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Rapid, Affordable, and Uncomplicated Production of Bacterial Cell-free Lysate

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TITLE:
Rapid, Affordable, and Uncomplicated Production of Bacterial Cell-free Lysate

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SUMMARY:
This protocol describes a rapid and simple method to produce bacterial lysate for cell-free gene expression, using an engineered strain of *Escherichia coli* and requiring only standard laboratory equipment.

ABSTRACT:
Cell-free gene expression offers the power of biology without the complications of a living organism. Although many such gene expression systems exist, most are quite expensive to buy and/or require special equipment and finely honed expertise to produce effectively. This protocol describes a method to produce bacterial cell-free lysate that supports high levels of gene expression, using only standard laboratory equipment and requiring minimal processing. The method uses an *Escherichia coli* strain producing an endolysin that does not affect growth but which efficiently lyses a harvested cell pellet following a simple freeze-thaw cycle.

The only further processing required is a brief incubation followed by centrifugation to clear the autolysate of cellular debris. Dynamic gene circuits can be achieved through heterologous expression of the ClpX protease in the cells before harvesting. An *E. coli* strain lacking the *lacZ* gene can be used for high-sensitivity, cell-free biosensing applications using a colorimetric or fluorescent readout. The entire protocol requires as few as 8–9 hours, with only 1–2 hours of hands-on labor from inoculation to completion. By reducing the cost and time to obtain cell-free lysate, this method should increase the affordability of cell-free gene expression for various applications.

INTRODUCTION:

Gene expression in cell-free lysates has several advantages over using live cells[1-4]. Lysates can be easily modified biochemically and used in conditions that could be detrimental to or impossible to achieve in live cells. Gene expression circuits do not have to contend or compete with host biological processes, and testing new genetic circuits is as simple as adding DNA. For these reasons, cell-free gene expression has found various applications, from biosensors[5,6] to rapidly prototyping synthetic gene circuits[7,8] to developing artificial cells[9]. Most cell-free gene expression utilizes cellular lysates that have been highly processed, generally requiring long and complex protocols, specialized equipment, and/or sensitive steps that can lead to significant variation between users and batches[10,11].

This paper describes a simple, efficient method for producing cell-free lysate that requires minimal processing and expertise (**Figure 1A**)[12]. The method relies on *E. coli* cells that are engineered to lyse following a simple freeze-thaw cycle. The cells express an endolysin from phage lambda that degrades the cell wall. As the cells are growing, this endolysin remains in the cytoplasm, sequestered from the cell wall. However, a simple freeze-thaw cycle disrupts the cytoplasmic membrane, releasing the endolysin into the periplasm, where it degrades the cell wall, resulting in rapid cell lysis. The protocol can be completed with only a few hours of hands-on work and requires only a freezer, a centrifuge capable of 30,000 × *g* (for optimal results. Lower speeds can be used with some loss of extract efficacy), a vortex mixer, and a simple buffer solution. Functional lysate can even be produced by freeze-drying the cells and rehydrating them *in situ*. However, this method produces lysates with lower activity, likely due to the remaining cell debris.

The lysates are highly active for cell-free gene expression, and they can be enhanced in various ways depending on the end-use. The rate of protein synthesis can be further increased by concentrating the lysate using standard spin concentrators. Linear DNA can be protected from degradation by adding purified GamS protein. Protein degradation, necessary for more complex circuit dynamics such as oscillation, can be achieved by co-expressing a ClpX hexamer in the autolysate-producing strain[13]. Finally, LacZ-based visual readouts are enabled by using an autolysate strain lacking *lacZ*. Overall, this method produces highly active cell-free lysate that is suitable for a wide range of applications.

PROTOCOL:

1. Prepare media and buffers.

1.1. Prepare 2xYTPG medium.

1.1.1. Mix 62 g 2xYT powder, 5.99 g potassium phosphate monobasic, 13.93 g potassium phosphate dibasic, and deionized water to 2 L.

1.1.2. Autoclave on liquid cycle with an exposure time of 30 min[14].

1.1.3. To 400 mL of 2xYTP media from 1.1.2, add 7.2 g D-glucose (dextrose) and mix until dissolved.

1.1.4. Filter-sterilize through a 0.2 µm filter.

1.2. Prepare S30A buffer.

1.2.1. Mix Tris-HCl (50 mM final concentration), potassium glutamate (60 mM final), and 14 mM magnesium glutamate (14 mM final).

1.2.2. Adjust the pH to 7.7 using 10 M KOH.

1.3. Prepare Solution 1 (see **Table 1**).

1.3.1. Resuspend 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) in 2 mL of water.

1.3.2. Adjust the pH to 8.0 using KOH.

1.3.3. Add all other components from **Table 1**.

1.3.4. Adjust the pH to 7.6 using 10 M KOH. Filter-sterilize.

1.4. Prepare 2.5x premix solution (see **Table 2**).

1.4.1. Mix all the components in **Table 2**.

1.4.2. Adjust the pH to 7.5 using KOH.

1.4.3. Aliquot and freeze at -80 °C.

NOTE: A 20 µL reaction uses 8.9 µL of premix.

2. Prepare cells.

2.1. Streak the autolysate cells onto LB agar plates containing 50 µg/mL ampicillin using an inoculating loop and grow at 37 °C.

2.2. Pick a single colony into a starter culture of LB/ampicillin medium using a pipet tip and grow at 37 °C overnight.

2.3. Inoculate 400 mL of 2xYTPG medium containing 50 µg/mL ampicillin with 400 µL of starter culture, and grow at 37 °C in a 1 L Erlenmeyer flask, shaking at 300 rpm.

2.4. Periodically measure the culture's optical density at 600 nm (OD_{600}) using a

spectrophotometer to read an optical cuvette with a 1 cm path length. When the OD_{600} exceeds 1, begin diluting the culture 5-fold before measurements to ensure that the measurements remain within the linear range of a typical laboratory spectrophotometer. Continue growing the cells until the 5-fold diluted culture reaches OD_{600} of 0.3 (corresponding to a culture OD_{600} of 1.5).

3. Prepare the lysate.

3.1. Prepare S30A buffer supplemented with 2 mM dithiothreitol (DTT). Mix 1 mL of S30A buffer with 2 µL of DTT stock solution at 1 M. Place on ice for use in step 3.7.

3.2. Harvest the cells by centrifuging at 1800 × *g* for 15 min at room temperature.

3.3. Discard the supernatant by pouring it off and using a pipet to remove any remaining liquid.

3.4. Resuspend the pellet in 45 mL of cold (4–10 °C) S30A buffer using a vortex mixer.

3.5. Weigh an empty 50 mL centrifuge tube, transfer the cells into it, and repeat steps 3.2–3.3 to wash the cells.

3.6. Weigh the pellet, subtracting the weight of an empty 50 mL tube. Make sure to carefully aspirate any remaining supernatant to ensure an accurate measurement of pellet weight.

NOTE: A typical yield is ~1.3 g of cell pellet from 400 mL of production culture.

3.7. Add 2 volumes of cold S30A buffer supplemented with 2 mM dithiothreitol, i.e., 2 µL of buffer for every 1 mg of cell pellet, and resuspend the cells by vigorously vortex mixing.

3.8. Freeze the cells. Place the cells in a -20 °C or -80 °C freezer until the pellet is thoroughly frozen.

NOTE: The freezing step is a good stopping point for the day.

3.9. Thaw the cells in a room temperature water bath.

3.10. Vortex vigorously for 2–3 min.

3.11. Incubate at 37 °C for 45 min with shaking at 300 rpm to further shear the genomic DNA and release the ribosomes.

3.12. Clear the sample of heavy cellular debris by centrifuging in transparent centrifuge tubes at 30,000 × *g* for 45 min at 4 °C.

NOTE: If a centrifuge capable of 30,000 x g is not available, centrifuge for 45 min at 21,000 × *g*,

and use additional caution in step 3.13, as the pellet will be less compact.

3.13. Carefully transfer the supernatant to a new tube with a pipet, avoiding disturbing the pellet as much as possible. If the transferred supernatant is contaminated with material from the pellet, repeat the previous step.

3.14. Transfer the supernatant to 1.5 mL centrifuge tubes and centrifuge once more at 21,000 × *g* (or the maximum speed of a tabletop centrifuge) for 5 min.

3.15. Aliquot the cleared autolysate into the desired volumes, carefully avoiding any remaining pellet, and freeze at -80 °C or use immediately.

NOTE: A single 20 µL reaction uses 8 µL of autolysate.

4. Cell-free gene expression

NOTE: The autolysate is now ready for any desired end-use. The following is an example standard protocol for cell-free gene expression.

4.1. For a 20 µL reaction, mix on ice 8 µL of autolysate and 8.9 µL of premix. See the NOTE at the end of the protocol section regarding the optimization of magnesium glutamate and PEG 8000 concentrations.

4.2. Add DNA (e.g., pBEST-OR2-OR1-Pr-UTR1-deGFP-T500 to a final concentration of 8 nM), any other reagents, and water to 20 µL.

4.3. Place the reaction in a 384-well microplate and measure the fluorescence time course and/or endpoints using a plate reader. For green fluorescent protein (GFP), use an excitation wavelength of 485 nm and an emission wavelength of 520 nm.

5. Protocol modifications

NOTE: The following modifications of the protocol enable it to serve other applications.

5.1. Cell-free gene expression using linear DNA templates

5.1.1. Perform the steps in section 4, supplementing the reaction with 2.2 µM purified GamS protein (expressed and purified as described[12]) before the addition of the linear DNA.

5.2. Cell-free gene expression incorporating protein degradation

5.2.1. In step 2.1, use autolysate cells containing the plasmid pACYC-FLAG-dN6-His (see the **Table of Materials**). In all growth media, additionally include 34 µg/mL chloramphenicol.

5.2.2. In step 2.3, include 40 μM isopropyl β-D-1-thiogalactopyranoside (IPTG) in the growth medium to induce expression from the plasmid.

5.2.3. Repeat steps 3.2–3.4 (washing) two additional times (for a total of three washes) to ensure the complete removal of chloramphenicol, which is a translation inhibitor. For the first two washes (step 3.4), substitute S30A buffer with phosphate-buffered saline (pH 7.4).

5.2.4. In step 4.2, supplement with an additional 3 mM ATP (added from a stock solution of 100 mM ATP in water, pH 7.2) and 4.5 mM magnesium glutamate (using a 1 M stock solution in water) (final concentrations) to compensate for high ATP use by ClpXP, as well as chelation of magnesium by the additional ATP.

5.3. Cell-free gene expression using LacZ-based readouts (including colorimetric)

5.3.1. In step 2.1, use autolysate cells that do not natively express LacZ (see the **Table of Materials**).

5.4. Alternatively, prepare the lysate directly from freeze-dried cells.

5.4.1. Perform all steps from 1.1 to 3.7.

5.4.2. Mix 8 μL of cell suspension with 8.9 μL of premix.

5.4.3. Add plasmid DNA (if desired), other custom reagents, and water to reach a final volume of 20 μL.

5.4.4. Transfer the reaction to a 384-well microplate and freeze-dry it.

NOTE: Freeze-dried samples can be stored for up to a week and possibly longer.

5.4.5. To begin the reaction, rehydrate it with 18 μL of deionized water supplemented with any desired DNA or other reagents.

5.4.6. Follow the fluorescence dynamics in a plate reader.

NOTE: Magnesium ions and PEG 8000 are critical for lysate performance. The base 2.5x premix, based on previously published data, contains 6 mM magnesium glutamate and 4.8% wt/vol PEG 8000, which become 2.4 mM and 1.9%, respectively, in the final reaction. The autolysate prepared with the protocol here typically performs best with an additional 5 mM Mg-glutamate and 1.5% PEG 8000 in the final reaction. However, this can be optimized in the range of an additional 0–10 mM Mg glutamate and an additional 0–3% PEG 8000 (compared to the base premix). To prepare the premix with the recommended additional 5 mM Mg-glutamate and 1.5% PEG 8000 (final concentrations), mix 380 μL of premix with 4.75 μL of magnesium glutamate at 1 M and 36.1 μL of PEG 8000 at 40% weight/volume.

REPRESENTATIVE RESULTS:

Representative results can be observed by using autolysate to express GFP from a constitutively expressing plasmid, here pBEST-OR2-OR1-Pr-UTR1-deGFP-T500, and recording a time course of GFP fluorescence in a plate reader (**Figure 1B**). A dilution series of plasmid DNA found strong expression even at 1 nM DNA. Compared to a commercially available lysate, the autolysate produced a greater total yield and achieved a greater maximum production rate, calculated as the time derivative of the GFP time course (**Figure 1C,D**). For additional results using this method, see Didovyk et al.[12]. This protocol has relatively few failure points; however, suboptimal results could be obtained if the autolysate is not sufficiently cleared of cell debris. Optimal results can also require optimizing the concentrations of PEG-8000 and Mg^{2+} for each batch of lysate (see the note at the end of the protocol).

FIGURE AND TABLE LEGENDS:

Figure 1: Representative results. (**A**) Visual representation of the protocol. (**B**) Time course of GFP expression from pBEST-OR2-OR1-Pr-UTR1-deGFP-T500 in a freeze-thaw autolysate. Maximum GFP production (**C**) and maximum production rate (**D**) of autolysate as compared to a commercial reference lysate (MYtxtl-70-960M from MYcroarray). This figure has been modified from [12]. Copyright 2017 American Chemical Society. Abbreviation: GFP = green fluorescent protein.

Table 1: Solution 1. Components of solution 1.

Table 2: Premix. Components of the premix solution.

DISCUSSION:

The protocol described here yields highly active bacterial lysate for cell-free gene expression. The key is to use cells carrying the plasmid pAD-LyseR, which expresses the lambda phage endolysin cytosolically. These cells are potentiated to lyse themselves upon permeabilization of the inner membrane, allowing the endolysin access to the cell wall, which the method achieves through a simple freeze-thaw cycle. Because the cells effectively lyse themselves, the product is referred to as autolysate. After the cells have lysed, the only remaining steps are incubation and centrifugation to clear the autolysate of cellular debris.

Compared to other methods for producing bacterial lysate, this approach is notably simple and rapid, yet it does not sacrifice the quality of the lysate. The protocol can be completed in 8–9 h after inoculating the production culture, with only 1–2 hours of hands-on labor. The only recommended piece of equipment that is not entirely standard for molecular biology laboratories is a centrifuge capable of achieving 30,000 × *g*. However, autolysate can be produced even with a lower-speed centrifuge; the user would just have to be more careful removing the lysate from the pellet, perhaps leaving behind slightly more liquid to ensure clean samples. This simplicity is not merely a matter of convenience; less complicated protocols tend to yield more reproducible results, with less variation when performed with different hands. The modification presented in step 5.4, in which cells are freeze-dried along with all other reagents, presents an even simpler

protocol, although with reduced protein production yields. Notably, in this modification, the centrifugation steps to clear the lysate of cellular debris are skipped, which further reduces processing labor; however, the remaining debris reduces expression from the lysate[12].

In recent years, many approaches to producing cell-free lysate have been published, summarized recently by Cole et al.[15]. These studies have explored various strategies for cell type, growth conditions, lysis methodologies, and post-processing. Most other methods for lysis have required specialized equipment such as a French press, homogenizer, bead beater, or sonicator. Fujiwara and Doi omitted this equipment in favor of a freeze-thaw cycle similar to the one described here, except that they rendered the cells susceptible to lysis by treating them with lysozyme rather than expressing an endolysin[16]. Although this is roughly as simple a protocol as the one described here, the lysozyme-treated cells must be washed while in their fragile state without disrupting them prematurely, which could require experimental finesse and introduce a source of variability.

In addition to lysate, cell-free gene expression requires a premix solution containing energy sources, RNA and protein monomers, and other small molecules. The premix recipe was described and optimized previously[17], with a few modifications. The premix used here contained approximately 4 times higher concentrations of amino acids, as well as additional magnesium glutamate and PEG 8000 corresponding to final reaction concentrations of 7.5 mM and 3.5% weight/volume, respectively. Optimal results may require adjusting the supplemental magnesium glutamate and PEG 8000 concentrations for each new batch of lysate, although the above concentrations consistently produce good results (see the note at the end of the protocol). Unique applications may require reoptimizing these concentrations, for example, when using ClpX-supplemented lysate[13].

A standard *E. coli* strain for producing cell-free lysate is BL21-Gold (DE3). A derivative of these cells containing the autolysis plasmid pAD-LyseR has been deposited in a strain and plasmid repository (see the **Table of Materials**). Also available are a derivative lacking genomic *lacZ* to reduce the background for circuits that use LacZ-based output and an expression plasmid pAD-GamS to be used for the purification of the GamS protein that can protect linear DNA from degradation. These cell strains and plasmids should be useful for a variety of applications in cell-free gene expression.

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DISCLOSURES:
The authors declare no conflicts of interest.

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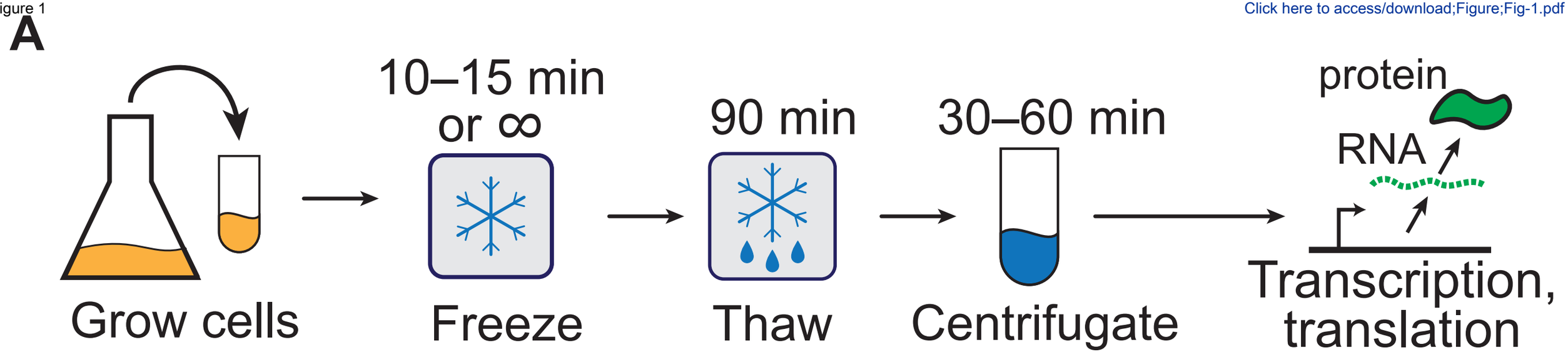
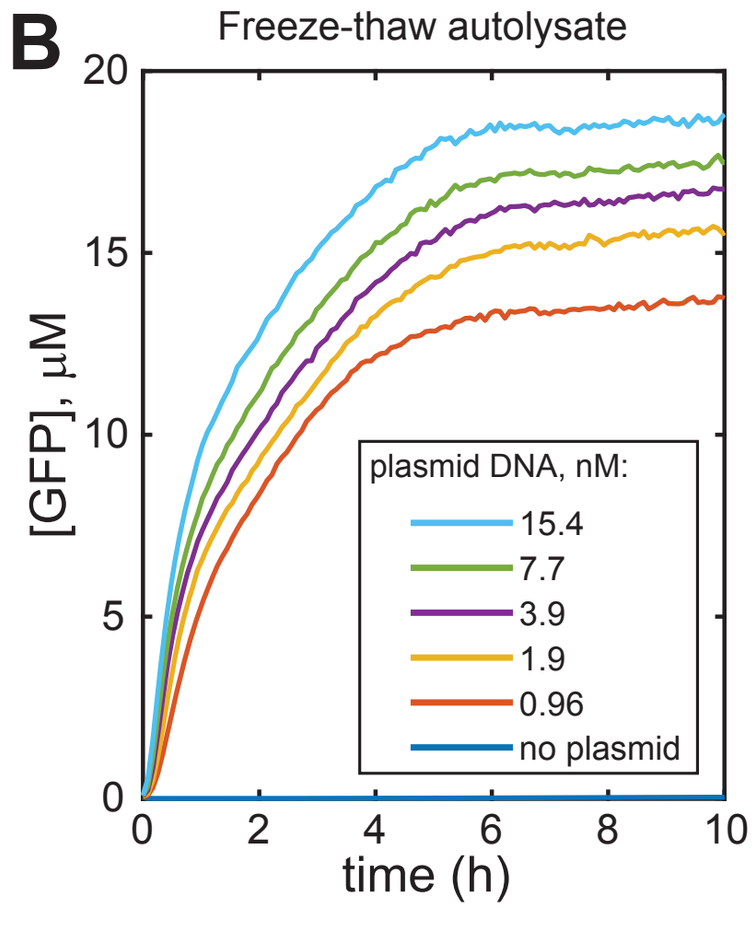
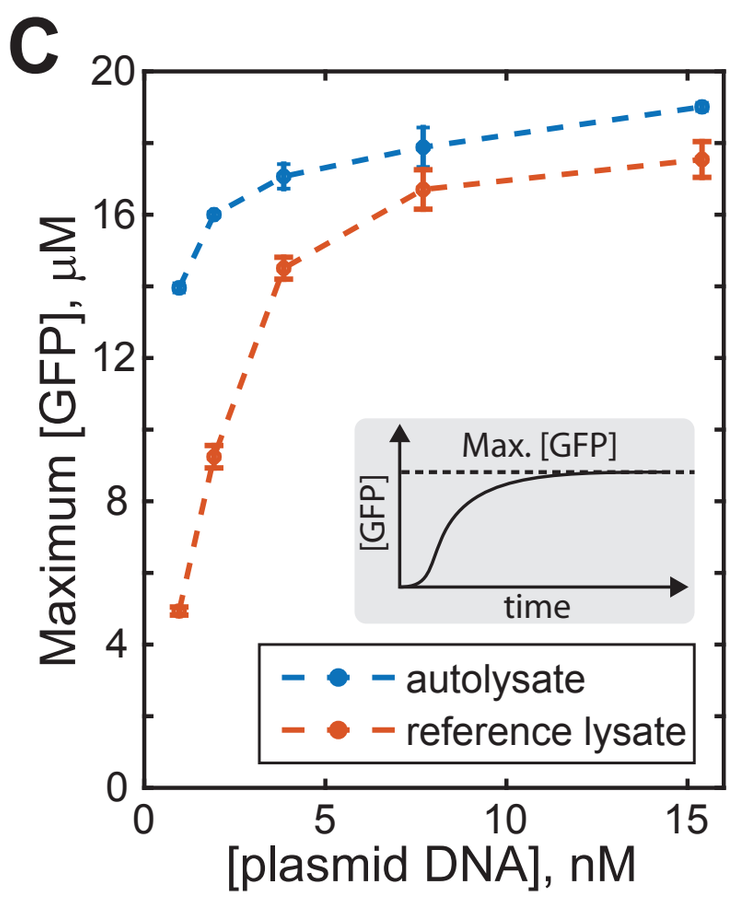
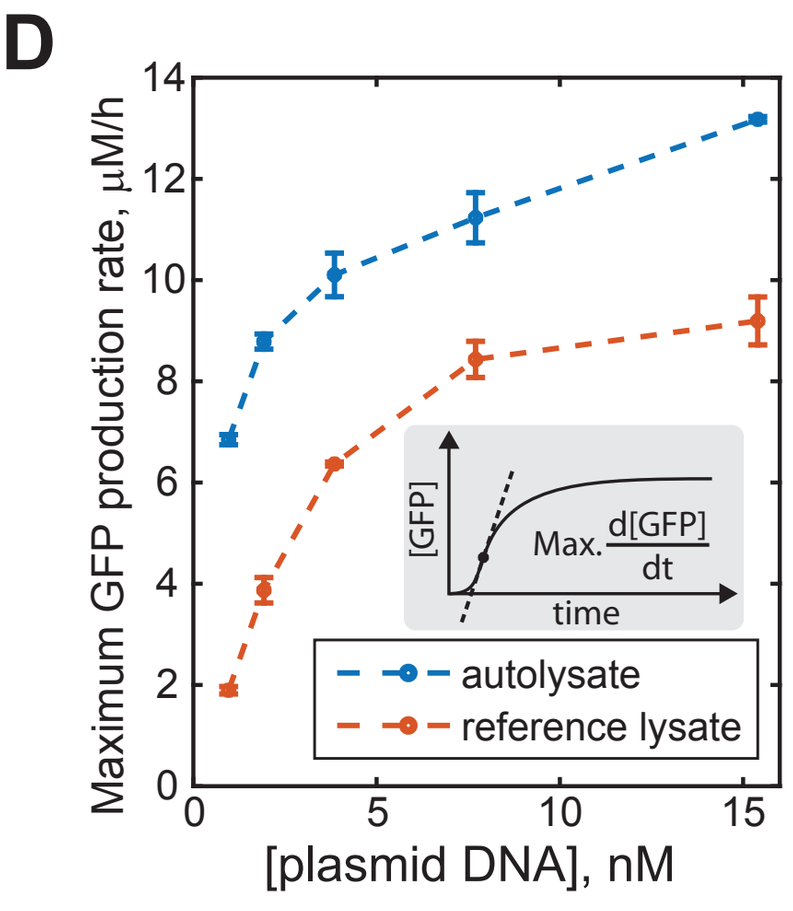
Figure 1









Solution 1: Add water to 4 mL total

Name	Weight (mg)
3-PGA	386.4
ATP	52
cAMP	13.8
CoA	11.2
CTP	28.4
Folinic acid	1.9
GTP	47.6
HEPES	667.2
NAD	12.3
Spermidine	8.1
tRNAs	11.2
UTP	29.5

2.5x Premix

Reagent
Amino acid mix containing 24 mM each, except for leucine, which is at 20 mM
dithiothreitol (DTT) at 100 mM
Magnesium glutamate at 1 M
PEG-8000 at 40% wt/vol
Potassium glutamate at 2 M
Solution 1 (see Table 1)

Volume (µM)
2500
100
25
5000
303
714.3

Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials (4).xls

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
2xYT media	EMD Millipore	4.85008	or equivalent
3-PGA	Sigma Aldrich	P8877	or equivalent
Amicon Ultra-15 centrifugal filter unit, 3 kDa cutoff	Millipore Sigma	UFC900308	optional, can be used to concentrate lysate, select concentrator capacity appropriate for the volume to be concentrated
ampicillin	Sigma Aldrich	A0166-5G	or carbenicillin, a more stable variant
ATP	Sigma Aldrich	A8937	or equivalent
cAMP	Sigma Aldrich	A9501	or equivalent
CoA	Sigma Aldrich	C4282	or equivalent
CTP	United States Biosciences	14121	or equivalent
D-glucose (dextrose)	Fisher Scientific	AAA1749603	or equivalent
dithiothreitol (DTT)	Sigma Aldrich	D0632-1G	or equivalent
E. coli BL21-Gold (DE3) carrying pAD-LyseR	Addgene	99244	
E. coli BL21-Gold (DE3) Δ*lacZ* carrying pAD-LyseR	Addgene	99245	
Folinic acid	Sigma Aldrich	F7878	or equivalent
GTP	United States Biosciences	16800	or equivalent
HEPES	Sigma Aldrich	H3375-25G	or equivalent
LB media	Fisher Scientific	DF0446075	or equivalent
magnesium glutamate	Sigma Aldrich	49605-250G	or equivalent
NAD	Sigma Aldrich	N6522	or equivalent
potassium glutamate	Sigma Aldrich	G1501-100G	or equivalent
potassium hydroxide (KOH)	Sigma Aldrich	221473-25G	for adjusting pH
potassium phosphate dibasic	Fisher Scientific	BP363-500	or equivalent
potassium phosphate monobasic	Fisher Scientific	BP362-500	or equivalent
Spermidine	Sigma Aldrich	85558	or equivalent
Tris-HCl	Fisher Scientific	9310500GM	or equivalent
tRNA mix	Roche	10109541001	or equivalent
UTP	United States Biosciences	23160	or equivalent

We thank the Editor and peer reviewers for their time and attention in reviewing this manuscript. We have addressed the concerns raised, as detailed below.

Editorial comments:

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

Confirmed

2. Please confirm whether the Filming location is “Kyoto, Japan”.

Confirmed

3. Please provide an institutional email address for each author.

Added email addresses.

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

Changed to passive voice.

5. Please revise the Introduction to include all of the following:
a) A clear statement of the overall goal of this method

This paper describes a simple, efficient method for producing cell-free lysate that requires minimal processing and expertise.

b) The rationale behind the development and/or use of this technique

The method relies on E. coli cells that are engineered to lyse following a simple freeze-thaw cycle. The cells express an endolysin from phage lambda that degrades the cell wall. As the cells are growing, this endolysin remains in the cytoplasm, sequestered from the cell wall. However, a simple freeze-thaw cycle disrupts the cytoplasmic membrane, thus releasing the endolysin into the periplasm where it degrades the cell wall, which results in rapid cell lysis.

c) The advantages over alternative techniques with applicable references to previous studies

Most cell-free gene expression utilizes cellular lysates that have been highly processed, generally requiring long and complex protocols, specialized equipment, and/or sensitive steps that can lead to significant variation between users and batches.[10]… The protocol can be completed with only a day’s work and requires only a freezer, a centrifuge capable of 30,000 x g (for optimal results; lower speeds can be used with some loss of extract efficacy), a vortex mixer, and a simple buffer solution.

d) A description of the context of the technique in the wider body of literature

Gene expression in cell-free lysates has a number of advantages over using live cells.[1-4] Lysates can be easily modified biochemically and used in conditions that could be detrimental to or impossible to achieve in live cells, gene expression circuits do not have to contend or compete with host biological processes, and testing new genetic circuits is as simple as adding DNA. For these reasons, cell-free gene expression has found a variety of applications, from biosensors[5,6] to rapidly prototyping synthetic gene circuits[7,8] to developing artificial cells.[9]

e) Information to help readers to determine whether the method is appropriate for their application.

Overall, this method produces highly active cell-free lysate that is suitable for a wide range of applications.

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For example: Tecan Infinite M200 PRO, Addgene, MYcroarray, etc.

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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.

Hedging words have been removed.

8. 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.

Detail has been added

9. Line 67: Please specify the conditions of the liquid cycle.

Exposure time and a reference for autoclave settings have been added.

10. Line 74: Please mention how the pH is adjusted.

The protocol now specifies to adjust pH using 10 M KOH.

11. For SI units, please use standard abbreviations when the unit is preceded by a numeral. Abbreviate liters to L to avoid confusion. Examples: 10 mL, 8 µL, 7 cm2 (Lines 76, 80, 81, 91, 92, 94, 99, 126, etc.)

The L in mL, etc., is now capitalized.

12. Line 83: Please mention how the optical density of the culture is measured. What is the volume of the culture used for measuring?

The line now specifies "using a spectrophotometer to read an optical cuvette with 1 cm path length"

13. Line 98-101/112-116: The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.

The discussions have been removed from the Protocol section.

14. Please 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 essential steps have now been highlighted.

15. Please remove the embedded figure(s) from the manuscript. All figures should be uploaded separately to your Editorial Manager account. Each figure must be accompanied by a title and a description after the Representative Results of the manuscript text.

The figure has now been removed.

16. 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

The Discussion covers these topics in 5 paragraphs.

17. Please do not use the &-sign or the word “and” when listing authors in the references. Authors should be listed as last name author 1, initials author 1, last name author 2, initials author 2, etc. Title case and italicize journal titles and book titles. Do not use any abbreviations. Article titles should start with a capital letter and end with a period and should appear exactly as they were published in the original work, without any abbreviations or truncations.

References are fixed to match JoVE formatting

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Permissions have now been uploaded and are cited in the Figure Legend

19. Figure 1: Please replace the hyphen between the numbers with en dash. Please replace the x axis unit in Figure 1B to “time (h)” instead of “time (hrs)”. Replace the y -axis unit of Figure 1D to “µM/h” instead of “µM/hr”.

These stylistic changes have been made.

20. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please sort the Materials Table alphabetically by the name of the material.

Materials Table is now sorted alphabetically

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The manuscript of Robert M. Cooper et al. describes a very useful, fast and easy protocol to obtain E. coli lysate for cell-free reactions. This protocol uses an autolysis strain of E. coli described in the paper: "Didovyk, A., Tonooka, T., Tsimring, L. and Hasty, J., 2017. Rapid and scalable preparation of bacterial lysates for cell-free gene expression. ACS synthetic biology, 6(12), pp.2198-2208." The protocol is described in detail, except for the premix composition, and required a minimal set of equipment to obtain E. coli lysate. This scientific method described in the manuscript is very useful, accurate and suitable for JOVE journal.

Major Concerns:

I have no major concern

Minor Concerns:
Line 20: What do you mean by "robust gene expression"? Did you compare different batches of lysate and saw identical or very close expression levels?

We were using robust here in the sense of strong and vigorous; i.e., the lysate supports high levels of gene expression that are comparable to or even exceed those from commercial lysate. However, we agree that in scientific contexts robustness often implies resistance to variation. We have replaced the word "robust" to avoid this confusion. Additionally, we will update the manuscript with reproducibility results from the filming experiments, which will be performed in a different lab and hemisphere once institutional approvals and JoVE scheduling are secured.

Line 24: I was not sure of the meaning of "dynamic gene circuits" I would cite an example here like the switches or oscillators or talked about reversible circuits like in your acs paper.

We have added a reference for producing oscillations in lysate.

Line 41: Need more ref about the issue of variations between batches

We have added an additional reference that describes the problem of batch-to-batch variation (Dopp et al).

Line 42: I am still note sure about the robustness of this protocol compared to previous one. As you will repeat the protocol for the JOVE video, It will be interesting to compare the new results with 2017 results and confirm the reproducibility of the approach

Although we personally have been able to reproduce the 2017 results in different hands within at least two separate research groups at University of California San Diego as well as others reported the use of the method in their publications, we agree, this will be one advantage of filming the protocol in a different lab (and part of the world). We will update the manuscript with these results after performing the filming. Dr. Tonooka is still waiting for institutional approval and coordination with JoVE to perform these experiments for filming, expected in May.

Line 49: Could you give an order of magnitude concerning the loss of efficacy when the lysate is obtained with at a low speed with a "classic" centrifuge. Is it around 2, 10 or 100 times less efficient?

We estimate the loss of efficiency to be closer to 2-3 fold, but we do not have directly comparable data for this as low speed centrifugation experiments were performed early in the development cycle of the 2017 protocol. We can make a direct comparison when we do the experiments for filming.

Line 55: Could you add an example of spin concentrator brand and put it in material/equipment in the annex?

An example is an Amicon Ultra centrifugal filter unit with 3 kDa molecular weight cut-off, and we have added this to the Table of Materials.

Line 74: Do you use acetic acid to reach ph 7.7?

We adjusted pH up using 10 M KOH, now added to the protocol.

Line 92: Do you mean "repeat step 3-1-3.2" and not 2.1-2.2?

Numbering has been corrected.

Line 92: You should add a step 3.4 bis in which you just say "weigh empty 50 ml centrifuge tubes"

We have added weighing the centrifuge tubes as an explicit direction.

Line 98: Can you give the concentration of DTT stock (is it 1M?)

We have added a step (3.1) describing preparation of S30A + DTT.

Line 112: Can you give the duration of the centrifugation at 21,000 g?

We have added this detail.

Line 126: Put a link to a reference for the premix composition or better add a detail protocol to prepare the premix here or in the annex. Compounds of the premix must be added to the material list in the annex.

We have added premix components to the Table of Materials, added 2 additional Tables for components of Solution 1 and the Premix, and added preparatory steps for mixing these solutions (Steps 1.3 and 1.4).

Line 138: Can you put here the plasmid number in addgene of the gamS plasmid and a method paper to purified it

JoVE has advised us that we cannot reference commercial providers, such as Addgene, in the Protocol section. Additionally, unfortunately despite lengthy troubleshooting, Addgene has been unable to propagate pAD-GamS plasmid in the BL21 expression strain, and so it has currently been discontinued. For this paper, we plan to resubmit the plasmid in a cloning strain. We will add the Addgene number of the gamS plasmid in cloning strain to the Table of Materials once it is available on Addgene.

Line 151: Step 2.1 and not 1.1

Numbering has been corrected.

Line 153: Step 5.4: is it freeze-dried lysate or cell-free reaction?

This alternative lyses the cells by freeze-drying them along with other reagents, and then begins the reaction by simply rehydrating them. This is not exactly "cell-free", because the cell debris is still present, but the reaction occurs in lysate released from the ruptured cells. We have added discussion regarding this to the Discussion section.

Line 154: There is no step 2.6 in your protocol

Numbering has been corrected.

Line 155: Is "cell suspension" a synonym of lysate?

Please see the response for the comment on Line 153.

Line 175: Step 4.1 not 3.1

Numbering has been corrected.

Reviewer #2:
Manuscript Summary:
This is a clear, well-written and easy-to-follow protocol describing the production of E. coli cell-free lysate for downstream protein productions. This rapid method is enabled by the pAD-LyseR plasmid.

Comments:
A SDS-PAGE to show the expression levels of GFP vs endogenous proteins (including protein products of the plasmids (pAD-LyseR, pAD-GamS) would be very helpful. I.e. have a lane with no GFP plasmid and a lane with over the time frame shown in Figure B. This is especially so if the system will be used to express proteins that don't give easy functional read outs.

A good suggestion. This was done in Didovyk et al. (2017), displayed as Figure S3. We have added a sentence directing readers to these additional results in the Representative Results section.

Given the importance of the pAD-LyseR plasmid and potential for its use in other E. coli strains, I suggest for this plasmid to be deposited to Addgene.

This has been deposited in Addgene (99244), now shown in the Table of Materials.

How long are the store lysate at -80C stable for? Can unused lysate be re-frozen after thawing and still maintain activity?

We have not explicitly tested how long the lysate can be stored at -80C, but we believe it should be good for at least more than a year. We also have not tested multiple freeze-thaw cycles, but we recommend storing the lysate in aliquots before freezing (step 3.15) to avoid potential loss of activity.

Reviewer #3:

Manuscript Summary:

The authors describe a modified method to prepare cell-free lysates for in vitro expression of biomolecules. The key message is to use an endolysin expressing E. coli strain for a subsequent autolysis procedure. The described protocol and results are mainly reprints from a previous publication of the authors. The article would go together with a video, nevertheless I would prefer to find all necessary information to reproduce the technique in the text part.

Major Concerns:

A table specifying the components, stocks and final concentrations of the premix should be included. In addition, the useful concentration and optimization range of the individual compounds should be given. Keeping the recommended total reaction volume of 20 µL in mind, examples for the described protocol modifications in part 5 including required stock concentrations of the optional compounds would be helpful.

Several other simple and highly efficient methods for E. coli lysate production have been described before and the protocol would clearly benefit if a more detailed comparison with these procedures would be given either in the introduction or discussion.

We have now added a table of the premix components and an additional note on the magnesium glutamate and PEG 8000 concentrations that can be optimized. We have also expanded discussion of other methods of lysate production in the Discussion section.

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Rapid and Scalable Preparation of Bacterial Lysates for Cell-Free Gene Expression

Author: Andriy Didovyk, Taishi Tonooka, Lev Tsimring, et al

Publication: ACS Synthetic Biology

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Date: Dec 1, 2017

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