Optimizing cell-free reaction composition to accelerate toehold switch sensor response time

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ABSTRACT

Recent developments in the field of synthetic biology have made robust and rapidly deployable sensors for traditional and evolving biological threats achievable at low cost and with low burden for the Warfighter. Through the use of cell-free expression systems (CFEs), we are developing a threat detection platform which will mitigate the shortcomings of current state-of-the-art approaches such as PCR-based analyses and lateral flow immunoassays (LFAs). By lyophilizing CFEs on paper tickets and incorporating software-designed RNA toehold switches which recognize distinct threat agent nucleic acids, our proposed device can be programmed for novel threats, and be rapidly deployed to austere environments without the need for cold-chain storage or laboratory conditions for processing. Speed to response is integral to a field-forward biological threat detection technology. This presents a major challenge in achieving a practical CFE-based device, as our planned form factor will also combine detection with extraction and amplification of target biological material.

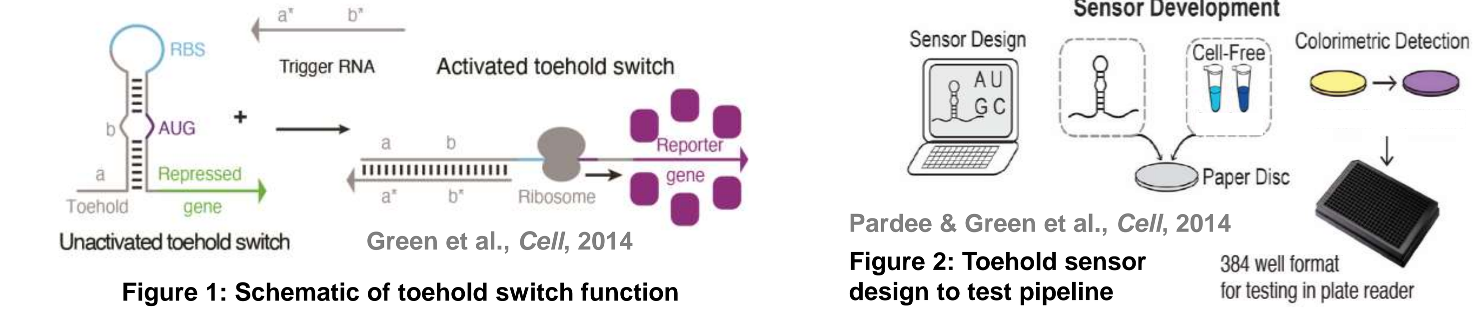

Figure 1: Schematic of toehold switch function

Figure 2: Toehold sensor design to test pipeline

Our current system utilizes an RNA toehold switch which regulates the expression of a lacZ gene, producing β-galactosidase, which in turn cleaves chlorophenol red-β-D-galactopyranoside (CPRG) substrate to produce an eye-readable colorimetric output.

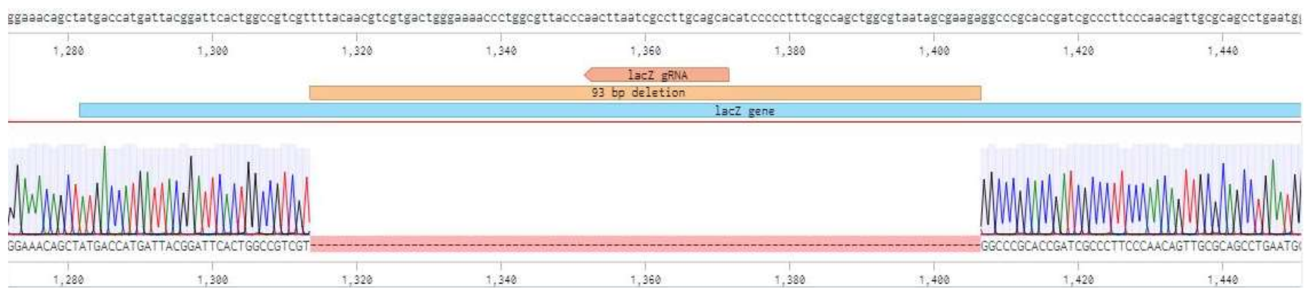

Figure 3: Alignment of DNA sequencing following the construction of the *ΔlacZα* strain of *E. coli*. The strain was created through CRISPR-Cas editing and the 93 base pairs deleted correspond to the LacZ α-subunit

Initial testing with the commercially available PURExpress (NEB) CFE system produced positive identification outside of our targeted timeframe of 30 minutes. Given this, we sought to increase reaction speed on paper tickets in a number of ways. In addition to exploring various reporter methods, we developed a LacZα knockout lysate as an alternative to the PURExpress system and to reduce background signal typically present with *E.coli*-based lysates. We conducted comparison studies in liquid reactions on plate readers and in freeze dried reactions on paper.

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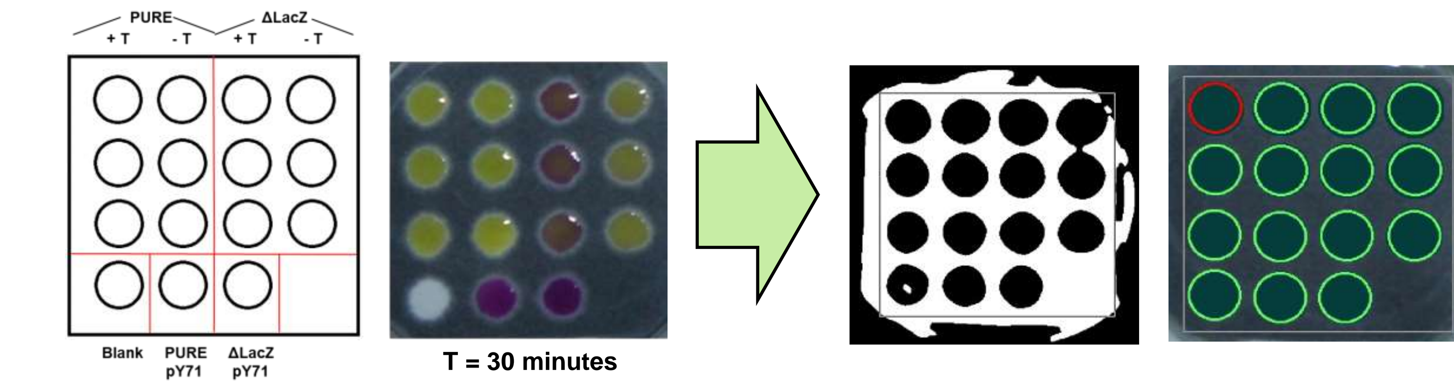
METHODS



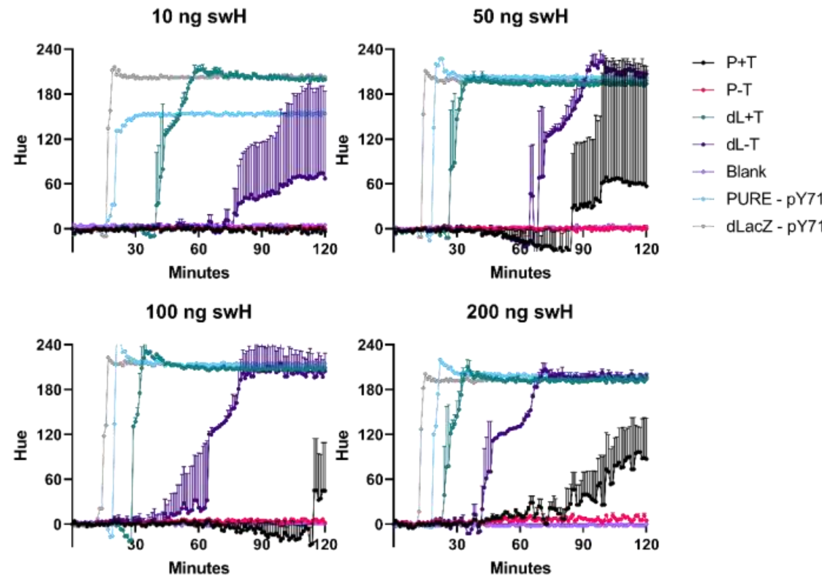


Figure 4 (above) : The paper ticket diagram and accompanying images show the basic image analysis pipeline. Reaction-spotted tickets are placed in a digital camera rig and images are captured at 1-minute intervals. Custom in-house designed software determines light thresholds for well position and employs edge-recognition analysis for hue change over time to quantify reaction progression. The ticket image shows a comparison of the PURExress system to our ΔlacZα lysate and indicates that our cell-free system is vastly superior to the commercially-available product in terms of signal speed.

Figure 5 (left): Change in hue over time data for the PURExpress vs ΔlacZα lysate is graphically displayed over a range of concentrations for our testing switch and trigger (T) pair, as well as our constitutively-expressing pY71-lacZ (+) controls.

REACTION OPTIMIZATION

Once we determined that our in-house lysate produced significantly faster results, reaction optimization studies were conducted to determine the ideal colorimetric substrate concentration, lyophilized reaction to rehydration volume ratio, and total lysate percentage in reaction mixtures.

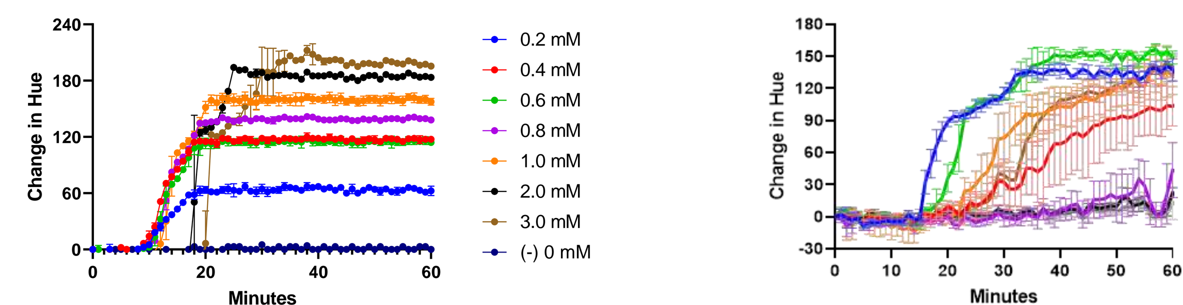


Figure 6: Change in hue over time data for CPRG concentration testing in ΔlacZα lysate on tickets with (+) controls DNA (pY71-lacZ) showing that while the amplitude of hue change increases with higher amounts of CPRG, higher concentrations inhibit reaction speed. A target CPRG concentration range was determined, as was a need to determine a balance between "eye-readability" and graphical image analysis signal times for the final device.

Figure 7: Change in hue over time for reaction/rehydration ratio test for ΔlacZα reactions with test switch (swH) and target (trH) DNA on paper. We determined that lyophilized reactions are faster if rehydrated with less DNA solution than the reaction volume spotted before drying. We found that although a 1.5:1 (reaction:rehydration) ratio has the fastest +trH signal, the slightly slower 1.75:1 ratio is superior based on the comparatively diminished –trH signal.

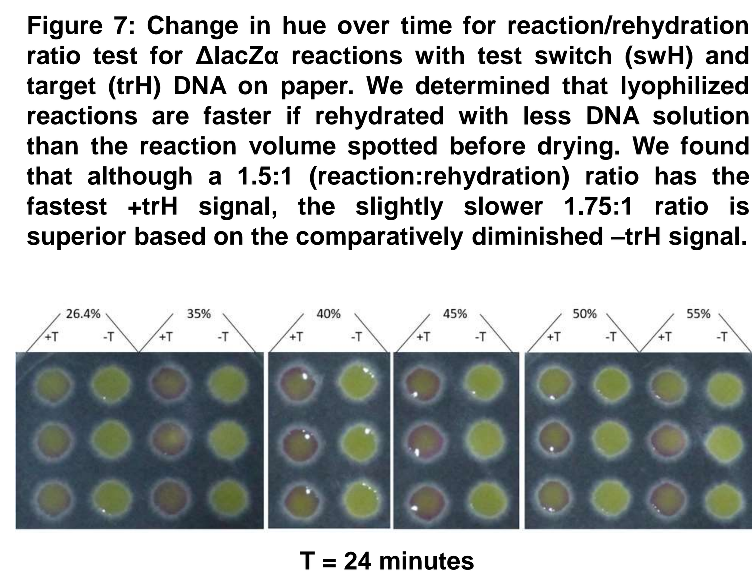


Figure 8: Ticket images of lysate percentage in reaction assay showing improved performance in the 40-45% range

ADDITIVE TESTING

Reaction additives to increase signal speed were also investigated, including supplementation with Triton X-100 and polyethylene glycol (PEG 8000), and by increasing the energy source adenosine 5`triphosphate (ATP).

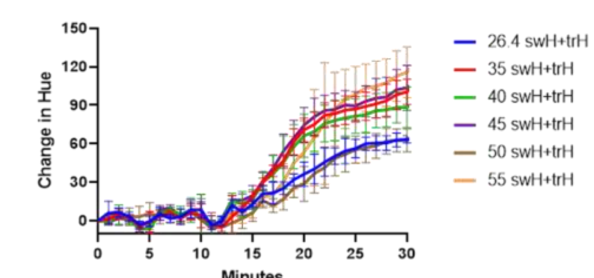


Figure 9: Varying lysate percentage in reaction data

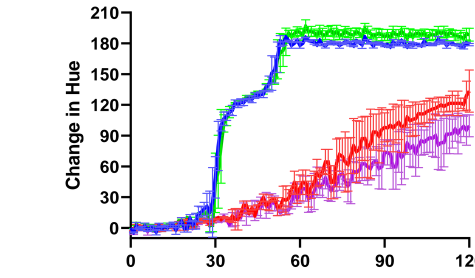


Figure 10: ATP supplementation data (units in μM)

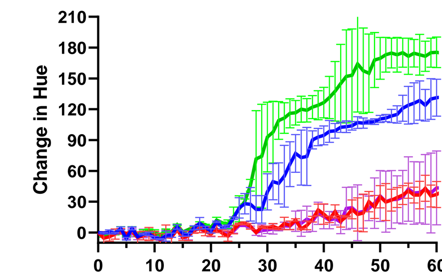


Figure 11: Triton X-100 addition to rehydration solution data

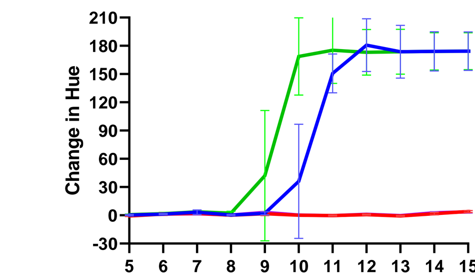


Figure 12: PEG supplementation data for control reactions

RESULTS

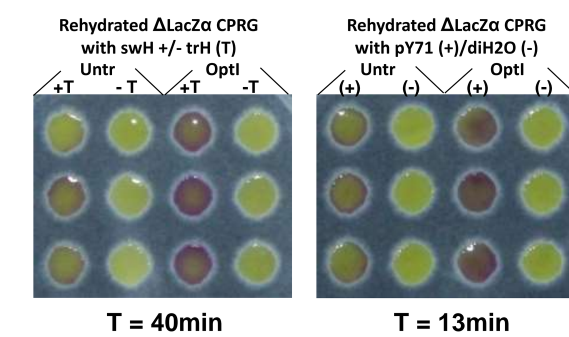


Figure 13: Ticket images showing optimized ΔlacZα lysate in comparison to "original recipe" reactions with test switch and controls

By combining our tested optimization strategies in our lysate system, we achieved a 20% reduction in time-to-signal in the control reactions and a 14% reduction in the toehold switch reactions, as compared to our initial lysate reaction conditions. With these efforts, the time to signal was reduced to ~25 min. This is a dramatic improvement over our preliminary testing utilizing the commercial system where the time to signal was ~1 hour for switch regulated reactions on paper.

ADDITIONAL SPEED-UPS

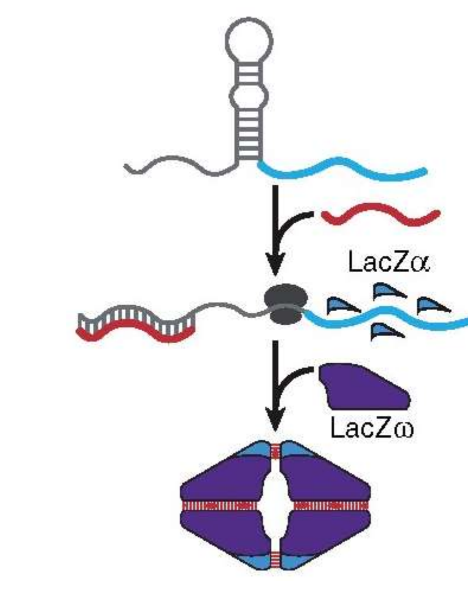
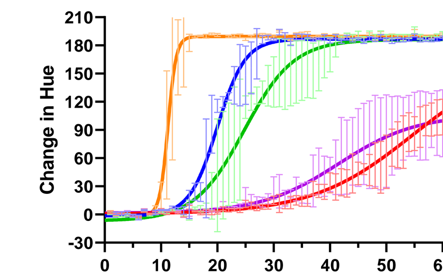


Figure 14: Schematic of α-complementation toehold for faster LacZ production



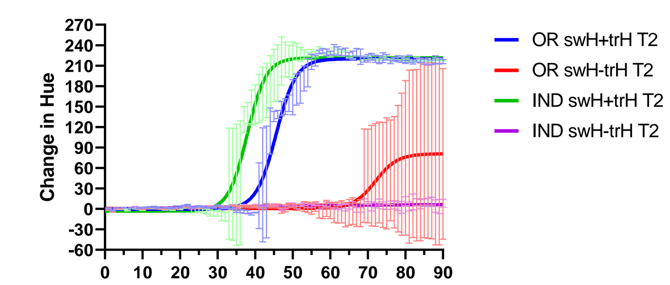


Figure 15, 16: Sigmoid fit data for α-complementation (top) and T7poly-induced lysate (bottom) ticket tests

DIAL-A-THREAT DEVICE INTEGRATION

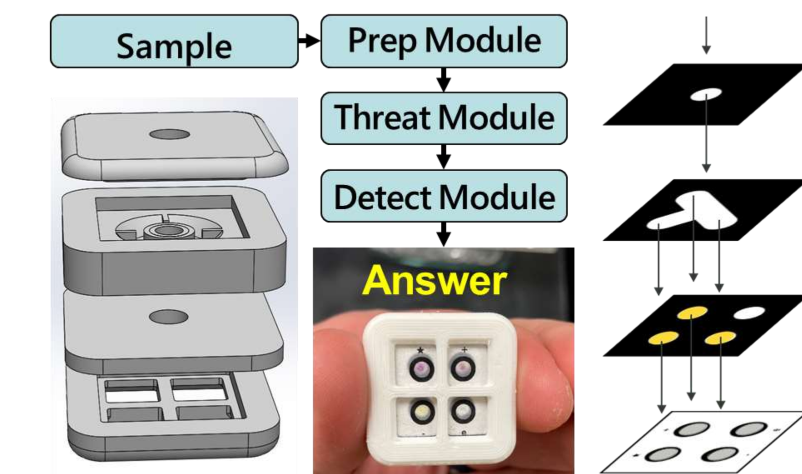


Figure 17: Schematic of prototype ticket cassette showing the stacked-ticket flow pattern and key components including a sample preparation module for DNA/RNA extraction and threat module for nucleic acid amplification. An actual prototype sensor device is pictured in the center.

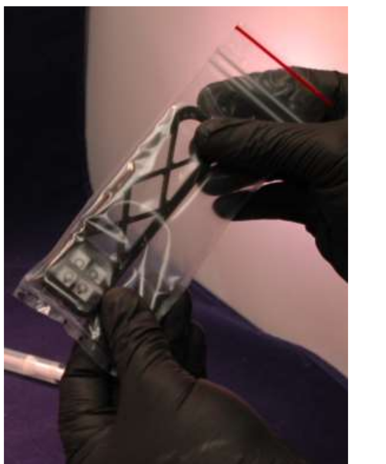


Figure 18: Sensor device in action with 3D-printed housing and support scaffold

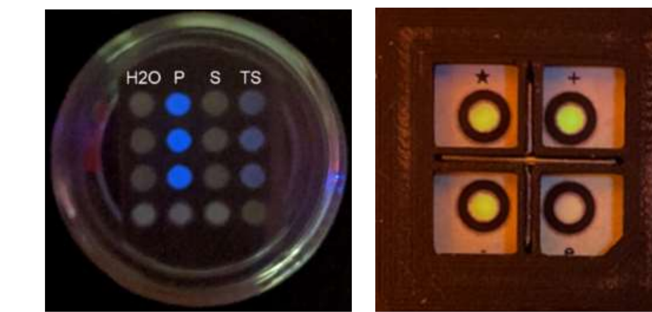


Figure 19 (left): Luciferase assay showing eye-readable luminescent signal on paper. Figure 20 (right): Utilizing fluorescein to investigate fluorescent signal in a prototype

In addition to developing novel toehold switches against threat agents for integration into fieldable sensors, we are also exploring alternative reporter systems and signal amplification methods. We ultimately aim to produce a multiplexed sensor platform that can be utilized on a variety of sample forms and in a variety of light conditions.

GOAL

Provide Warfighters bio-detection solutions for in-field use with:

- **Low cost, low burden, and minimal training**
- **No cold-chain requirements**
- **Simplicity and response time of LFIs**
- **Sensitivity and specificity of PCR**
- **Potential for rapid targeting and deployment for emerging threats**
- **No environmental release of modified organisms**

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