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# IMPACT OF CRUDE BACTERIAL CELL LYSATE ON PERFORMANCE OF COMMERCIAL CELL-FREE EXPRESSION SYSTEM

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**14. ABSTRACT:**
Pardee et al. described a novel approach to synthetic biology in which several engineered gene networks were freeze-dried onto paper with in vitro expression machinery and then rehydrated. The results showed high performance. These paper-based gene networks exhibited transformative potential for synthetic biology and its applications within the Department of Defense by shifting the power of engineered gene circuits from the fragile and challenging world of the cell onto stable, reproducible paper substrates. Paper-based gene networks offer a potential future for biodetection that is inexpensive; disposable; stable; multiplexible over targets and modalities (ribonucleic acid/small molecule/protein); rapid to design and manufacture; and embeddable in paper, clothes, and other porous materials. However, two major concerns challenged the potential of this brand new technology: (1) whether its sensitivity could be augmented by gene-network amplification circuits and (2) whether cellular lysis could be embedded into the system, which would allow built-in sample preparation and maintain robust performance. In this study, we focused on the second concern and investigated whether paper-based gene networks exposed to bacterial lysate would function normally after being subjected to mechanical lysis.

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## EXECUTIVE SUMMARY

The use of cells to detect broad-spectrum threats through synthetic biology has long been an attractive prospect because of the range of stimuli, sensitivities, and specificities that organisms exhibit naturally. However, even when these sensing capabilities can be harnessed for military or other applications, keeping cells alive in the field can be problematic. Research studies have shown that as an alternative, cell-free expression systems can be freeze-dried onto paper substrates and rehydrated when needed. This opens up potential applications for biological sensing modalities in real-world applications, especially in far-forward, resource-poor environments. In one application in biodetection, current techniques typically use cold-chain or bulky instrumentation that requires a high level of training as well as sample preparation. As a step toward developing technology for far-forward field use, we explored the robustness of these reactions to crude cellular lysate to simulate a minimal sample preparation step. We found that inhibition of the reactions in the presence of lysates did occur, although not to a crippling extent. Our results highlight an additional advantage to the approach, namely, that more developed versions are expected to be at least partially favorable to cellular contaminants in real-world use. We also demonstrated the reactions that occurred on paper tickets and thus, laid the groundwork for further testing.

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## PREFACE

The work described in this report was authorized under grant number R.0015510.9.2.7 by the U.S. Army Edgewood Chemical Biological Center (ECBC; Aberdeen Proving Ground, MD) through the ECBC Seedling Program. This work was started in May 2015 and completed in September 2015.

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## Acknowledgments

We would like to acknowledge Keith Pardee (University of Toronto; Toronto, Canada), Alex Green (Arizona State University; Tempe, AZ), and James Collins (Massachusetts Institute of Technology; Cambridge, MA) for graciously facilitating the transition of their technology to our laboratory. In particular, we thank Keith Pardee for taking the time to teach us his methodologies in person. We also thank Alex Miklos, Tricia Buckley (ECBC), and Randy Hoffman (Excet, Inc.; Springfield, VA) for fruitful discussions.

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# IMPACT OF CRUDE BACTERIAL CELL LYSATE ON PERFORMANCE OF COMMERCIAL CELL-FREE EXPRESSION SYSTEM

## 1. INTRODUCTION

The transformative potential of synthetic biology has been highly touted since 2000;[1–2] however, its progress has been restricted by several technical challenges.[3] Within the complex environment of even the simplest bacterial cells, synthetic biology can be challenging because of interactions between the engineered circuit, the cell, and growth conditions. Simple proof-of-concept circuits may function well in a laboratory environment, but moving circuits to well-controlled, industrial fermenter environments can immediately disrupt their functions. Transitioning synthetic circuits from the laboratory into real-world environments, especially in the face of competition from endemic microbial communities, is a daunting challenge.

Cell-free systems can be used to circumvent the issues that arise from working in living cells.[4] These systems contain all of the necessary components for gene expression and can be obtained either as cellular lysate or as kits of purified components. Cellular lysates have the advantage of being extremely cost-effective, but there can be inconsistency between batches, and to confound matters, an abundance of other unnecessary components can be present in the cellular milieu, especially ribonucleases. Purified components perform far better in terms of expression and reproducibility but are expensive. Both types of cell-free systems require cold-chain storage at –20 ºC to function, which severely limits real-world applications.

In 2014, Pardee et al. described a new approach to address the issue of cold-chain storage by freeze-drying cell-free systems onto paper.[5] Through this groundbreaking work, they demonstrated that a variety of gene network circuits could be implemented in this format and that functionality of the freeze-dried cell-free systems could be maintained at room temperature for at least a year. To substantiate their applications, they demonstrated the detection of ribonucleic acid (RNA) sequences and small molecules. As further proof, they built and tested a strain-specific Ebola detector in under 12 h. The work performed by Pardee et al. opens the door for a range of real-world applications. It provides a practical format that can be used for engineered gene circuits. Furthermore, coupling the application of gene networks onto wax-printed, paper-based microfluidics presents even more possibilities. The use of these paper-based gene networks as low-cost, multiplexed sensors is particularly attractive and has the potential to meet Department of Defense requirements in far-forward fieldable detection.

Although the work performed by Pardee et al. has great potential, two significant issues must be addressed before the technology is fieldable. (1) Initial demonstrations of detection showed low sensitivity. Several obvious approaches to building signal amplification into the circuits are possible but have not as yet been explored. (2) It is important to determine whether a practical application of paper-based assays in the field could be bolstered by the ability to directly analyze environmental samples with minimal or no sample preparation. In this preliminary study, we investigate the second issue and probe the performance of a simple paper-based gene network in the presence of unrelated cellular lysate. This study addresses

the basic science around potential interference between environmental samples and embedded cell-free expression systems that remain open.

## 2. MATERIALS AND METHODS

### 2.1 Wax Printing of Paper Tickets

All paper tickets were prepared by wax-based printing on Whatman number 1 chromatography paper (Carolina Biological Supply Company; Burlington, NC) using a Phaser 8560 printer (Xerox Corporation; Norwalk, CT). Numerous patterns were tested, and the format shown in Figure 1 was selected. After the tickets were printed, they were baked in an oven at 110, 120, 130, and 140 °C for 1, 2, 5, and 10 min, respectively. Individual tickets were then cut out by hand and incubated overnight at room temperature in 5% w/v aqueous bovine serum albumin (MilliporeSigma; St. Louis, MO), as described by Pardee et al. Finally, the tickets were dried, and cell-free expression system and circuit deoxyribonucleic acid (DNA) template were applied.

### 2.2 Cell-Free Expression

We used the PURExpress system from New England BioLabs, Inc. (Ipswich, MA) in all of the experiments in accordance with the instructions included with the kit to determine the concentration of each component. For the plate reader experiments, total volumes were 10 μL, whereas for the paper-based experiments, total volumes were 2 μL. All of the tests were performed using a plasmid that contained super-folder green fluorescent protein (sfGFP) expressed from the T7 RNA polymerase promoter.[6]

### 2.3 Interfering Lysate Preparation

Lysates were prepared by growing laboratory-strain *Escheria coli* (DH5α competent cells) in 10 mL of Luria-Bertani broth at 37 °C with shaking at 220 rpm to an optical density of 1.4 absorption units. The cultures were pelleted at 4000 rpm for 10 min and resuspended in 10 mL of deionized water. The resuspended cells were cooled to 0 °C and lysed using an ultrasonic homogenizer (10 × 10 s) to produce the cell lysates, which were used as is and stored at 4 °C. To prepare a concentrated solution of the lysate, 10 mL of the dilute lysate was pelleted at 4000 rpm for 10 min and resuspended in 1 mL of Tris buffered saline.

### 2.4 Fluorescence Measurements

Liquid-phase experiments were performed on a BioTek (Winooski, VT) Synergy 4 multi-detection microplate reader with 485 nm excitation and 510 nm emission filters. Fluorescence was read every 5 min at 37 °C. Paper-based fluorescent imaging was performed on a custom imaging rig consisting of an EOS Rebel T3 digital single-lens reflex camera (Canon Inc.; Tokyo, Japan) with the same filters mounted in an incubator with a blue light trans-illuminator. Time-lapse images were taken every 5 min. The incubator was set to 37 °C for the duration of the time-lapse experiments.

## 3. RESULTS

### 3.1 Paper Ticket Optimization

We tested numerous wax-printed patterns to create spots of various sizes that acted as "wells" in the paper within hydrophobic wax barriers, as demonstrated by Lu et al.[7] After the tickets were printed, they were baked to allow the wax to permeate the paper, which created the wells. All tested maps were based on a 24 × 28 mm format so that they were compatible with a portable reader that was being developed in parallel for a separate but similar purpose. We varied the number of wells per ticket, the size of the wells, the thickness of the wax barriers, and the spacing between the wells. Different candidate formats were printed, baked at different temperatures (120–140 °C) for different amounts of time (1–15 min), and cooled. Dye was then pipetted onto the wells to investigate bleed-through between the neighboring wells. Several volumes of dye were tested for each candidate ticket. Ultimately, wells with 5 mm spacing, 2 pt lines, and 3.8 mm dots with 5 min of baking at 135 °C were selected for subsequent testing because these wells had the capability to hold at least 2 µL of dye without showing any bleed-through. Figure 1 shows each side of a sample ticket before and after baking.

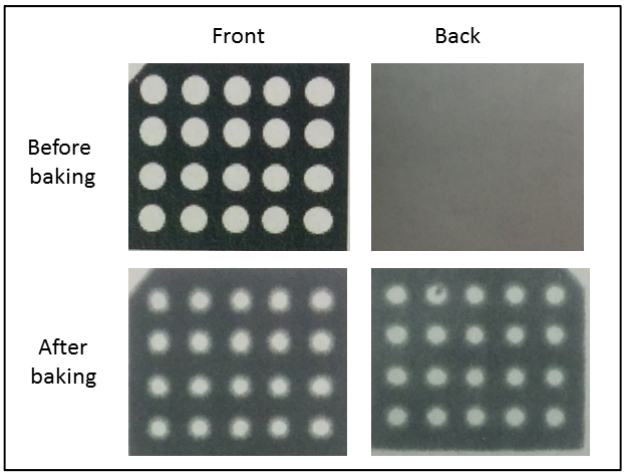


Figure 1. Sample test ticket maps. Front and back of a sample ticket before and after baking.

Wax was deposited on the front of the ticket by the wax printer and was then allowed to flow through the paper during baking to create hydrophobic wells. This arrangement of wells was selected from numerous candidates to show 2 µL of deposited liquid in the wells with no bleed-through.

### 3.2 Performance of Cell-Free Expression System in the Presence of Lysate

We first tested the performance of the PURExpress system using a plate reader. Two variables were tested: the concentration of template DNA and the presence or absence of cellular lysate (Sections 2.2 and 2.3). Figure 2 shows timecourse trajectories of triplicate wells containing 0, 12.4, or 124 ng of template DNA with and without cellular lysate. In both (with and without lysate), optimum performance of the assay was observed with 12.4 ng of DNA template. On the other hand, using 124 ng of DNA template actually appeared to inhibit the assay. When unconcentrated lysate was added to the reaction, there was a moderate reduction in activity for the 12.4 and 124 ng reactions.

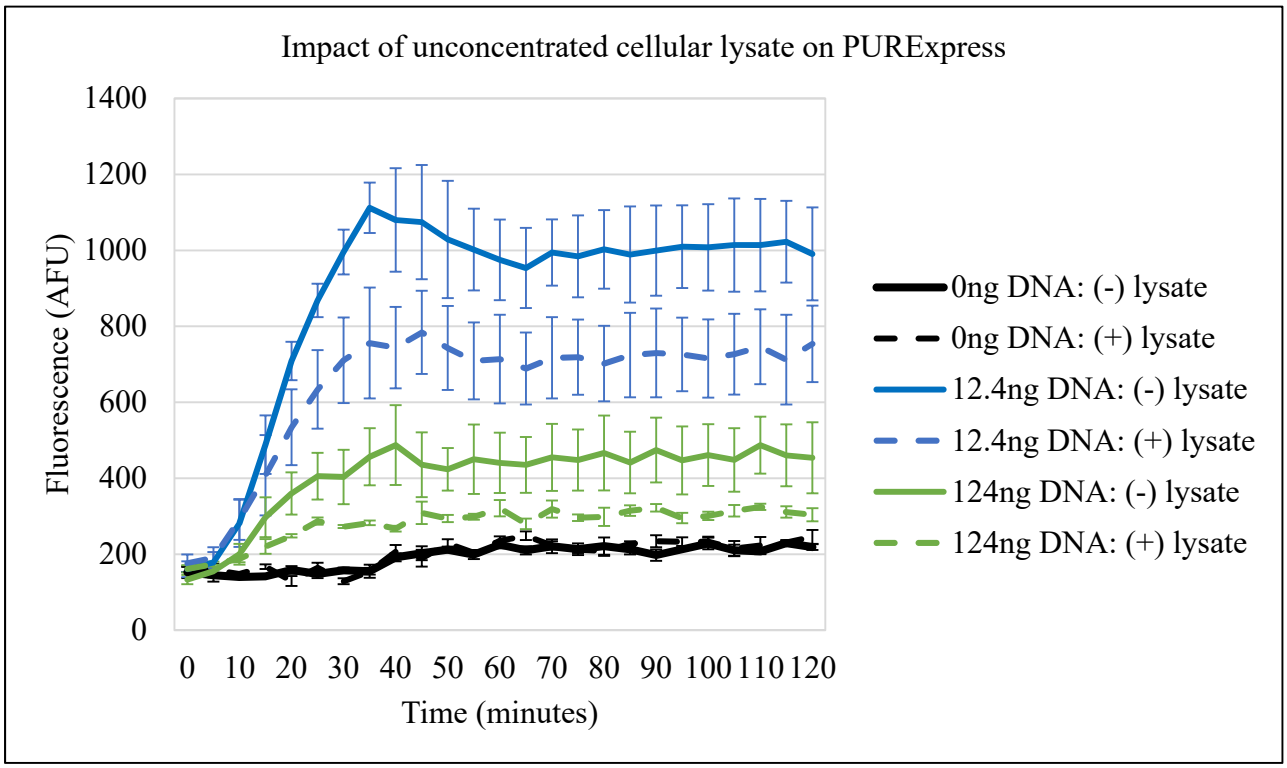


Figure 2. Impact of unconcentrated cellular lysate on PURExpress system. Time points of fluorescent trajectories on plate reader containing PURExpress system with (dashed lines) and without (solid lines) unconcentrated lysate and 0 ng (black lines), 12.4 ng (blue lines), or 124 ng (green lines) of template DNA. Error bars indicate standard error for triplicate samples.

Because the unconcentrated lysate demonstrated a moderate decrease in the performance of the PURExpress system, we repeated the tests using a 10× higher concentration of cellular lysate (Section 2.3). Figure 3 shows time course trajectories at different template concentrations (0, 12.4, and 124 ng), with and without concentrated lysate. The reaction with 12.4 ng of DNA performed the best; it showed a continued suppresion of the assay at the higher DNA amount. The concentrated lysate had a similar effect on the assay performance as the unconcentrated lysate, which showed a slight reduction in assay activity.

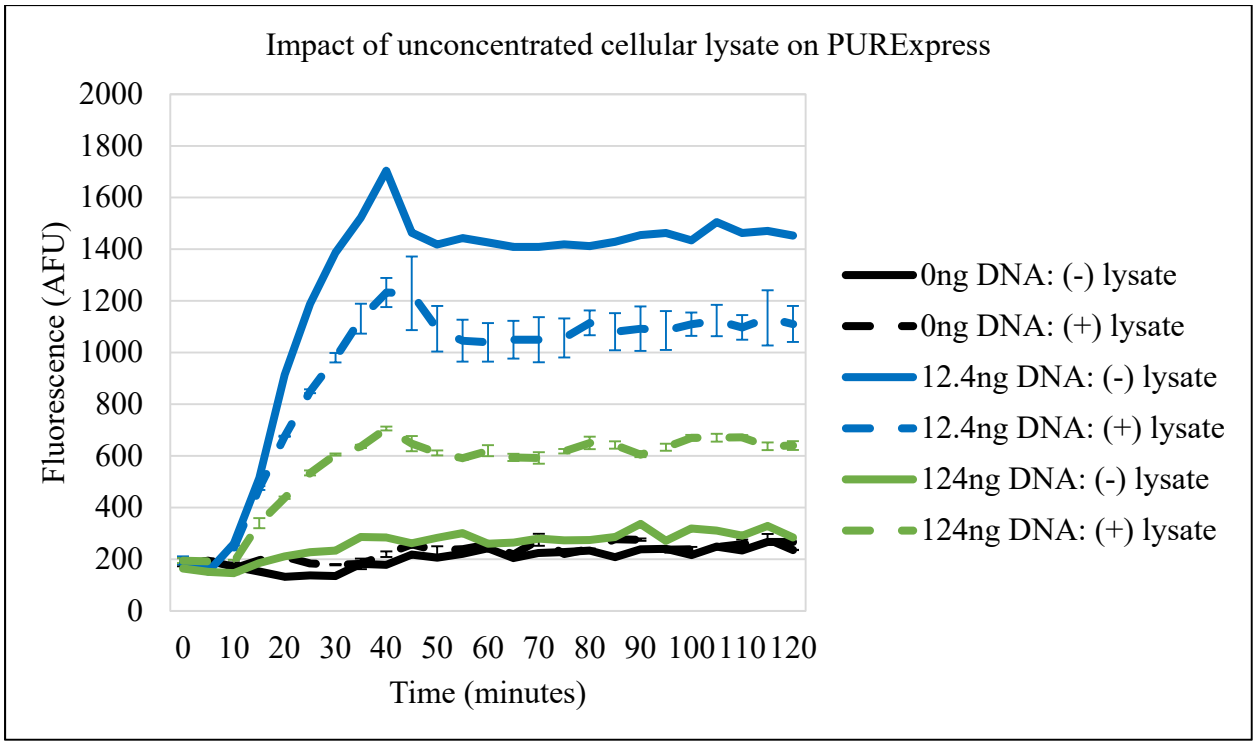


Figure 3. Impact of concentrated cellular lysate on PURExpress system. Time points of fluorescent trajectories on plate reader containing PURExpress system with (dashed lines) and without (solid lines) concentrated lysate and 0 ng (black lines), 12.4 ng (blue lines), or 124 ng (green lines) of template DNA. Error bars indicate standard error for triplicate samples. Traces without error bars were not performed in triplicate because of technical limitations.

### 3.3 Preliminary Demonstration of Functionality on Paper

Experiments that evaluated the performance of the PURExpress system with sfGFP plasmid were performed after components were freeze-dried on paper to determine whether the minimal effects of concentrated lysate that were observed in the plate reader experiments could be transferred. Figure 4 presents three images from a time-lapse experiment. The images show increasing fluorescence in appropriate spots. All images were recorded using the custom imaging rig (Section 2.4). Although the fluorescent change was not quantified at this point, no obvious difference was apparent.

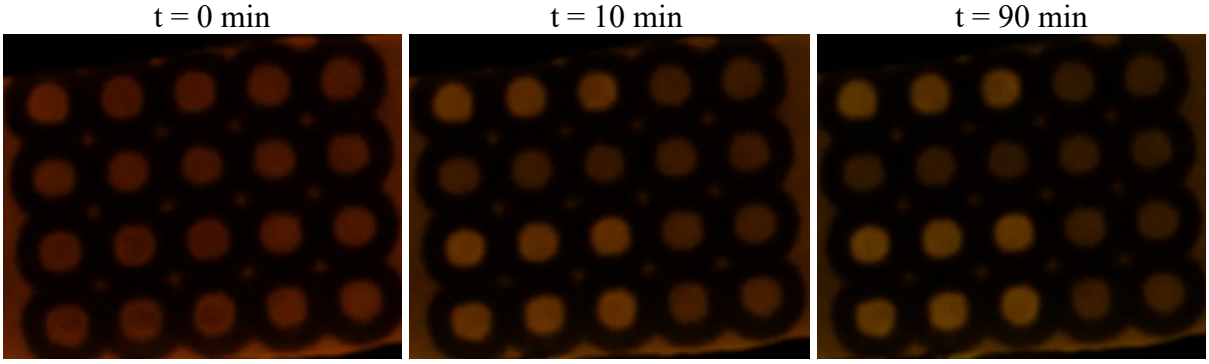

Figure 4. Fluorescent expression on paper tickets. Spots in row 1 contain no lysate, spots in rows 3 and 4 contain concentrated lysate, and spots in row 2 and columns 4 and 5 contain nothing.

## 4. CONCLUSIONS

The preliminary results presented in this study suggest that paper-based gene networks may react well to the contents of cellular lysates present in real-world samples. Only assay robustness against the presence of *E. coli* lysate was tested. No other organisms or environmental contaminants were present; therefore, a significant amount of testing will be necessary to determine the overall robustness of these systems. Ongoing work includes adapting image analysis software to provide a quantitative analysis of the system performance, which will result in a greater understanding of the effect of cell lysates on paper-based gene networks. In addition, this project focused on the simplest possible gene network to streamline initial tests. Impacts on performance for complex circuits may be more pronounced and may limit applicability of future paper-based assays. An important and surprising observation was that the concentration of template DNA had a huge impact on system performance. Future applications of paper-based gene networks will therefore need to be carefully optimized. The results of this project demonstrate that the presence of bacterial lysate from crude sample preparation does not inhibit functionality of cell-free expression systems that are freeze-dried on paper, which encourages further exploration of these systems for real-world use.

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## ABBREVIATIONS AND ACRONYMS

| | |
|---|---|
| DNA | deoxyribonucleic acid |
| RNA | ribonucleic acid |
| sfGFP | superfolder green fluorescent protein |
| DH5α | competent *Escheria coli* cells |

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