Journal of Visualized Experiments

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--Manuscript Draft--

Article Type:	Invited Methods Collection - JoVE Produced Video
Manuscript Number:	JoVE62728R1
Full Title:	Rapid, enzymatic methods for amplification of minimal, linear templates for protein prototyping using cell-free systems
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Additional Information:	
Question	**Response**
Please specify the section of the submitted manuscript.	Bioengineering
Please indicate whether this article will be Standard Access or Open Access.	Standard Access ($1400)
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TITLE:

Rapid, Enzymatic Methods for Amplification of Minimal, Linear Templates for Protein Prototyping using Cell-Free Systems

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SUMMARY:

The study describes a protocol for creating large (µg-mg) quantities of DNA for protein screening campaigns from synthetic gene fragments without cloning or using living cells. The minimal template is enzymatically digested and circularized and then amplified using isothermal rolling circle amplification. Cell-free expression reactions could be performed with the unpurified product.

ABSTRACT:

Here the protocol describes the design of a minimal DNA template and the steps for enzymatic amplification, enabling rapid prototyping of assayable proteins in less than 24 h using cell-free expression. After receiving a vendor, the gene fragment is PCR-amplified, cut, circularized, and cryo-banked. A small amount of the banked DNA is then diluted and amplified significantly (up to 10^6x) using isothermal rolling circle amplification (RCA). RCA can yield microgram quantities of the minimal expression template from picogram levels of starting material (mg levels if all starting synthetic fragment is used). In this work, a starting amount of 20 pg resulted in 4 µg of the final product. The resulting RCA product (concatemer of the minimal template) can be added directly to a cell-free reaction with no purification steps. Due to this method being entirely PCR-based, it may enable future high throughput screening efforts when coupled with automated liquid handling systems.

INTRODUCTION:

Cell-free gene expression (CFE) has emerged as a powerful tool with many applications. Such applications include disease detection[1–6], micronutrient and small molecule detection[7–12], biomanufacturing[13–18], education[19–21], manufacturing difficult proteins[17,22–27], and variant screening[23,28–33]. This is due to the open nature of cell-free systems and the flexibility they confer. Many great review articles offer historical education and future perspectives on the technology[34–44].

A typical cell-free reaction consists of three major components: cell extract, energy mix, and genetic template. Active cell extract contains all the necessary machinery for transcription and translation (TXTL) and can be processed in a variety of ways[36]. Glycolytic intermediates, electrolytes, amino acids, and cofactors in the energy mix support the TXTL process. It is a major source of variability in cell-free experiments[45] and can be prepared in many ways[34,46]. Preparation of the genetic template has seen fewer improvements since traditional cloning methods result in plasmids with excellent expression characteristics. The downside to these traditional methods is the turnaround time and amount of biological expertise needed to construct and propagate them. Recent optimization efforts have resulted in simple 24-hour workflows for cell extract preparation[47,48] that can be performed in parallel with energy mix preparation[49,50]. However, traditional cloning adds multiple days to the CFPS prototyping timeline (**Table 1**)[23]. Quickly amplified PCR products from the commercial gene fragment can be used directly[51], but this limits the number of prototyping experiments as only 1 µg of DNA is produced, which corresponds to approximately five reactions (traditional 15 µL volumes). With these additional steps of circularization and isothermal amplification, greater than milligram quantities of the DNA is possible (~5,000 reactions for 1 mg). This dramatically increases the number of tests that can be made in high-throughput screening of proteins or combinatorial enzyme networks (cell-free metabolic engineering); it also allows for effective preservation of the linear template library as high concentration DNA. Furthermore, an increased amount of template would be necessary to prototype larger quantities of protein needed for material science applications (protein-based fibers and hydrogels). Some limitations of linear templates can be overcome by using an extract from BL21 DE3 Star or using recently discovered methods to protect linear templates from degradation[52–54]. However, this does not address having limited stocks of vendor-produced DNA for PCR amplification or the issue of biological expertise and equipment needed for cloning.

This work presents a protocol explicitly designed to increase the amount of expression template that can be obtained from small quantities of vendor-produced gene fragments (typically 500–1000 ng of lyophilized powder). The described method does not require the skills necessary to perform traditional cloning in plasmids or transforming and propagating in living cells. Upon receiving a gene fragment in the mail, a user can produce enough templates for many cell-free reactions by employing isothermal rolling circle amplification (RCA) (**Figure 1**)[23]. While the amount of DNA received from the vendor may be enough for limited screening efforts, it is quickly depleted, and re-purchasing gene fragments is time consuming and costly. The method is also especially well-suited for genes that are toxic and difficult to clone in *E. coli*.

PROTOCOL:

1. Designing the gene fragment

NOTE: The gene fragment should have all the necessary genetic elements for transcription/translation, including promoter, ribosome binding site (RBS), start codon, the gene of interest, and terminator. While the terminator is not necessary for a linear expression template (LET), it will be important if the user decides to insert the sequence into a plasmid. These sequences were lifted from the pJL1-sfGFP plasmid[55] (gift from Michael Jewett's lab),

which uses a T7 promoter. In addition to these necessary genetic elements, a restriction enzyme cut site is added six base pairs before the promoter (5' cut site) and another six base pairs after the terminator (3' cut site), in this case using HindIII (other restriction enzymes can be used, but it is helpful to standardize the sequences with one high fidelity restriction enzyme to reduce the number needed to keep in the library). Primer sites are added ten base pairs upstream of the 5' cut site and ten base pairs downstream of the 3' cut site, in this case using standardized M13 primer sequences (primers are inexpensive stock items). The restriction enzyme site and primers used are at the discretion of the user. However, the user must ensure the sequences are not present anywhere else in the template (do not want to create unwanted cuts or sites of amplification initiation). The sequences for the templates used in this work are detailed in the supplemental material. These steps are used to modify from this base template.

1.1. Determine the desired gene to be expressed and obtain the amino acid sequence or the genetic sequence if it has been expressed in *E. coli*.

1.2. If it is an amino acid sequence, optimize the sequence codon for *E. coli* using one of many standard vendor tools[56]. If using the template provided in the supplement, ensure the optimized sequence has no HindIII restriction sites (AAGCTT). In the case that it does, continue to optimize the sequence until there is no longer a HindIII site.

1.3. Copy the sequence and paste it into the provided template for **Supplementary Sequence #1** where the gene of interest is indicated. If expressing sfGFP, use **Supplementary Sequence #1** as is. If expressing subtilisin, use **Supplementary Sequence #2** as is.

1.4. Order the minimal template and the necessary primers from the preferred DNA synthesis service.

2. Resuspending the gene fragment and the primers

NOTE: Upon receipt of the gene fragment, follow the manufacturer's protocols for resuspension or use this simple guide to create a DNA stock.

2.1. Centrifuge the tube (300 x *g* for 5 s) to collect the DNA pellet at the bottom.

2.2. Add double distilled water (ddH_2O) to make the final concentration to 10 ng/µL of DNA template.

2.3. Vortex the solution on a medium setting for 5–10 s.

2.4. Dissolve the entire pellet by incubating at 50 °C for 20 min.

2.5. Briefly vortex again

2.6. Centrifuge at 300 x *g* for 5 s to collect the solution at the bottom of the tube.

2.7. Store at -20 °C or use in PCR.

2.8. Prepare a 100 µM primer stock by resuspending the primers in nuclease-free water. To determine the amount of water to add, multiply the nanomolar amount of lyophilized primer by 10. For example, if the tube contains 45 nM of lyophilized primer, add 450 µL of ddH_2O and vortex the solution.

2.9. Store the primer stock solutions at -20 °C or continue to perform the amplification.

3. Amplifying the gene fragment via PCR

NOTE: Decide which PCR is right for the gene of interest. Smaller genes (<1,000 kb) may be more amenable to a cheaper Taq polymerase, while larger genes (≥1,000 kb) may benefit from high fidelity polymerase to reduce errors. It is important to note that this initial PCR amplification is not necessary if the user is not concerned with preserving the initial gene fragment (It provides multiple attempts at circularization and allows for comparative studies of LET vs. RCA product). It is also important to note that this PCR amplified LET can be used directly in reactions; however, as mentioned in the introduction, it would only allow for a limited number of reactions if the further amplification steps were disregarded. Digestion and ligation can be performed on the resuspended gene fragment directly[57] (if one is certain, they will not need more LET to perform additional circularization stocks). If this is the case, skip section 3 and continue to section 4. For performing PCR, follow these steps.

3.1. Use the 100 µM stocks from step 2.8 to create 10 µM working solutions. Many PCR kit protocols call for 10 µM solutions of primers.

3.2. Program the thermal cycler to conduct the reaction according to the kit manufacturer's protocols. Different kits call for slightly varied cycling parameters. For the kit listed in the **Table of Materials**, the conditions are 94 °C for 30 s of initial denaturation; 30 cycles of 94 °C for 30 s of denaturing, 45 °C for 30 s of primer annealing, and 68 °C for 60 s of extension; with a final extension at 68 °C for 5 min; and finally, a 10 °C indefinite hold.

3.2.1. Ensure to select the correct elongation time (variable depending on the length of the gene to be amplified). Have an elongation time of 1 min for every 1,000 bp.

3.2.2. Ensure to enter the correct annealing temperature for the primers. Use an online Tm calculator that uses both primers as inputs to determine the best annealing temperature[58]. An annealing temperature of 45 °C is sufficient when using M13 primers.

3.2.3. When determining the number of cycles, refer to the manufacturer's protocol, but 30 cycles will most often result in sufficient amplification.

3.3. If performing PCR, thaw and vortex the dNTPs. Use the PCR buffer provided in the kit.

3.4. In a single PCR tube, combine all the kit components as directed in the manufacturer's protocol. To ensure successful amplification, add 1 µL of resuspended DNA stock (step 2.6).

3.5. Gently homogenize the mixture by vortexing on medium setting for 5–10 s. Alternatively, pipette half the volume up and down 10–20 times to vortex.

3.6. Perform the PCR reaction.

3.7. If the PCR protocol did not include a final cooling step, allow the reaction to cool for 5 min at 10 °C before removing to drive condensation to the bottom of the tube.

3.8. Purify the reaction using a PCR clean-up kit following the vendor's instructions.

3.8.1. In a 1.5 mL tube, add DNA binding buffer and PCR sample at a ratio of 5:1, respectively.

3.8.2. Transfer this mixture to the spin column and centrifuge at 16,000 x *g* for 1 min. Discard the flow-through.

3.8.3. Add 200 µL of DNA wash buffer to the column and incubate at room temperature for 1 min.

3.8.4. Centrifuge for 1 min at 16,000 x *g* and discard the flow-through.

3.8.5. Repeat steps 2.8.3 and 2.8.4 without the 1 min incubation step.

3.8.6. Centrifuge for an additional 1–2 min at 16,000 x *g* to remove any remaining buffer.

3.8.7. Elute the DNA in 46 µL of ddH_2O.

3.9. Quantify the purified DNA using a spectrophotometer.

3.10. Store the purified DNA at -20 °C or proceed to the next step.

4. Digestion and circularization

NOTE: Further amplification can be achieved by circularizing the DNA followed by RCA. Digest the DNA to prepare the template for circularization. This will remove the primer sequences and create sticky ends at both the 5' and 3' ends of the template. Reattach these ends via ligation reaction.

4.1. In a PCR tube, combine 5 µL of the necessary buffer, 20 U of HindIII, and 45 µL of the purified DNA from step 3.8.

4.2. Gently homogenize this mixture with a pipette.

4.3. Incubate the mixture in a thermal cycler for 15 min at 37 °C.

4.4. Heat inactivate HindIII by incubating for 20 min at 80 °C.

4.5. Allow the reaction to cool to 10 °C before removing to drive condensation to the bottom of the tube.

4.6. Add 5 µL of T4 ligase buffer and 800 U of T4 ligase to the newly digested DNA.

4.6.1. Use T7 ligase, if desired.

4.7. Gently homogenize this mixture with a pipette.

4.8. Incubate the mixture for 1 h at 25 °C to perform the circularization reaction.

4.9. Purify the reaction using a PCR clean-up kit following the vendor's instructions. Use the same protocol detail in step 3.8.

4.10. Quantify the DNA using a spectrophotometer. The expected values are ~20 ng/µL.

4.11. Store at -20 °C or proceed to the next step.

5. Isothermal rolling circle amplification

NOTE: The Rolling circle amplification (RCA) can be performed using a commercial kit or with individually purchased components. Following the manufacturer's protocol will ensure a successful amplification. Kits typically contain a sample buffer, reaction buffer, and strand displacing polymerase, such as ϕ29 polymerase. Multiple reaction tubes can be combined to produce a large amount of DNA for cell-free expression (4 µg from 20 pg of starting material). The following protocol works efficiently.

5.1. In a single tube, combine 20 µL of the sample buffer, 20 µL of the reaction buffer, 0.8 µL of the enzyme, and 1 µL of the circular expression template (CET) from step 4.9.

NOTE: This will have a total DNA mass of ~20 ng, but RCA can work with picogram amounts, thus allowing the dilution of the CET and extreme enzymatic amplification if there is a significant need for material in the high throughput screening.

5.2. Homogenize the mixture with a pipette and aliquot 10 µL of the mixture into four separate tubes.

5.3. Incubate at 30 °C for 4–18 h.

5.4. Heat inactivate the enzyme by incubating at 65 °C for 10 min. Reduce the temperature to 12 °C for 5 min to encourage condensation at the bottom of the tube.

NOTE: It's easiest to combine all temperature steps in an automated protocol on a thermal cycler.

5.5. Dilute the resulting solution by adding 15 µL of ddH_2O to each tube.

5.6. Combine all tubes and add directly to a cell-free reaction. Use a PCR clean-up kit to purify the product and elute it in 36 µL of ddH_2O to quantify. Ensure that the template concentration is ~100 ng/µL.

6. Cell-free reaction

NOTE: Perform cell-free expression by combining energy buffer, extract, and RCA template. A typical cell-free reaction using the PANOx-SP energy buffer consists of 1.2 mM ATP, 0.85 mM each of GMP, UMP, and CMP, 30 mM phosphoenolpyruvate, 130 mM potassium glutamate, 10 mM ammonium glutamate, 12 mM magnesium glutamate, 1.5 mM spermidine, 1 mM putrescine, 34 µg/mL of folinic acid, 171 µg/mL of *E. coli* tRNA mixture, 2 mM each of 20 unlabeled amino acids, 0.33 mM NAD, 0.27 mM Coenzyme A (CoA), 4 mM potassium oxalate, 57 mM HEPES-KOH buffer (pH 7.5), 0.24% volume of the *E. coli* extract, and variable amounts of DNA[23,49]. The volume of reaction can vary but 15 µL reactions can save on reagent usage and are small enough for use in a 384 black-walled microplate[49,50].

6.1. If expressing a fluorescent protein such as sfGFP, prepare a plate reader to read at the desired excitation/emission, temperature, and agitation.

6.2. If using a 384-well plate, aliquot 60 µL of H_2O into the wells bordering an empty sample well to maintain humidity and reduce the edge effect.

6.3. Add the various required components into a tube for each sample. Add enough to perform triplicates. Replicates within the plate can help identify causes of variability.

6.3.1. Add the extract, the energy buffer and then the DNA.

6.3.2. Dilute to the final desired volume with ddH_2O.

6.4. Mix this solution thoroughly by pipetting half of the solution volume up and down 10–20 times.

6.5. Transfer the reaction mixture in 15 µL aliquots to the desired wells in the microtiter plate.

6.6. Seal the plate with a colorless sealing film to maintain humidity and prevent evaporation.

6.7. Place the sealed plate in the plate reader and allow the reaction to complete.

6.7.1. If expressing a protein that does not have the capability of being monitored live, use another temperature-controlled apparatus such as a thermoblock to incubate the plate.

7. Subtilisin assay

NOTE: If expressing the subtilisin BPN' (SBT(n)) gene in **Supplementary Sequence #2**, follow this protocol to assay the activity.

7.1. Prepare a 10 µM stock solution of N-succinyl-Ala-Ala-Pro-Phe p-nitroanilide in dimethylformamide (DMF).

7.2. Set a plate reader to measure absorbance at 410 nm every 20 s for 10 min while maintaining a temperature of 25 °C.

7.3. In a flat bottom, colorless 96-well plate, aliquot 94 µL of ddH_2O and 1 µL of N-succinyl-Ala-Ala-Pro-Phe p-nitroanilide from step 7.1.

7.4. Add 5 µL of the finished cell-free reaction from step 6.7 and read using a plate reader set to the protocol described in step 7.2.

REPRESENTATIVE RESULTS:

Expression of sfGFP from RCA templates was comparable to that of the pJL1 plasmid when using only 0.30 µL of unpurified RCA DNA in a 15 µL reaction (**Figure 2A**). In fact, doubling and tripling the amount of template appears to offer no benefit in BL21 DE3 Star extract, suggesting already saturated levels of the template at 0.30 µL per reaction. Conversely, there appears to be a benefit to increasing the amount of RCA template when added to cell extract sourced from the SHuffle strain (**Figure 2B**)[28]. For some proteins, results can be observed very quickly, which compresses the entire workflow (amplification and assaying) to under 24 h. However, some proteins require lower temperature or have slower folding times, which will increase the time until results are obtained but will affect the workflow presented here. This can be observed when expressing subtilisin (SBT(n)) where assaying after 4 h of expression was not long enough for SBT(n) maturation (**Figure 3A**, example of a failed result). Allowing the reaction to continue to 16 h can lead to detectable levels of SBT(n) (**Figure 3**B). This improvement may be temperature-dependent, as observed in literature where optimized temperature conditions were explored[59,60].

FIGURE AND TABLE LEGENDS:

Figure 1: A representative schematic of the minimal genetic template and the process it undergoes after the initial PCR amplification step. The templates are digested with HindIII, circularized with T4 ligase, and further amplified with ϕ29 polymerase to create large concatemers.

Figure 2: Results of the cell-free reactions with unpurified 5 nM of plasmid (pJL1), 5 nM of linear template (LET), and varying concentrations of unpurified RCA. RCA #1, #2, and #3 contained 0.3 µL, 0.6 µL, and 0.9 µL (respectively) of unpurified RCA product in a 15 µL reaction incubated at 30 °C (n = 3). Error bars represent ± 1 SD from the mean. The y-axis is fluorescence, and the x-axis is the time that has passed during the reaction. The kinetics of sfGFP expression are represented in (**A**) BL21 DE3 Star and (**B**) T7 SHuffle.

Figure 3: Cell-free reactions with 5 nM of SBT(n) LET and varying concentrations of unpurified RCA product. RCA ng and pg correspond to the concentration of the DNA used to perform rolling circle amplification. Unpurified RCA product was used in a 15 µL reaction incubated at 30 °C (n = 3). Error bars represent ± 1 SD from the mean. The y-axis is the absorbance at 410 nm, and the x-axis is the amount of time that has passed in the assay. Reactions were performed for (**A**) 4 h and (**B**) 16 h.

Table 1: A comparison of the timeline between a simplified traditional cloning protocol and the RCA protocol covered herein.

Supplemental File: The supplemental file lists the sequences. Sequence #1 is sfGFP (999 base pairs) and sequence #2 is subtilisin BPN' (1344 bp).

DISCUSSION:

The gene of interest can be any desired protein, but it is best to start with a fluorescent protein as a convenient reporter for real-time or end-point readout on a well plate reader for new adopters of this method. For new protein sequences, copy the amino acid sequence of the desired protein and paste it into the desired codon optimization tool[61,62]. There are usually many available organisms and strains of *E. coli* in the codon optimization tool, but choosing the general *Escherichia coli* option will be suitable. After optimization, double-check the gene to ensure the previously chosen cut site and primer sequences are not present. If so, the sequence can be optimized until the sequences are no longer present. In some situations, it may be necessary to replace the HindIII cut sites with a different restriction site if repeated optimization consistently results in an internal HindIII cut site. It is best to use the same cut site as often as possible to keep the process standardized and reduce the cost of keeping multiple restriction enzymes on hand.

Before beginning amplification, choose the PCR kit that best suits the LET. An LET with <1,000 base pairs can be amplified with a simple Taq polymerase while an LET with ≥ 1,000 base pairs may require a higher-fidelity polymerase[63]. Set up the protocol for amplification according to the kit manufacturer and the annealing temperature of the desired primers. The annealing temperature is critical to a successful amplification. A general rule of thumb is to select an annealing temperature that is 5 °C lower than the Tm of the primers. There are free online tools that will provide an optimized annealing temperature based on the sequence of the primers[58]. Using the correct annealing temperature is critical to producing high-quality DNA template.

Cell-free gene expression has seen a renaissance in recent years due to its speed, simplicity, and utility for synthetic biology prototyping. This work has outlined a method to increase the speed

and ease when testing a large library of new, functional proteins. While traditional cloning methods can take days or weeks, this protocol can be done in less than 24 h. **Table 1** outlines typical time ranges for both traditional cloning and the RCA protocol. Note that the cloning protocol has been simplified, and some optional steps have been left out, such as gel isolation. **Table 1** also refers only to the common restriction digestion protocols; there are many other cloning methods, but these require a similar amount of time. The upper range of this enzymatic amplification protocol requires less time than the lower range of the cloning method, especially when considering an 8-hour workday. This is largely due to the removal of overnight incubations and lack of sequencing confirmation. The RCA protocol is based entirely on PCR and RCA, which require less specialized skills than traditional cloning, transformation, and cell culture techniques. This method makes cell-free expression accessible for labs that have no prior experience in cloning or access to capital necessary to transform and grow cells. This method is also well suited for projects focused on cytotoxic proteins, where cloning and propagation of the genes with traditional cloning is difficult due to toxicity in the living cell. This RCA protocol is also capable of amplifying very small amounts of circular DNA (picograms of DNA) to the levels necessary for CFE. In **Figure 3B**, the circularized SBT(n) template was diluted to 20 ng/µL and 20 pg/uL prior to RCA. While the observed rates of degradation were different, both reactions resulted in the same amount of substrate degradation within 10 min. The proposed method is not meant to replace cloning; plasmid propagation can produce quantities of DNA for non-toxic genes that cannot be matched. Rather, this is a convenient prototyping tool at a massive library screening phase (enzymatic steps are amenable to automation with standard fluid handler) that would help identify which sequences should be cloned for archiving and further propagation.

When designing a cell-free reaction, there is much flexibility, but there are some factors that must be taken into consideration to ensure success. Smaller reactions have a greater surface area to volume ratio, which is great for gas exchange, but the reaction needs to completely cover the bottom of the well for accurate fluorescence measurements. The temperature of the reaction can also vary depending on the gene of interest[59]. There are multiple choices for users when it comes to the selection of an energy buffer[34,46]. Some recipes are more expensive than others, but the decision is left to the user. There are also many potential sources for *E. coli* extract[36]. Users should familiarize themselves with extract production protocols and decide which is best for their purposes[28,48,50,64–67].

When using RCA to amplify templates for cell-free expression, the choice of bacterial strain is very important. The expression profile tends to be better than that of LETs. The popular BL21 DE3 Star strain handles this well, with RCA performing ~1.4x better than LET and the pJL1 plasmid performing ~1.2x better than the best RCA concentration (**Figure 2A**). On the other hand, some strains exhibit template degradation and perform poorly due to the presence of native nucleases[23,28]. In this case, it appears that the SHuffle strain benefits from an increased RCA template. Previous literature has shown that SHuffle extract does not perform well with PCR products, but the highest concentration of unpurified RCA product used in this study outperformed the pJL1 plasmid (**Figure 2B**). The expression of functional SBT(n) (**Figure 3**) is an example of a cytotoxic protein that is conveniently made by cell-free but difficult to prototype in living cells due to toxicity (unable to clone this expression template in plasmid and propagate in

E. coli). Unlike sfGFP, SBT(n) activity cannot be observed after only 4 h of expression (**Figure 3A**). The signal is detectible after 16 h of expression and maturation (**Figure 3B**). The unpurified DNA used in these examples came from 100 µL, which was four 11 µL RCA reactions diluted and combined. This one stock can support 333 cell-free reactions if only using 0.30 µL per reaction.

The compatibility of RCA templates with CFE needs to be further explored with various extracts. Potential complications with linear products may be alleviated by adding short oligos containing the *E. coli* chi sequence or the GamS protein[52,53]. Literature suggests that the addition of chi oligos can provide greater protection to linear templates than the GamS protein[53]. If using an extract known to provide low yields from linear templates, the user should analyze the cost of each protective agent and use the one that best suits their purposes. Another limitation is the inability to convert RCA template concentrations to molarity due to the nature of the resulting concatemer. This means one can add the same number of minimal templates based on mass, but they will have varying concatemer lengths, which might affect expression levels. The authors have not found this to be an issue in screening/prototyping as the averaged expression levels from each well are the same (low variance) but could be an issue if expressing in smaller volumes (e.g., microdroplets).

Cell-free gene expression has the potential to be used as a significant tool in protein prototyping and design-build-test workflows. However, most cell-free expression workflows still rely on traditional plasmids as the genetic template. This slows down the process and keeps cell-free expression from being utilized to its fullest extent for screening/prototyping purposes. Amplifying templates and subsequently performing RCA can quickly produce enough genetic templates for many reactions, producing enough protein for downstream characterization and functional testing.

ACKNOWLEDGMENTS:

The authors acknowledge NIH 1R35GM138265-01 and NSF 2029532 for partial support of this project.

DISCLOSURES:

Nigel Reuel serves on the scientific advisory board of BigHat Biosciences Inc., a company that uses cell-free systems for the design of antibodies.

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Figure1

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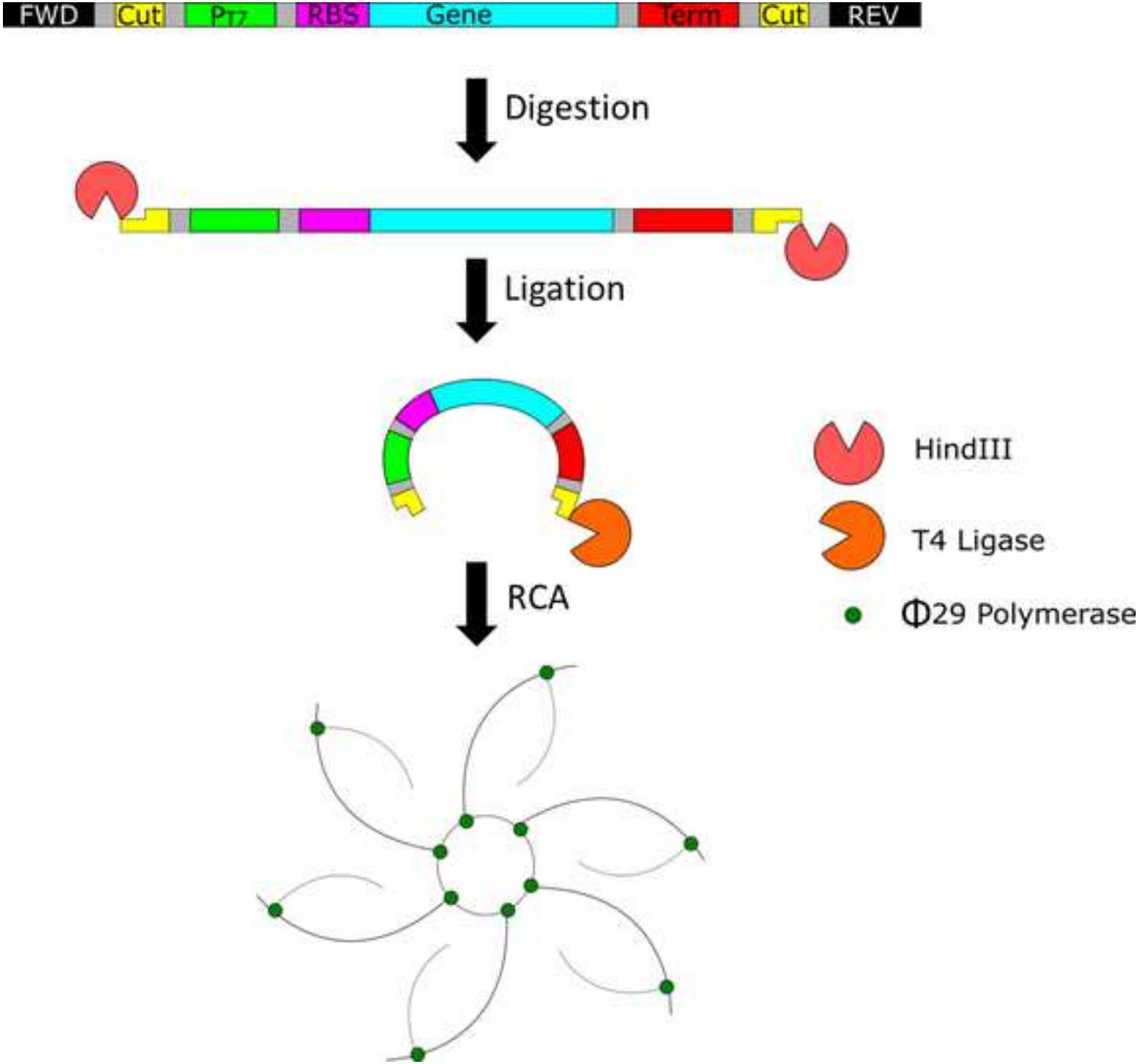

Figure2

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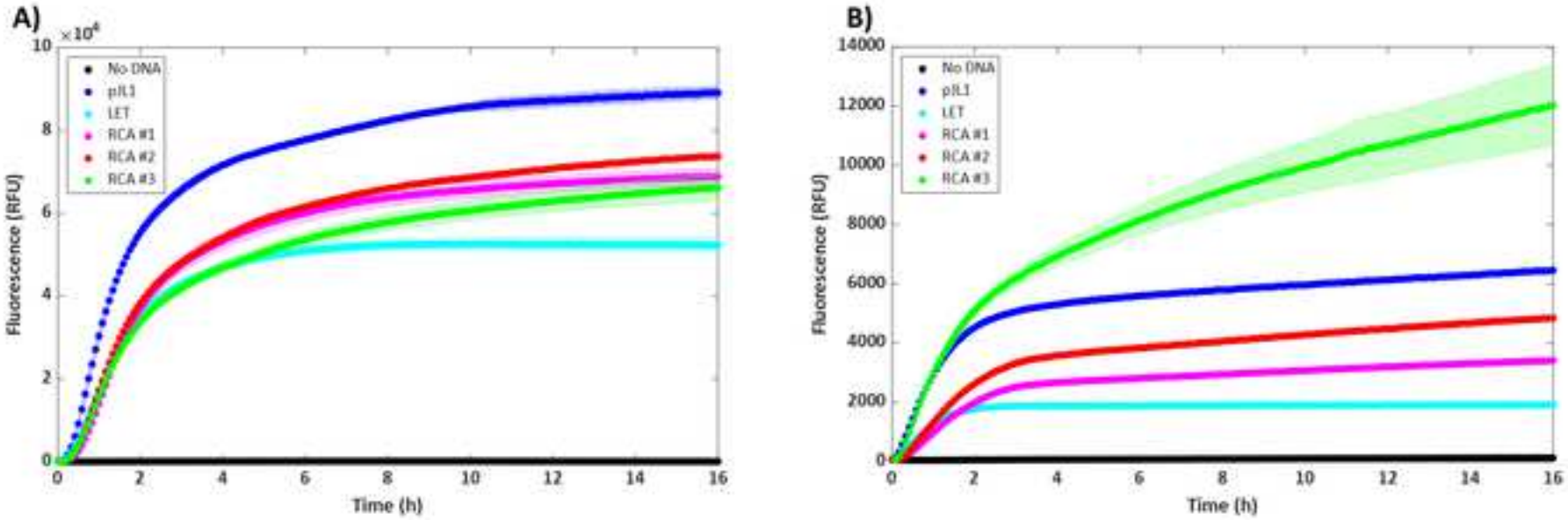

Figure3

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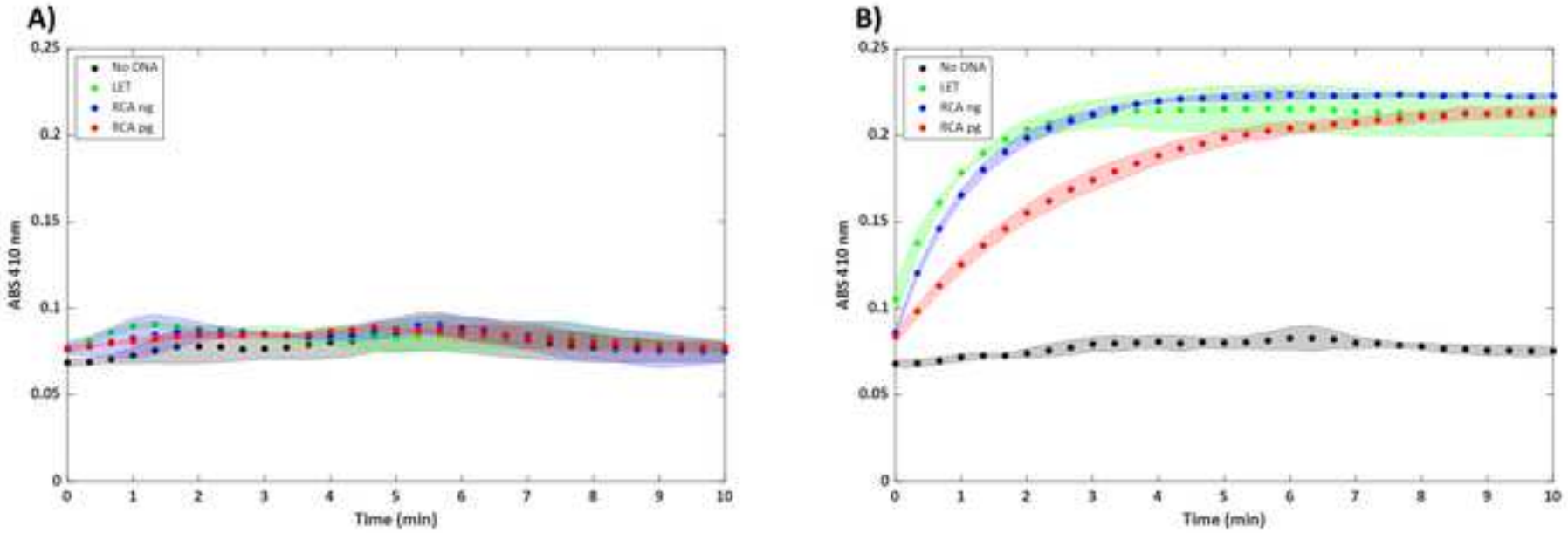

Table1

	Cloning Method
PCR	2 -4 h
Plasmid Digestion	35 min
Ligation	1 h
Transformation	2 h
Overnight Incubation	16 h
Sequencing	24 - 48 h
Glycerol Stock Prep	16 h
Growth and Purification	16 h
Total Time	46 - 72 h

	RCA Method
PCR	2 - 4 h
Digestion	35 min
Ligation	1 h
RCA	4 - 18 h
CFE	4 - 16 h
Total Time	12 - 40 h

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Alaline	Formedium	DOC0102	
Ammonium glutamate	MP Biomedicals	MP21805951	
Arginine	Formedium	DOC0106	
Asparagine	Formedium	DOC0114	
Aspartic Acid	Formedium	DOC0118	
ATP	Sigma	A2383	
Axygen Sealing Film	Corning	PCR-SP	
CMP	Sigma	C1006	
Coenzyme A	Sigma	C3144	
CutSmart Buffer	NEB	B7204S	Provided with HindIII
Cysteine	Formedium	DOC0122	
DNA Clean and Concentrator Kit	Zymo Research	D4004	Used for purifying DNA
dNTPs	NEB	N0447	
E. coli tRNA	Sigma (Roche)	10109541001	
Folinic Acid	Sigma	47612	
Gene Fragment	IDT		
Glutamic Acid	Formedium	DOC0134	
Glutamine	Formedium	DOC0130	
Glycine	Formedium	DOC0138	
GMP	Sigma	G8377	
HEPES	Sigma	H3375	
HindIII-HF	NEB	R3104L	
Histidine	Formedium	DOC0142	
Isoleucine	Formedium	DOC0150	
Leucine	Formedium	DOC0154	
Lysine	Formedium	DOC0158	

Magnesium glutamate	Sigma	49605	
Methionine	Formedium	DOC0166	
Microtiter Plate (384 well)	Greiner	781906	
Microtiter Plate (96 well)	Greiner	655809	
Multimode Plate Reader	BioTek	Synergy Neo2	
NAD	Sigma	N8535	
NanoPhotometer	Implen	NP80	
OneTaq DNA Polymerase	NEB	M0480	
PCR Tube	VWR	20170-012	
Phenylalanine	Formedium	DOC0170	
Phosphoenolpyruvate	Sigma (Roche)	10108294	
Potassium glutamate	Sigma	G1501	
Potassium oxalate	Fisher Scientific	P273	
Proline	Formedium	DOC0174	
Putrescine	Sigma	P5780	
Serine	Formedium	DOC0178	
Spermidine	Sigma	S0266	
T4 DNA Ligase	NEB	M0202S	
T4 DNA Ligase Reaction Buffer	NEB	B0202S	Provided with T4 DNA Ligase
TempliPhi Amplification Kit	Cytiva	25640010	Used for RCA
Thermal Cycler	Biorad	C1000 Touch	
Thermoblock	Eppendorf	ThermoMixer FP	
Threonine	Formedium	DOC0182	
Tryptophan	Formedium	DOC0186	
Tyrosine	Formedium	DOC0190	
UMP	Sigma	U6375	
Valine	Formedium	DOC0194	

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May 5, 2021

Dr. Amit Krishnan
Review Editor
Journal of Visual Experiments

Dr. Amit Krishnan,

We are submitting our significantly revised and expanded manuscript entitled 'Rapid, enzymatic methods for amplification of minimal, linear templates for protein prototyping using cell-free systems,' authored by Jared L. Dopp and myself. The previous reviews correctly highlighted our inadequate job of formatting according to author guidelines; one reviewer also questioned why this method was needed when there are existing cloning methods the result in large amounts of DNA. In this revised manuscript, we have expanded our protocol and we have carefully reworked the text and title to focus on the purpose of this new method – that it is a simple, functional process that will enable those without cloning experience to produce large quantities of DNA. Moreover, we have expanded the results and discussion section to include an example of a cytotoxic protein (subtilisin BPN'), an example that was amplified from only 20 pg of starting material, and expression in another source of cell extract. This work is an in-depth demonstration of a process we have previously published in *Biotechnology and Bioengineering* [1].

We are confident this improved manuscript will be of significant interest to your readership; it can be applied in the many growing applications of cell free systems (sensors, pathway prototyping, production of cytotoxic products, etc.). We hope that this protocol will be of use to those who not only wish to express toxic proteins, but also to those who wish to perform large combinatorial screening efforts that may require many iterations. In this way, researchers may be able to save time and money by limiting the number of stock gene fragments purchased.

We have provided an itemized response to the reviewers (copied below for your convenience) as well as a manuscript marked with changes. We look forward to hearing back on your editorial decision and are happy to make further edits you deem necessary.

Please contact me with any questions.

Sincerely,

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1. Dopp JL, Rothstein SM, Mansell TJ, Reuel NF (2019) Rapid prototyping of proteins: Mail order gene fragments to assayable proteins within 24 hours. *Biotechnology and Bioengineering*, 116(3):667–676. https://doi.org/10.1002/bit.26912

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

2. Please provide the complete address of the affiliation.

Added

3. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

All these pronouns have been removed.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.

We have removed all commercial labeling and moved them to the list of reagents.

For example: Roche, etc.

All have been removed and listed in the table of reagents.

5. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes.

The manuscript has been formatted to fit this requirement.

6. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.

We have removed the paragraphs and broken everything down into steps.

7. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

We have reworded the manuscript to use this language.

8. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g., button clicks for software actions, numerical values for settings, etc.) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

We have simplified our steps and added much more information

9. Line 63-83/99-113/116-130/132-138/156-165: The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion. Please ensure that the Protocol section consists of numbered steps. We cannot have non-numbered paragraphs/steps/headings/subheadings.

These sections have been heavily reworked. Paragraphs introduce each section, but do not contain the steps. Steps are exclusively numbered.

10. For SI units, please use standard abbreviations when the unit is preceded by a numeral throughout the protocol. Abbreviate liters to L to avoid confusion. Examples: 10 mL, 8 µL, 7 cm2

We have implemented this change.

11. For time units, please use abbreviated forms for durations of less than one day when the unit is preceded by a numeral. Do not abbreviate day, week, month, and year. Examples: 5 h, 10 min, 100 s, 8 days, 10 weeks (Lines: 88, 94, 96, etc.)

We have implemented this change.

12. Please include a one-line space between each protocol step and then highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

The spaces have been added and we believe 3 pages of steps have been highlighted.

13. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

b) Any modifications and troubleshooting of the technique

c) Any limitations of the technique

d) The significance with respect to existing methods

e) Any future applications of the technique

14. Figure 2: Please revise the x-axis legend in Figure 2A to “Time (h)” instead of “ Time (hr)” and the figure labels in Figure 2B to “4 h” and “16 h”.

This has been adjusted.

15. Please sort the Table of Materials in alphabetical order.

The table has been sorted.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This paper reported an RCA-based method for the preparation of template DNA used for cell-free protein synthesis. This protocol paper is mainly based on a previous publication (Ref. 13) by the same group. The primary claim is that template DNA preparation with RCA is easier than traditional molecular cloning. However, many cell-free systems, particularly those based on E. coli, are compatible with linear DNA, which means that a rapid PCR reaction without additional time-consuming RCA is enough for producing

a large amount of template DNA from minimal starting materials. I cannot find any advantage in applying their proposed method instead of using a conventional linear DNA template. Besides, JoVE requests providing negative results in the Representative Results; however, the authors completely missed this point. Due to the poor writing, confusing logic, few advantages over existing methods, and lack of research integrity, I think this paper should not be accepted for publication in JoVE. I would like to offer my comments and suggestions to the authors for improving the paper in the future.

Major Concerns:

First, to my knowledge, the writing style for the protocol does not follow JoVE's format, making reproducing the experiment difficult. Too many experimental details were missed, and some important experiment parameters were too broad. Second, the authors made many points without explicit support from experimental results or references. Some arguments that would be important for the whole story are very vague and unspecific. These issues are all fatal for a research paper, even without considering the novelty. For example, "assayable quantities" in the Summary cannot tell the reader any useful information about the quantity. The authors must specify the quantity required by specific assays as different assay schemes must require highly different quantities of protein samples. Also, no direct experimental results supported "this can yield microgram quantities of the minimal expression template from picogram levels of starting material" mentioned in the Abstract. Other significant issues are also listed as the following:

We have reformatted the manuscript to follow the author guidelines provided by JoVE and made the protocol easier to replicate. Regarding DNA concentration we have reported this in the revised manuscript. In response to novelty, this is the purpose of JoVE, taking a previously published method and contributing it again in a visual format. We were asked to present this work specifically by the special issue editors and notified that novelty was not a necessity for the journal (although it does show good consistency in our results, as this data was all generated new for this manuscript).

Line 31-34: The authors missed a lot of representative studies published previously.

Thank you for the suggestion. We have added multiple references that readers can use to educate themselves on these applications. Let us know if some seminal publication has been missed. The new line numbers are 37-41

Line 46-47: Add a reference to support the claim "Extract and energy mix can be prepared in parallel within 24 hours."

We have reworded this phrase to make it seem less misleading to a new practitioner. We have also added references to support the claim of a 24-hour workflow of cell growth to protein expression. We have also added references for energy mix preparation. While there is no amount of time listed in the literature for energy mix preparation, we find it possible to make while growing cells. These new lines are 52 and 53.

Line 47: Linear DNA template can be used in a variety of cell-free expression systems. The authors should make their claim more specific.

While it is true that linear DNA can be used in many cell-free expression systems, the yield does suffer. Expression from linear templates in most cell-extracts results in a fraction of the protein produced when using plasmids. There are a few ways around this, but it generally requires extra steps and extra cost to the user, such as the use of chi containing oligos and Gam protein. This is also already addressed in the text and supported by the listed references. Please see Fig 2B for an observation.

Line 55-57: The template of 500-1000 ng is generally enough for prototyping works. The authors should first state the reason somewhere for why we need to increase the amount of template DNA.

Thank you for pointing this is out because this is a distinction that we wished to make but were not successful in making clear in this work. It is common to work with genes that are toxic and cause

problems during cloning (enzymes, antimicrobial peptides, etc.). We have added an explanation at the end of the introduction section. Also, this helps preserve the initial gene fragment stock. It only requires 1 microliter to undergo a PCR that results in microgram quantities of product. Since, RCA can be used from picrogram amounts, this can result in thousands of cell-free reactions. This becomes very useful in large combinatorial screening campaigns. See lines 54-63

Line 67: The full name of the principal investigator should be provided.

We have entered Michael Jewett's full name to avoid any confusion. We have followed typical practice of using PI last name and reference but leave it to editor to determine their preferred format.

Line 75-76: The protein-coding sequence of pJL1-sfGFP plasmid has already been optimized and demonstrated for protein expression using E. coli extract. Why did you need to re-optimize the sequence again in this study?

We did not optimize the sequence from the pJL1 plasmid but used the expression cassette verbatim in our minimal template. We suggest new users hone their skills with an easy-to-use reporter such as sfGFP. We also highlight use a codon optimization tool. We have reworked these sentences so that they are easier to understand.

Line 77-78: The information regarding the tool that was exactly used in this paper should be provided.

We use the IDT codon optimization tool but are not allowed to list companies in the manuscript as per JoVE's policies. We have cited them, and they are now in the references section of the manuscript.

Line 79-80: Why not optimizing the sequence prior to determining the restriction site? Some bioinformatic tools also support excluding the undesired restriction sites during the sequence optimization.

This is a good point and we have included it in the discussion. Since this is a methods paper and we recommend using our template before designing your own, we suggest keeping the HindIII restriction site. Trying to make this simple to use for non-biology focused labs that might not have a large library of restriction enzymes, or budget to get one. Lines 91-95

Line 81-82: Was the restriction site information helpful for this paper?

Upon further consideration we found it unnecessary and perhaps confusing to add the restriction information for the pJL1 plasmid. Thank you for bringing this to our attention. It has been removed.

Line 83: The final sequence of 999 bp should be provided in the supplementary materials. Again, the sfGFP LET herein can be used for protein expression directly, without performing the following steps 2 to 4. The authors must give a clear and convincing statement regarding the necessity or advantage.

We agree that the sequence used should be included and the lack of a supplement was an oversight on our part. We have included more information in the supplement section. Regarding the novelty and advantage of the added steps for circularization and isothermal amplification we refer you to lines 55-63 which we have added to the introduction.

Line 102-104: This may sound backward. In general, increasing the annealing temperature can improve the amplification specificity.

This is a good catch, thank you for pointing it out. We have removed that text and replaced with an emphasis on the importance of annealing temperature. Lines 363-368

Line 111: How did this value (i.e., 92) be calculated?

This is a good question. We generalized this to 100x instead of providing a concrete value. We also explained that this is the amount of total DNA at the end of the reaction compared to the amount of DNA initially added.

Line 120-121: The incubation at 12 °C sounds unconventional and empirical. Please provide reference support if possible and add the information about the incubation time.

Good point. We have changed it to 10 °C. This is not empirical but is from the protocol for both OneTaq and Phusion by NEB. We perform the PCR protocol as per supplier instructions and suggest others do the same.

Line 133-134: The information about the manufacture of kits or individual components should be provided in the reagent list.

These have already been included in the table of reagents Excel sheet but more have been added that were previously overlooked such as buffers.

Line 142-143: How to validate the lifetime of the diluted stock?

This is a good point. We don't have a method than can reliably measure such a small concentration. We have removed the sentence on validation and do not suggest prolonged storage of such a small amount of DNA. Instead, we recommend the circular template to be stored in its purified, concentrated state. Once diluted the material should all be used for RCA amplification or discarded.

Line 146: If overnight herein is allowed, conventional plasmid transformation to E. coli cells followed by cell culture and plasmid extraction can also produce a large amount of circular DNA template with a similar timetable. In particular, there are some fast sub-cloning methods in state of the art for the preparation of plasmid DNA. Again, the authors should clearly state the advantage of the RCA-based method over the previous ones in the Introduction.

We completely agree that the advantage of this approach was not clearly articulated and thank you for emphasizing this again. We have added Table 1 to compare the timelines. While we agree that someone experienced in cloning could clone gene fragments to plasmids and transform within a very busy 24 hour period, we also acknowledged that the clones would need to be sequenced and another overnight waiting period will be required for growth and purification. This is much longer than the proposed method RCA method, and again is not well suited for labs that want to use cell free as a method to avoid some of the complexities of working with living cells.

In terms of why increased amounts of template are needed, we refer you to the added lines 55-63 of the intro.

Line 156: "PANOx-SP" is not a well-known term for general readers. Please describe how to obtain or prepare it?

Since this is not a how to perform cell-free experiments, preparation of energy mix is outside the scope. However, we have added multiple literature sources to educate the reader and offer multiple energy buffer recipes. Lines 261-262

Line 160: Why add the manufacture information only for a few numbers of chemicals? The writing style should be unified.

Thank you for pointing this out. We have removed all manufacturer information to fit the JoVE author guidelines.

Line 164: How to obtain or prepare the E. coli S12 extract?

Again, this is outside of the scope of what we were invited to provide. We have provided references to how we prepare the extract as well as how others prepare extract. Lines 262-264

Line 165: A general cell-free protein synthesis reaction would stop after 4~5 hours. Why can the reaction sustain such a long time? Were there any efforts in this study that enabled a much longer reaction?

This statement is only true for higher temperature reactions. It is in fact very common to extend the reaction when reducing the temperature. We have gathered new data at 30 °C and replotted everything. We have made no changes from literature to achieve a prolonged reaction.

Line 170-171: Please give a timetable in parallel with the LET and plasmid-based conventional methods to support this claim.

That is a good idea. We have added Table 1 that compares a simplified traditional cloning timeline with the timeline proposed in this manuscript.

Line 177: Figure 1 may have never been cited in the main text.

Thank you for noticing this. We have referenced figure 1 near the end of the introduction as originally intended.

Line 182: The quantity of the protein synthesized from the RCA-based template was lower than the plasmid-based conventional CFPS. This result sounds like no advantage would be obtained by this method.

Excellent point. As stated in the manuscript, the advantage is not the yield. The advantage is the amount of DNA that can be generated in such a small amount of time. This is especially useful when it becomes necessary to generate a large amount of DNA for a gene that may be toxic and cause issues when cloning. It can also be much easier for those who have no experience with cloning. It is also advantageous when performing multiple rounds of screening that will require large amounts of DNA to be produced quickly.

Line 190: This paper did not show any library results.

We understand the desire to see new library results, but the JOVE format is focused on highlighting a method (protocol paper), in this case steps from receiving a small amount of gene fragment to expressing large amounts of protein. It is common to list potential uses for a method in the discussion section of an article. If the reviewer is interested, we have used this method to explore putative proteins in past literature.

Dopp, Jared Lynn, et al. "Rapid prototyping of proteins: Mail order gene fragments to assayable proteins within 24 hours." Biotechnology and bioengineering 116.3 (2019): 667-676.

Dopp, Jared L., and Nigel F. Reuel. "Simple, functional, inexpensive cell extract for in vitro prototyping of proteins with disulfide bonds." Biochemical Engineering Journal 164 (2020): 107790.

Line 193-194: It is overstated since PCR, RCA, restriction digestion, and ligation are all molecular biology techniques.

Thank you for pointing this out. This has been reworded to focus on the lack of cloning experience in particular. Lines 68-76 and 380-382 also address this.

Line 197: No results supported this claim "The expression profile tends to be very similar to that of LETs."

We have added experimental data from an LET and have reworded this section. Lines 395-409

Line 198: No results supported this claim "there is no appreciable difference in expression levels."

We have reworded this statement to reflect the actual results in a quantitative manner.

Line 201: No results supported this claim "the chi oligos to be cheaper and more effective."

This is true and the statement has been reworded to be less misleading. A reference to literature showing the increased effectiveness of the chi oligo over GamS was also added. The reference to cost was removed since that may involve mentioning brand names in the text.

Line 209-210: Again, many cell-free systems, including cell extracts and reconstituted ones, are compatible with linear DNA templates.

Agreed, and we have made the statements to align better with the central thesis of the paper, that this is a biochemical method of amplifying a gene fragment to enable large campaign prototyping without plasmid cloning and living cell propagation. This enables a robotic assisted workflow more readily than the cloning route. Single amplification and production of linear templates can only generate so much DNA. As stated in the manuscript, 1 x 50 uL reaction may only produce 1 ug of DNA but 1 uL from this amplification can result in over 4 ug of RCA product. This is one of the disadvantages of using linear templates. Further amplifying linear templates using this method generates much more DNA and results in fewer amplifications of the original gene fragment. We have changed the wording to focus more on this point.

Reviewer #2:

Manuscript Summary:

The manuscript by Jared Dopp and Nigel Reuel describes a method of rapid DNA template production that can be utilised for cell free (CF) transcription-translation reactions. The main benefit of which, is the quick turnaround from delivery of synthesised genes to usable DNA-template in a medium to high throughput capacity. A second benefit is that method allows for rapid screening of multiple genes of interest. A further benefit is that the method seems to be fairly straightforward to those who may be unfamiliar with standard molecular-techniques required for cloning of plasmid templates.

The paper briefly introduces the topic of CF, highlighting the need for alternatives to plasmid based CF reactions which otherwise slow the progression of protein expression testing. Linear expression templates are described, as well as their apparent pitfalls such as template degradation and limited strain adaptability. The authors showcase the method of rolling circle amplification for rapid generation of a gene template as well as the genetic modules controlling transcription and translation. Further, the use of the rolling circle templates produce comparable amounts of protein to plasmid derived templates. The protocol would be of interest to those who utilise CF for medium to high throughput studies.

Major Concerns:

No major concerns

Minor Concerns:

A couple of minor points that need to be addressed.

Line 90:

Is double distilled water considered as nuclease free? Perhaps it is enough to say double distilled?

Excellent point. We removed the “nuclease free” and kept double distilled.

Line 168

"Great results" is subjective terminology. Comparatively, sfGFP expression from the plasmid template was the same, and while the use of rolling circle templates may be as good, the result should be reported as such but not analysed by subjective terminology. sfGFP expression was comparable for reactions using 0.3 µL, 0.6 µL or 0.9 µL but a little lower than plasmid templates.

Thank you for pointing this out. We have re-worded the section to be more objective and comparative between templates. Lines 395-409

Lines 196-201

Question: do templates derived from rolling circle amplification have problems in extracts derived from cell-lines other than BL21(DE3) Star? This is something which I feel is not clearly defined here.

Excellent question. Other extracts do have issues with the product and treat it like a linear template. As such, we've added expression data from NEB's SHuffle strain to demonstrate the challenges. We have also added experiments that use protective agents. Lines 395-409

Question

Do the authors have any reference to genes other than sfGFP? Perhaps a line to address this.

Thank you for the suggestion. As much as we like to use sfGFP, we understand that it isn't representative of all proteins that can be expressed. As such, we have included a cytotoxic protein, subtilisin BPN' that has much different expression and maturation properties than sfGFP.

Extra point

The manuscript makes reference to researchers who may not understand the finer nuances of molecular biology. It is a suggestion by this reviewer that the authors include a line recommending any gene ordered from a vendor be checked for encoded restriction sites (RES) that are to be used for downstream steps, and that those gene-specific RES's be removed. If the gene of interest contains the same RES that are also incorporated into the minimal genetic template, then that will be problematic for ligation and rolling circle amplification.

Thank you for pointing this out. We originally mentioned it in passing but agree that this is a very important issue, so we took more time to expand on it. Lines 86-95

Reviewer #3:

Manuscript Summary:

Dopp& Reuel describe the detailed protocol of their rapid DNA amplification strategy associated for in vitro transcription/ translation.

Major Concerns:

In general, the purpose of the protocol is clear, and the steps are described well. However, I am wondering if the level of detail is deep enough for readers with no prior experience in cloning as claimed in line 57. In particular, the PCR amplification step could be described in a bit more detail and maybe some references for troubleshooting ideas could be included in case people are struggling with this step. As far as I can see, there is also no reference to the need for a PCR cycler and a (microtiter plate) incubator in the list of materials and equipment, which will be important information for researchers without cloning experience.

Thank you for the suggestion. We have reformatted our manuscript to fit the JoVE template and have added much more detail in the protocol section. We've also added the missing equipment to the reagent list.

Another conceptual question that I have is whether the DNA amplification step is specific for downstream use in E. coli lysates or whether it could be used for generating templates other (less common) lysates? Of course, the example provided is specific for expression in E. coli (codon optimization, promoters and terminators) but I could imagine that the template amplification could also be more broadly applicable.

This is a good point, and we are currently working on implementing this in another bacterial system. However, it's very challenging to express from PCR products in extracts that do not have nuclease removal or suppression, and RCA products suffer from the same issues that other PCR products suffer from. See the new Fig 2B for an example in the SHuffle strain of E. coli.

Minor Concerns:

Introduction - maybe also mention V. Noireaux's work on using linear DNA templates (10.1021/sb400131a, 10.1021/acssynbio.5b00296) and the recent application thereof (10.1101/2020.08.27.258715)?

Thank you for the suggestion. We've added references for these papers to the introduction.

Line 71: "[…] but ensure […] who is the subject of that sentence? The sequences or the user?

Thanks for pointing that out. We have reworded this sentence to make it more clear.

Line 82: should it be "DNA synthesis service"?

Yes, that is probably the best way to phrase it. Thank you for pointing that out.

Line 99: Is PCR strictly required or would it also be possible to use the synthetic DNA directly for the following steps?

Good catch! It is not necessary, and we have included a reference to a paper that skips the initial amplification step.

Line 104: since you provided the specific example of using M13 primers in step 1, maybe provide the annealing temperature that you would use for your example. How many cycles do you suggest for the PCR? Please also briefly mention the need to adapt the elongation time. Given that you state in the introduction that no cloning skills are necessary for this protocol, you should also assume that your audience doesn't know the best practices of PCR.

Thank you for pointing that out. You are correct. We have added more information on annealing, elongation, and number of cycles. Lines 154-158

Line 109: "use a kit" - "PCR clean-up kit"?

Correct. However, we have reworked this section to match the format of the journal.

Line 118: "CutSmart" refers to a specific brand of enzymes, maybe make this more general.

Great catch. We've generalized this to "necessary buffer" and added CutSmart to the reagent list.

Line 121: "This" refers to the 12°C step - please rephrase to generate the correct logical connection. Or move the sentence to the introductory part of the paragraph (e.g. after sentence 1)

Thanks for bringing this to our attention. This has been reworked into a step-by-step breakdown.

Line 127: "Purification is not […] at this time." This time suggests clean-up after ligation, but I assume it is meant to refer to after the enzyme digest.

You are correct. Thank you for bringing this to our attention. This section has been reworked to fit the style of the journal.

Line 128: purified with PCR clean-up kit?

Yes, this has been edited.

Line 150: when does the solution become viscous, maybe you can already "warn" the reader at that specific step in the protocol.

Thank you for pointing this out. This was added by mistake and has been removed.

Line 151: purified by PCR clean-up kit?

Yes, we have added this.

Line 156: purified or non-purified?

Thank you for pointing this out. For this manuscript, unpurified but purified constructs can work as well.

Line 165: what's the final volume of each reaction? Is there an optimum to how full the wells should be? How do you seal the plates? Do you simply place the plate in a 37°C incubator or do you use a plate reader/ microtiter plate incubator for this step?

Thank you for asking these questions. This section has been reworked to fit the style of the journal and these questions have been answered. The lines for this are 257-262 and 290.

Line 173: Can you rephrase the last sentence? I had to read it 5 times to understand it's meaning.

Yes, upon returning to this, it is not clear. We have expanded on it and added references that discuss temperature optimization. This information now resides in lines 318-324

Line 201: typo "convert"?

Yes, thank you for pointing this out.

Figures: the quality of the figures is quite bad. I assume that has to do with the PDF conversion?

Yes, we apologize for that. The pictures were uploaded as .Tiff but did not convert well. We hope they appear with higher quality in the revision.

Table of materials: add PCR clean-up kit (or is that the last item on the list?); maybe mark TempliPhi kit as the one needed for RCA; please add PCR cycler and incubator to the list.

Yes, that is the last item on the list. We have updated the list and added comments, so readers know what the product is used for.

Supporting Information

Rapid methods for amplification of minimal, linear templates for protein prototyping using cell-free systems

Jared L. Dopp and Nigel F. Reuel*

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Supplementary Sequences

Sequence #1

sfGFP – 999 base pairs

GTAAAACGACGGCCAGTAGCGCTATTAAGCTTCGAAATTAATACGACTCACTATAGGGAGACCACAACGGTTTCC
CTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGAGCAAAGGTGAAGAACTGTTTACCGGCGTT
GTGCCGATTCTGGTGGAACTGGATGGCGATGTGAACGGTCACAAATTCAGCGTGCGTGGTGAAGGTGAAGGCGA
TGCCACGATTGGCAAACTGACGCTGAAATTTATCTGCACCACCGGCAAACTGCCGGTGCCGTGGCCGACGCTGGT
GACCACCCTGACCTATGGCGTTCAGTGTTTTAGTCGCTATCCGGATCACATGAAACGTCACGATTTCTTTAAATCTG
CAATGCCGGAAGGCTATGTGCAGGAACGTACGATTAGCTTTAAAGATGATGGCAAATATAAAACGCGCGCCGTTG
TGAAATTTGAAGGCGATACCCTGGTGAACCGCATTGAACTGAAAGGCACGGATTTTAAAGAAGATGGCAATATCC
TGGGCCATAAACTGGAATACAACTTTAATAGCCATAATGTTTATATTACGGCGGATAAACAGAAAAATGGCATCAA
AGCGAATTTTACCGTTCGCCATAACGTTGAAGATGGCAGTGTGCAGCTGGCAGATCATTATCAGCAGAATACCCCG
ATTGGTGATGGTCCGGTGCTGCTGCCGGATAATCATTATCTGAGCACGCAGACCGTTCTGTCTAAAGATCCGAACG
AAAAACGGGACCACATGGTTCTGCACGAATATGTGAATGCGGCAGGTATTACGTGGAGCCATCCGCAGTTCGAAA
AATAATAAGTCGACCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACT
AGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAAGCGAGACTAAGCTTTAAACTTCGG
GTCATAGCTGTTTCCTG

Sequence #2

Subtilisin BPN' – 1344 bp

GTAAAACGACGGCCAGTAGCGCTATTAAAGCTTCGAAATTAATACGACTCACTATAGGGAGACCACAACGGTTTCC
CTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGGCAGGTAAAAGTAACGGCGAGAAAAAATA
CATCGTTGGCTTCAAACAAACGATGTCGACCATGAGCGCAGCGAAAAAGAAAGATGTCATCAGCGAAAAAGGCG
GTAAAGTGCAGAAACAATTCAAATACGTTGACGCGGCCAGTGCCACCCTGAATGAAAAAGCAGTGAAAGAACTG
AAGAAAGATCCGTCCGTGGCGTACGTTGAAGAAGACCATGTTGCTCACGCGTATGCCCAGTCCGTTCCGTACGGT
GTCTCACAAATTAAAGCACCGGCTCTGCATTCGCAGGGCTATACCGGTAGCAACGTTAAAGTCGCGGTGATTGATA
GCGGCATCGACAGTTCCCACCCGGATCTGAAAGTTGCGGGCGGTGCCAGCATGGTGCCGAGCGAAACCAATCCGT
TCCAGGACAACAATAGCCATGGCACGCATGTGGCGGGTACCGTTGCAGCTCTGAACAATTCTATTGGCGTCCTGG
GTGTGGCACCGTCTGCTAGTCTGTATGCGGTTAAAGTCCTGGGCGCCGATGGCTCTGGCCAGTACAGTTGGATTAT
CAACGGTATTGAATGGGCGATCGCCAACAATATGGATGTGATCAATATGAGCCTGGGCGGTCCGTCCGGTTCAGC
CGCACTGAAAGCAGCTGTCGACAAAGCAGTTGCTTCCGGTGTGGTTGTTGTGGCCGCAGCCGGTAACGAAGGCAC
GTCAGGCTCATCGAGCACCGTGGGTTATCCGGGCAAATACCCGTCGGTTATTGCGGTCGGTGCCGTGGATTCTAG
TAATCAGCGTGCGAGCTTTTCCTCAGTTGGCCCGGAACTGGACGTTATGGCCCCGGGTGTCTCTATTCAAAGTACG
CTGCCGGGTAACAAATATGGCGCGTACAATGGTACCAGCATGGCATCACCGCATGTGGCTGGTGCTGCGGCCCTG
ATCCTGAGCAAACACCCGAACTGGACGAATACCCAGGTTCGCTCGAGCCTGGAAAACACCACGACCAAACTGGGC
GATTCTTTCTATTACGGCAAAGGTCTGATCAATGTTCAGGCAGCTGCGCAATAATAAGTCGACCGGCTGCTAACAA
AGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACG
GGTCTTGAGGGGTTTTTTGCTGAAAGCGAGACTAAGCTTTAAACTTCGGGTCATAGCTGTTTCCTG

Needed Genetic Elements by color code:

T7 Promoter

RBS

Start

Protein Sequence –

Stop

T7 Terminator

Circularization site – HindIII Digest

Primer Sequences