synthesis (CFPS) has recently become very popular in the field of synthetic biology due to its numerous advantages. Using linear DNA templates for CFPS will further enable the technology to reach its full potential, decreasing the experimental time by eliminating the steps of cloning, transformation, and plasmid extraction. Linear DNA can be rapidly and easily amplified by PCR to obtain high concentrations of the template, avoiding potential *in vivo* expression toxicity. However, linear DNA templates are rapidly degraded by exonucleases that are naturally present in the cell extracts. There are several strategies that have been proposed to tackle this problem, such as adding nuclease inhibitors or chemical modification of linear DNA ends for protection. All these strategies cost extra time and resources and are yet to obtain near-plasmid levels of protein expression. A detailed protocol for an alternative strategy is presented

here for using linear DNA templates for CFPS. By using cell extracts from exonuclease-deficient knockout cells, linear DNA templates remain intact without requiring any end-modifications. We present the preparation steps of cell lysate from *Escherichia coli* BL21 Rosetta2  $\Delta recBCD$  strain by sonication lysis and buffer calibration for Mg-glutamate (Mg-glu) and K-glutamate (K-glu) specifically for linear DNA. This method is able to achieve protein expression levels comparable to that from plasmid DNA in *E. coli* CFPS.

## INTRODUCTION:

Cell-free protein synthesis (CFPS) systems are increasingly being used as a fast, simple, and efficient method for biosensor engineering, decentralized manufacturing, and prototyping of genetic circuits<sup>1</sup>. Due to their great potential, CFPS systems are used regularly in the field of synthetic biology. However, so far CFPS systems rely on circular plasmids that can limit the technology from reaching its full potential. Preparing plasmid DNA depends on many time-consuming steps during cloning and large amounts of DNA isolation. On the other hand, PCR amplification from a plasmid, or a synthesized DNA template, can be used to simply prepare CFPS templates within a few hours<sup>2,3</sup>. Therefore, application of linear DNA offers a promising solution for CFPS. However, linear DNA is rapidly degraded by exonucleases naturally present in cellular extracts<sup>4</sup>. There are solutions that address this problem, such as using the  $\lambda$ -phage GamS protein<sup>5</sup> or DNA containing Chi sites<sup>6</sup> as protective agents, or directly protecting the linear DNA by chemical modification of its ends<sup>2,7,8,9</sup>. All these methods require supplementations to the cell extract, which are costly and time-consuming. It has been known for a long time that the exonuclease V complex (RecBCD) degrades linear DNA in cell lysates<sup>4</sup>. Recently, we showed that linear DNA can be much better protected in lysates from cells knocked out for exonuclease genes (*recBCD*)<sup>10</sup>.

In this protocol, steps for the preparation of cell-free lysates from the *E. coli* BL21 Rosetta2  $\Delta recBCD$  strain by sonication lysis are described in detail. Sonication lysis is a common and affordable technique employed by several labs<sup>11,12</sup>. The extracts produced from this strain do not need the addition of any extra component or DNA template modification to support expression from linear DNA templates. The method relies on the essential step of buffer optimization for cell extracts specifically for linear DNA expression from native *E. coli* promoters. It has been shown that this specific buffer optimization for linear DNA expression is the key for native  $\sigma 70$  promoters to yield high protein production without GamS protein or Chi DNA supplementation, even avoiding purification of the PCR products<sup>10</sup>. The optimal concentration of Mg-glu for linear DNA expression was found to be similar to that for plasmid DNA. However, the optimal concentration of K-glu showed a substantial difference between linear and plasmid DNA, likely due to a transcription-related mechanism<sup>10</sup>. The functionality of proteins expressed using this method has been demonstrated for several applications, such as rapid screening of toehold switches and activity assessment of enzyme variants<sup>10</sup>.

This protocol provides a simple, efficient, and cost-effective solution for using linear DNA templates in *E. coli* cell-free systems by simply using mutant  $\Delta recBCD$  cell extracts and specific calibration for linear DNA as template.

## PROTOCOL:

### 1. Media and buffer preparation

#### 1.1. Preparation of 2xYT+P medium (solid and liquid)

1.1.1. Prepare liquid and solid media as described in **Table 1**, and then sterilize by autoclaving.

NOTE: This protocol requires one 2xYT+P agar plate containing chloramphenicol (10 µg/mL). The volume of liquid media is proportional to the volume of the lysate required. Here, a starting volume of 5 L of liquid 2xYT+P media is used. However, the volume can be adjusted as needed, knowing that 1 L of the cell culture results in 2 to 3 mL of the final cell lysate.

#### 1.2. Preparation of chloramphenicol (34 mg/mL)

1.2.1. Weigh 0.34 g of chloramphenicol, add ethanol to 10 mL, and dissolve by mixing. Aliquot 1 mL each into several tubes, and store at -20 °C to use later.

#### 1.3. Preparation of S30A buffer

1.3.1. Prepare S30A buffer according to **Table 2**, aiming for 2 L of this buffer.

NOTE: Once again, the volume of this buffer is proportional to the volume of the lysate required.

1.3.2. Before autoclaving, adjust the buffer's pH to 7.7 using glacial acetic acid.

1.3.3. Autoclave the buffer.

1.3.4. Keep this buffer at 4 °C after autoclaving.

1.3.5. Before use, add DTT (filtered by a 0.22 µm membrane filter) to the S30A buffer to reach a final concentration of 2 mM (2 mL of stock 1 M DTT to 1 L of S30A buffer).

NOTE: Once again, the volume of this buffer is proportional to the volume of the lysate required.

#### 1.4. Preparation of energy solution

NOTE: This energy solution is based on the Sun et al. paper<sup>13</sup> (14x stock concentration). **Table 3** recapitulates the components needed for the preparation of the energy solution stock, with catalog numbers for all the chemicals used in this protocol.

1.4.1. Prepare the stock solutions according to **Table 3** and keep them on ice.

1.4.2. Calibrate the pH as requested for the indicated components, either with Tris buffer 2 M (60.57 g of Tris base in a 250 mL volume of sterile water) or KOH 15% (15 g of KOH in a 100 mL volume of sterile water) as indicated in **Table 3**.

1.4.3. In a 15 mL tube, add the volume of each component in the order indicated in **Table 3**.

1.4.4. Aliquot the energy solution, 150  $\mu$ L per tube.

1.4.5. Flash freeze aliquots on dry ice and store at -80 °C.

#### 1.5. Preparation of amino acid (AA) solution

NOTE: Amino acid solution is prepared by the RTS Amino Acid Sampler kit. Each of the 20 amino acids is provided in this kit as a 1.5 mL volume at 168 mM, except for leucine which is at 140 mM. Here, the prepared stock solution is 4x concentrated, rather than the working solution required. The final concentration of the amino acid solution is 6 mM for all amino acids, except leucine which is at 5 mM.

1.5.1. Thaw the 20 amino acid tubes by vortexing and incubate at 37 °C until they are completely dissolved.

NOTE: Cys may not dissolve fully.

1.5.2. In a 50 mL tube, add 12 mL of sterile water and 1.5 mL each of the amino acids in the order indicated in **Table 4**.

1.5.3. Vortex until the solution is fairly clear, incubating at 37 °C if necessary.

NOTE: Cys may not dissolve fully.

1.5.4. Aliquot 500  $\mu$ L of the amino acid solution per tube on ice.

1.5.5. Flash-freeze the aliquots on dry ice and store at -80 °C.

#### 1.6. Preparation of solutions for buffer calibration

1.6.1. Prepare the stock solution for Mg-glu (100 mM) and K-glu (3M) as indicated in **Table 5**. Make a serial dilution (in 1 mL volume) from these stocks for Mg-glu (0 to 20 mM) and K-glu (20 to 300 mM) as indicated in **Supplementary File 1** for calibration steps.

NOTE: These stock concentrations are for the calibration step and may need to be changed when setting up the final experiment. The highest concentrations of the stocks tested for this protocol are 1 M and 4.5 M for Mg-glu and K-glu, respectively.

## 1.7. Preparation of PEG8,000 solution stock

1.7.1. Prepare 50 mL of 40% PEG8,000 solution (20 g of PEG8,000 in a 50 mL volume of sterile water).

## 2. Cell culture and lysate preparation (4 day experiment)

### 2.1. Day 1

2.1.1. Streak strain BL21 Rosetta2  $\Delta recBCD$  (**Table 6**) from  $-80\text{ }^{\circ}\text{C}$  glycerol stock onto a 2xYT+P agar plate that contains  $10\text{ }\mu\text{g/mL}$  chloramphenicol. Alternatively, BL21 Rosetta2  $\Delta recB$  (**Table 6**) may also be used<sup>10</sup>.

2.1.2. Incubate overnight at  $37\text{ }^{\circ}\text{C}$ .

### 2.2. Day 2

2.2.1. Inoculate a single colony from the above agar plate into 10 mL of 2xYT+P supplemented with  $10\text{ }\mu\text{g/mL}$  chloramphenicol.

2.2.2. Incubate overnight at  $37\text{ }^{\circ}\text{C}$  with 200 rpm shaking.

### 2.3. Day 3

2.3.1. Make a subculture by diluting the overnight culture 100 times into 4 L of fresh 2xYT+P media containing  $10\text{ }\mu\text{g/mL}$  chloramphenicol.

NOTE: For example, 40 mL of the overnight culture is added into 3960 mL of fresh media. After dilution, the 4 L media is divided into 4 flasks (5 L volume) such that each flask contains 1 L.

2.3.2. Incubate at  $37\text{ }^{\circ}\text{C}$ , 200 rpm for about 3 to 4 h of growth to reach an  $\text{OD}_{600}$  of 1.5–2.0. Dilute the culture by 4x if measuring  $\text{OD}_{600} > 0.8$ .

NOTE: The exact incubation time may vary according to the strain, initial inoculum, labware, and the instruments used. The  $\Delta recB/\Delta recBCD$  knockout strains have growth rates comparable to the BL21 Rosetta2 parental (**Supplementary Figure 1**).

2.3.3. Put the culture on ice.

2.3.4. Spin down the cells at  $5,000 \times g$  for 12 min at  $4\text{ }^{\circ}\text{C}$ , and then discard the supernatant by decanting.

2.3.5. Resuspend the cell pellets in 800 mL of chilled S30A+DTT.

NOTE: The volume of S30A+DTT is 5x less than the original cell culture volume. For example, for a starting volume of 1 L of the cell culture, resuspend cell pellets in 200 mL of S30A+DTT. It is also recommended to carry out this step in a cold room at 4 °C, as well keeping all materials on ice.

2.3.6. Spin down the cells at 5,000 x *g* for 12 min at 4 °C, and then discard the supernatant by decanting.

2.3.7. Repeat steps 2.3.5 and 2.3.6.

2.3.8. Resuspend cell pellets in 160 mL of chilled S30A+DTT.

NOTE: This time the volume of S30A+DTT is 25x less than the original cell culture volume. For example, for a starting volume of 1 L of the cell culture, resuspend cell pellets in 40 mL of S30A+DTT. Again, it is recommended to carry out this step in a cold room at 4 °C, as well to keep the materials on ice.

2.3.9. Transfer the cells to chilled and pre-weighed 50 mL tubes.

2.3.10. Spin down the cells at 2,000 x *g* for 8 min at 4 °C, and then discard the supernatant by decanting.

2.3.11. Spin down the cells at 2,000 x *g* for 4 min at 4 °C, and then remove the remaining supernatant carefully by using a pipette.

2.3.12. Re-measure the weight of the tube to calculate the weight of the cell pellet.

2.3.13. Keep the cell pellets at -80 °C.

2.4. Day 4

2.4.1. Take the cell pellets from -80 °C and thaw them on ice for 1–2 h.

2.4.2. Resuspend the cell pellets in 0.9 mL of S30A+DTT buffer per gram of the pellets' weight. Pipette slowly to resuspend the cells, avoiding top froth as much as possible, if any.

2.4.3. Place 1 mL aliquots of the resuspended cells into 1.5 mL microtubes, and keep them on ice or a pre-chilled cold block at 4 °C (it is preferable to use metal blocks).

NOTE: If the last aliquot is much less than 1 mL, it is better to discard it than to sonicate a sub-optimal volume. The optimal volume (1 mL) for sonication was determined for this setup (tube, sonicator, and probe), and may vary for a different one.

2.4.4. Sonicate each tube in a sonicator (3 mm probe, frequency 20 kHz), with a setup of 20% amplitude for three cycles (30 s sonication, 1 min pause).

NOTE: The total energy delivered over the three cycles was ~266 Joules (~80–110 Joules per cycle). During this step, keep the tubes on the cold block that is surrounded by ice. Alternate between two cold blocks to avoid overheating of the probe (the standby cold block is also kept cold on ice). Both the cold blocks were pre-chilled in the fridge the day before the sonication.

2.4.5. Spin down the lysate at 12,000 x *g* for 10 min at 4 °C.

2.4.6. Collect the supernatant (cell lysate) with a pipette and transfer to a 50 mL tube.

2.4.7. Incubate the cell lysate at 37 °C at 200 rpm agitation for 80 min.

2.4.8. Spin down the lysate at 12,000 x *g* for 10 min at 4 °C.

2.4.9. Collect the supernatant and aliquot 30 µL each in pre-chilled 1.5 mL microtubes, while keeping all tubes on ice.

2.4.10. Flash-freeze the lysate aliquots on dry ice and store at -80 °C.

### 3. Cell-free buffer calibration for linear DNA

NOTE: Cell-free buffer was calibrated for optimal Mg-glu and K-glu concentrations as described in Sun et al.<sup>13</sup>. **Supplementary File 1** is needed for the calibration steps. Reactions were set to a final volume of 10.5 µL each. Extracts were calibrated using 1 nM of linear (see section 4 below) or plasmid DNA. The experiments can be performed on the same day the buffers are prepared, or the prepared buffers can be frozen at -80 °C to perform the experiment on another day. For all calibration steps, each component was thawed on ice before mixing and pipetting into a 384-well plate.

3.1. Prepare a Master Mix, according to the 'Buffer Preparation' tab in the Excel file **Supplementary File 1**, containing: (a) cell extract (33% of the total reaction volume), (b) reporter DNA (as linear and/or plasmid DNA) to a final concentration of 1 nM, (c) cell-free buffer (PEG8,000, AA solution, and energy solution), and (d) K-glu to a final concentration of 80 mM.

3.2. For a reaction volume of 10.5 µL, take 1.05 µL (10% total reaction) of different concentrations of Mg-glu 10x concentrated stocks (final concentration ranges: 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, and 20 mM) and add 9.45 µL (90% total reaction) of the Master Mix. Mix gently.

NOTE: Use PCR eight-strip tubes to prepare the dilutions and mix with a multichannel pipette. If needed, a different range of Mg-glu concentrations may be used.

3.3. Pipette 10 µL of the above reactions into a 384-well square-bottom microplate, cover it using an adhesive plate seal, and measure gene expression as fluorescence output. Record



fluorescence data with a plate reader (Ex: 485 nm; Em: 528 nm) at regular intervals (e.g., 5 min) for 8 h of incubation at 30 °C with continuous orbital shaking at 307 cpm.

NOTE: If needed, spin down the 384-well microplate (<2,500 x g, 1 min, room temperature) before starting data acquisition. End-point measurements were used to compare GFP expression in the different buffer compositions. The fluorescence values can be converted to standardized units for plotting (see below).

3.4. Identify the Mg-glu concentration that results in the highest fluorescence value at the end-point.

NOTE: If unsure about the optimal Mg-glu concentration obtained, repeat step 3.2 with more diverse concentration ranges.

3.5. With the optimized Mg-glu concentration, proceed to K-glu calibration for a range of concentrations (20, 40, 60, 80, 100, 120, 140, 160, 180, 200, 220, and 240 mM), using the same volumes indicated in step 3.2. Run the experiment and identify the highest fluorescence value from among the K-glu concentrations tested. This indicates the optimal buffer composition for Mg-glu and K-glu for this lysate.

NOTE: If unsure about the optimal K-glu concentration obtained, repeat the step with more diverse concentration ranges.

3.6. Once the Mg-glu and K-glu values are established, prepare stock tubes of the optimized buffer composition according to the batch volume obtained. Use the 'Buffer Preparation' tab in **Supplementary File 1** to calculate the number of buffer tubes needed, aliquot 38 µL per tube, and store at -80 °C.

#### 4. Linear DNA preparation

NOTE: For lysate calibration, plasmid P70a-deGFP is used. This plasmid was maintained in *E. coli* KL740 cl857+ (**Table 6**) for miniprep using the Plasmid Miniprep Kit, or maxiprep using the Plasmid Maxiprep Kit. Linear DNA fragments used as expression templates in the cell-free reactions were PCR amplified using the primers and templates listed in **Table 7** and **Table 8**.

4.1. PCR amplify from plasmid P70a-deGFP using DNA polymerase with oligo primers listed in **Table 7**. Set up the PCR reactions in a 50 µL volume, according to the manufacturer's protocol, using the thermocycler program: [98 °C for 2 min; 40 cycles x (98 °C for 30 s → 66 °C for 30 s → 72 °C for 60 s); 72 °C for 10 min; 4 °C for ∞].

4.2. Digest the template DNA in the PCR reactions by adding 1 µL of DpnI restriction enzyme per 50 µL PCR reaction and incubate at 37 °C for 1 h. Next, purify the PCR reaction using a PCR & DNA cleanup Kit and elute in nuclease-free water.

4.3. Verify the PCR products on a 1% agarose gel (1x TAE) prior to use.

4.4. Quantify the purified PCR products (or the plasmid preps) using Nanodrop (ND-1,000).

NOTE: If using a DNA polymerase/buffer that is directly compatible with the downstream cell-free reaction, the purification step (step 2) can be skipped entirely and the concentration of unpurified DNA measured by a fluorometric assay with DNA-binding dye instead (see Batista et al.<sup>10</sup>).

## 5. Experimental execution

NOTE: In addition to using **Supplementary File 1** to calibrate the buffer stock for each lysate batch prepared (above), it is recommended to use the 'Reaction Preparation' tab in the file to set up the subsequent cell-free reactions. As before, the reactions' volume is set at 10.5  $\mu$ L per reaction, of which only 10  $\mu$ L is finally pipetted into the 384-well plate for data acquisition. Here, 5 nM of each template is used for cell-free expression. It is important to be consistent when pipetting the reactions—use the same pipette and the type of tips. Dispense carefully to avoid any bubbles in the well or any liquid sticking to the walls of the well. If needed, spin down the plate (<2,500 x *g*, 1 min, room temperature).

5.1. Calculate the volumes to pipette by inputting the sample descriptions and replicates needed per sample into the 'Reaction Preparation' tab of **Supplementary File 1**.

NOTE: If a reaction volume other than 10.5  $\mu$ L is required, that can also be inputted into **Supplementary File 1**. The file will calculate the number of tubes of previously optimized buffer stocks and the cell extract tubes to be thawed on ice.

5.2. Label a 1.5 mL microtube for the Optimal Master Mix preparation (separately for linear and plasmid DNA), add the correct volumes of the Buffer and Lysate, and mix gently.

NOTE: Due to differences in optimal K-glu concentrations in their buffers, copies of the 'Reaction Preparation' tab should be made to be used separately for linear and plasmid DNA.

5.3. Pipette the DNA samples first, followed by nuclease-free water, and finally the Optimal Master Mix (MM) from step 5.2. Mix the cell-free reaction gently with the pipette just before adding to the plate reader and avoid any bubbles.

NOTE: Use a PCR eight-strip tube to mix the reaction volume for an additional replicate. For example, if technical triplicates are intended, prepare a mix for four reactions and leave a dead volume in the tube in order to reduce pipetting errors while adding the samples to the plate.

5.4. Set up the reactions in the plate reader (Ex 485 nm; Em 528 nm). Kinetic runs recorded fluorescence data at regular intervals (e.g., 5 min) for 8 h of incubation at 30 °C, with continuous orbital shaking at 307 cpm.

NOTE: End-point measurements were reported as the 8 h time-point. The fluorescence values collected are in arbitrary units (a.u.), but they can be converted to standardized units for plotting (see below).

## 6. FITC and GFP relative quantification

NOTE: GFP expression is exported by the plate reader in arbitrary fluorescence units (a.u.). However, it is recommended to use standardized units of measurement in order to compare fluorescence values between different settings (batches, equipment, users, and laboratories). Presented here are detailed steps to convert the fluorescence values (a.u.) to FITC-equivalent and eGFP ( $\mu\text{M}$ ) values, using standard curves of NIST-FITC and recombinant eGFP. Store NIST-FITC stock solution at 4 °C and store recombinant eGFP at -20 °C. Ensure that the stock solution and serial dilutions are protected from light. Upon delivery, it is recommended to aliquot the recombinant eGFP into smaller volumes to avoid multiple freeze-thaw cycles.

6.1. Prepare a solution of 100 mM sodium borate, pH 9.5, and store at room temperature or at 4 °C.

6.2. Prepare 12 dilutions (70  $\mu\text{L}$  each) for the standard curve, 2x per step (50, 25, 12.5, 6.25, 3.125, 1.562, 0.781, 0.390, 0.195, 0.097, 0.048, and 0  $\mu\text{M}$ ) of NIST-traceable FITC standard from the 50  $\mu\text{M}$  stock using the sodium borate solution. Prepare the dilution series in triplicate.

6.3. Similar to step 6.2, prepare serial dilutions (70  $\mu\text{L}$  each) from the recombinant eGFP using the sodium borate solution (1.2, 0.3, 0.075, 0.0188, 0.0047, 0.0012, 0.,0003, 0.,00007, and 0  $\mu\text{M}$ ). For molarity calculation, consider the full size of the protein (28 kDa) and the concentration of the purified eGFP (1 g/L). Prepare the dilution series in triplicate.

6.4. Add 20  $\mu\text{L}$  of each dilution (nine wells each = three dilutions series x three technical replicates) into a 384-well square-bottom microplate, covered by an adhesive plate seal, and incubate in a plate reader for measurement.

NOTE: Here, the settings used were Excitation: 485/20, Emission: 528/20, with gain 50. However, additional wavelength/gain combinations can also be used to calibrate for other fluorescent molecules and/or gain settings. To calculate the conversion factor, the same wavelength and gain settings are required to acquire the GFP fluorescence data from the cell-free experiments and the standard curve data.

6.5. Use the linear signal range (here, for example,  $[\text{FITC}] \leq 0.781 \mu\text{M}$ ) to fit the slope [ $y = ax$  and  $R^2$ ] for each condition.

NOTE: The range may differ for different machines and standard solutions used.

6.6. Save the data for FITC and eGFP calibration to calculate relative measurements for the lab by following the example in **Table 9**. Divide the values obtained in fluorescence arbitrary units (a.u.) by the curve slope “*a*” value ( $y = ax$ ) to estimate GFP production in terms of FITC or eGFP.

#### **REPRESENTATIVE RESULTS:**

Representative results are shown here after calibration of the lysate for optimal Mg-glutamate and K-glutamate levels separately for linear and plasmid DNA (**Figure 1**). The Mg-glutamate optimal concentration is similar across  $\Delta recB$  and  $\Delta recBCD$  extracts at 8 mM (**Figure 1B**). However, the optimal K-glutamate concentration for plasmid DNA is 140 mM, whereas the optimal K-glutamate concentration for linear DNA for the same extract is 20 mM (**Figure 1C**). In a comparative analysis of GFP expression in extracts from WT and  $\Delta recBCD$  cells, it was seen that the buffer composition of extracts must be specifically calibrated for optimal expression from linear and plasmid DNA used in exonuclease V deleted extracts.

After calibration steps, levels of GFP expressions were compared from linear and plasmid DNA (5 nM) in the WT and  $\Delta recBCD$  extract (**Figure 1D**). GFP expression from linear DNA reached 102% and 138% of the expression from plasmid DNA in extracts from  $\Delta recB$  and  $\Delta recBCD$  strains, respectively. Together, these results show that expression from linear DNA, using a native *E. coli*  $\sigma 70$  promoter, can reach the same level as that from plasmid expression when the cell lysate is prepared from such mutants and the cell-free buffer is specifically calibrated for linear DNA.

#### **FIGURE AND TABLE LEGENDS:**

**Figure 1: Differential buffer optimization for linear and plasmid DNA in  $\Delta recB/\Delta recBCD$  extracts.** (A) A 1361 bp linear DNA amplicon was amplified from plasmid p70a-deGFP and used as a template for gene expression for buffer calibration. (B) Mg-glutamate buffer calibration with 1 nM of each DNA type using different concentrations from 0 to 20 mM (with K-glutamate fixed at 80 mM). Square boxes indicate concentration points chosen. P = plasmid DNA, L = linear DNA. (C) After selecting the optimal Mg-glutamate concentration for each lysate, K-glutamate was titrated from 20 to 240 mM. Square boxes indicate concentration points chosen. P = plasmid DNA, L = linear DNA. Data shown in the heat-maps (B) and (C) are from a single experiment. (D) After buffer calibration, cell-free reactions were performed with 5 nM of each DNA type. Extract BL21 Rosetta2 does not support linear DNA expression, whereas differentially optimized extracts from  $\Delta recB$  and  $\Delta recBCD$  strains exhibit linear DNA (green) expression similar to plasmid DNA (blue) levels. Endpoint data shown are mean  $\pm$  SD for three replicates done on the same day and collected after 8 h of incubation.

**Figure 2: Visual representation of the protocol.** Workflow for cell lysate preparation and lysate optimization for linear DNA as a template in CFPS synthesis.

**Table 1: List of components for preparation of liquid and solid 2xYT+P media.** The quantity (in grams) of each component is specified for 1 L of media. Scale up proportionally as needed. Media must be autoclaved before use.

**Table 2: List of components for preparation of buffer S30A+DTT.** The quantity (in grams) of each component is specified for 1 L of buffer S30A. Scale up proportionally as needed. S30A buffer must be autoclaved and then chilled (4 °C) before use. The DTT buffer must be prepared and filtered separately as indicated (in 15 mL tube) and stored in -20 °C. Right before using buffer S30A, add 2 mL of DTT (from 1 M stock) to 1 L of S30A buffer.

**Table 3: List of components for energy solution preparation.** The quantity (in grams) or the volume of each component is specified (in column G) for the preparation of a specified volume (in column H) of stock solutions for the indicated concentration (in column F). The pH of each component must be adjusted if needed, as it is indicated (in column I and J). 14x energy solution stock is prepared by mixing the listed volume of each component (in column K) in the order listed.

**Table 4: List of components for preparation of amino acid stock solution.** For preparation of the 4x amino acid stock solution, all of the amino acids must be mixed in the order listed.

**Table 5. List of components for preparation of lysate calibration solutions.** The quantity (in grams) of Mg-glutamate and K-glutamate stock solutions are specified to prepare 50 mL of the indicated concentration of each buffer.

**Table 6: List of bacterial strains used in this protocol.**

**Table 7: List of primers used in this protocol.**

**Table 8: Plasmid used in this protocol for lysate calibration and as a template for linear DNA amplification by PCR.**

**Table 9: FITC and eGFP conversion.**

#### **DISCUSSION:**

Here, we show that cell lysate prepared from *E. coli* BL21 Rosetta2 with a genomic knockout for either *recB* or *recBCD* operon supports high protein expression from linear DNA templates. This protocol elaborates a step-by-step lysate calibration procedure specific for linear DNA templates (**Figure 2**), which is a critical step that leads to the high expression from  $\sigma_{70}$  promoters in linear DNA, reaching near-plasmid levels for equimolar DNA concentrations. This protocol highlights the importance of buffer calibration according to the type of DNA (linear or plasmid) to be used in the final application.

The protocol can help avoid plasmid cloning, which provides significant gains in time and cost. Linear DNA fragments can be synthesized by commercial providers and/or amplified by PCR in the laboratory. The same process using plasmid DNA would take at least 1 week longer, taking into account cloning, sequence verification, and DNA preparation steps<sup>10</sup>. It is recommended to prepare large volumes of the cell extract and linear DNA templates to minimize batch-to-batch variability between experiments<sup>14,15</sup>.

This method has been shown to be robust and used across laboratories<sup>10</sup>. Results from these extracts have been consistent across biological replicates, different extract batches, as well as across different strain backgrounds<sup>10</sup>. The robustness of the protocol has also been tested across different experimental parameters: cell lysis methods, temperatures, DNA templates, and different DNA concentrations. The method has already been used for rapid screening and characterization of genetic constructs for two relevant applications: toehold switch library characterization and enzyme activity screening<sup>10</sup>.

Although high expression levels were obtained in this study from linear DNA in  $\Delta recB/\Delta recBCD$  extracts from the highly productive parental strain *E. coli* BL21 Rosetta2, the methodology would require genome engineering for a new strain of interest. In contrast, supplementation with protective agents for linear DNA, like GamS<sup>5</sup> or Chi DNA<sup>6</sup>, is easier to apply for a new strain, but costlier and more time-consuming.

The protocol provided here will facilitate the use of linear DNA templates in cell-free systems and accelerate the design-build-test cycles for the synthetic biology community. Easier cell-free expression from linear DNA templates in  $\Delta recB/\Delta recBCD$  extracts can be deployed for the production of small molecules of interest, biosimilars, and other on-demand point-of-care applications.

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#### **DISCLOSURE:**

None

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