**A cell-free paper-based system for the detection of antibiotic**

**resistance mRNA via signal amplification**

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**Significance**

The discovery and usage of antibiotics to combat infection is responsible for saving many lives. However, this strong selective pressure has, over time, led to the emergence of resistance through mechanisms such as evolution and horizontal gene transfer1. In one sense this is not surprising, for both antibiotics and genetic reservoirs of resistance have existed in the environment for millions of years2. Further, while the broad action of many antibiotics derives from their ability to disrupt highly conserved processes, it also means that resistance to these actions are likely to have been broadly selected for3.

While antibiotics with both similar and diverging modes of action have been developed to get around this resistance, the continued success of this model requires a steady stream of novel antibiotics. Unfortunately, in the past 30 or so years, the number of new antibiotics in the pipeline has fallen to near zero, and while the approval of new antibiotics has slowed to nearly a halt, the emergence of antibiotic resistance has not4. Within the past decade, cases have emerged of infections that are totally resistant to all existing available antibiotics, rendering once-treatable infections deadly5. Worse still, a pan-resistance phenotype has been found to be conferred by a plasmid capable of being transmitted among Gram-negative members of the human microbiome6. While the development of new, safe antibiotics, must be a priority, it is also imperative to preserve the usefulness of existing options. This involves, among other strategies, improving the global surveillance of drug resistance and the promotion of rapid diagnostics to reduce the unnecessary use of antibiotics7. Hospitals in particular are loci for the development and maintenance of antibiotic resistance due to the presence of patients with weakened immune systems and the constant application of antibiotics, and antibiotic resistance bacteria are thought to kill at least 23,000 people each year in the U.S. alone8,9. Detecting the presence of genotypes for resistance to carbapenems, an antibiotic used when infections are known or suspected to be caused by multidrug resistant bacteria, enables precautionary measures to be taken to limit its spread10. Rapid antibiotic resistance identification has additionally been shown to reduce unnecessary antibiotic usage in hospital settings11,12. Many diagnostic applications of nucleic acid detection require attomolar (aM) sensitivity, but existing techniques for achieving this, such as PCR detection and immunoassays, are expensive and require special training to perform13,14.

Cell-free detection systems based on the isolation of cellular machinery in vitro, including engineered genetic circuits, are capable of performing bio-sensing and reporting mechanisms quickly and cheaply15. Remarkably they are also very stable, having been shown to maintain activity over the course of a year at room temperature when stored as freeze-dried pellets16. However, these systems are generally not sensitive enough for diagnostic applications15. Two recently described cell-free systems are utilized for the detection of antibiotic resistance genotypes as a proof of concept, with the primary purpose remaining the introduction of novel biochemical systems16,17. I propose taking advantage of signal amplification techniques drawn from both systems to develop a rapid and affordable cell-free system that is sensitive enough for the detection of antibiotic resistance in clinical settings and patient samples. The sensitivity of the paper-based toehold system as originally described is limited by the binding kinetics of the toehold switch to the target mRNA and/or the fact that each detection event only results in the translation of beta-galactosidase from a single transcript. In the Cas13a detection system, fluorescence is produced only upon a target mRNA binding event and is therefore directly associated with the amount of target mRNA present, limiting sensitivity. In the proposed system, each binding event results in the translation of multiple T7 RNA Polymerases, that are each capable of producing multiple transcripts that code for the Cas13a system. Each transcript can, in turn, generate multiple Cas13a enzymes that can each cleave multiple sacrificial RNAs, leading to the liberation of many fluorophores via collateral cleavage of RNase Alert, resulting in a larger final signal for each initial binding event.

**Innovation**

This project is innovative in that it seeks to overcome the sensitivity limitations of existing cell-free systems for the purpose of identifying the presence of one or more drug resistance genotypes in clinical settings to monitor resistance and inform antibiotic prescription choices. The components of this system were chosen to enable rapid, sensitive, affordable, and accessible detection of antibiotic resistance genes, and to my knowledge they have not been previously combined for this purpose. Specific detection of mRNAs will be accompanied by a cascade of signal amplifications to obtain the attomolar sensitivity that clinical applications require. The system will be cheap to produce and easy to use in an hour or less, requiring only a plate reader to detect the fluorescence. We envision this tool initially being used to monitor hospital settings for the presence of target antibiotic resistance genes. For all the reasons described, we would like it to eventually be applied to patient samples to help inform the antibiotic prescription choices of doctors.

**Approach**

**Aim One – Assemble and optimize cell free system components for resistance mRNA detection**

The proposed system hopes to achieve its goal of increased sensitivity by incorporating multiple signal cascades to allow a small number of target mRNA binding events to produce a detectable fluorescent output. An initial description of the system composition will be followed by an exploration of the experiments needed to test its effectiveness.

The initial detection of target mRNAs will be based on their binding to toehold switches via complementary base pairing. Toehold switches are similar to regular mRNA sequences in that they contain the traditional features that enable translation: a ribosome binding site (RBS), a translational start site, (the AUG codon) site, a coding sequence for the T7 RNA Polymerase, and a hairpin terminator that takes the place of a stop codon. However, they also contain an upstream sequence that is complementary to the bases around the start codon but not the codon itself. Additionally, there is a ‘toehold’ tail sequence that does not bind the start site but can be engineered to be complementary to any desired sequence. In the absence of this sequence, the start codon is sequestered and no translation can occur. In the presence of a complementary RNA sequence, toehold binding unwinds the hairpin and enables translation of T7 RNA Polymerase to occur18. The next step in the system will be transcription by T7 RNA Polymerase of a gene construct that codes for the Cas13a protein from *Leptotrichia wadei* under the control of a T7 promoter. Cas13a is a Cas9 ortholog that cuts RNA instead of DNA. However, after cutting its initial target, it nonspecifically cleaves nearby non-target RNAs as well19. This feature has been exploited for the detection of mRNA by having Cas13a cut a target mRNA in the presence of RNase Alert v2 (Thermo Fischer), an RNA-quencher hybrid that produces a fluorophore upon cleavage17. The gene will also code for a guide RNA for the Cas13a. Upon translation of Cas13a by the cell-free translational machinery, this guide RNA will direct the protein to bind and cleave a sacrificial mRNA supplied to the system. This system will also utilize RNase Alert v2 to generate fluorophores with excitation/emission maxima at 490/520 nm. The output fluorescence will be measured at 37 degrees Celsius using a BioTek plate reader over one hour, with confidence in detection being determined via a two-tailed Student t-test between the signal of a negative control (background) and the background-subtracted signal of the sample17.

The cell-free system will therefore require transcriptional and translational machinery, toehold switch mRNAs, a high copy amount of the Cas13a/gRNA gene construct, sacrificial RNA complementary to the gRNA sequence, and RNase Alert v2. The transcriptional and translational machinery will be supplied by the commercially available PT7 expression system20. I will also add Protector RNase Inhibitor (Sigma-Aldrich 3335399001) to the system to minimize nonspecific RNA cleavage. Components will be combined, flash frozen in liquid nitrogen (-80 degrees Celsius), and freeze dried overnight before being used, which has been shown to not eliminate their activity. While the construction of each these components individually is trivial, the primary challenge will lie in finding the optimal ratios of toehold switch mRNA, Cas13a/gRNA gene copy number, sacrificial RNA, and RNase Alert to ensure that the output of any amplification step is not limited by its inability to fully process the input from the previous step. Different concentrations of these components will be tested based on their ability to produce fluorescence above background in the presence of 3000nM ampicillin resistance mRNA introduced in the presence of the buffer for the cell-free expression system described by Shimizu et al20. This target mRNA and concentration produced the highest signal of any resistance mRNA/concentration combination test by Pardee et al. in the development of the paper-based toehold switch detection system16. For each concentration to be varied, I will start with the reported concentrations of each and explore a two-level design space that ranges from 0.1x-10x these initial concentrations using a full factorial design of experiments approach, resulting in a total of 16 experiments ((2 levels for each component) ^ (number of components)). Ideally, these experiments will identify components whose reported concentrations are limiting for detection in this new system and therefore need to be increased for optimal functioning. If the entire design space yields no differences in detection response (including total failure to detect ampicillin mRNA in any condition), I will continually repeat the 16 experiments while broadening the concentration design space by 10-fold on the high and low ends until detection is successful.

**Aim Two – Explore sensitivity of single and multiplex detection at diagnostically-relevant concentrations and in environmental and patient samples**

With a better understanding of how the different cascades interact, I will seek to challenge the sensitivity of the system by titrating the concentration of ampicillin resistance mRNA down from 3000nM to 1aM and checking if the system is still able to generate fluorescence distinguishable from background. Paper-based versions of the toehold switch and Cas13a detection systems were capable of 3nM and 20fM sensitivity, respectively16,17. If the system fails to detect the mRNA at these concentrations, I will initially return to a design of experiments approach to attempt to further optimize relative component concentrations to increase sensitivity. However, if this fails, I will test the implementation of a reverse transcription/recombinase polymerase amplification step which was found to reduce the paper-based Cas13a system’s limit of detection from 20fM to 20aM17. In this approach, samples containing target mRNA will be pre-treated using TwistAmp Basic and TwistAmp Basic RT kits. In these reactions, target mRNA is reverse transcribed into DNA, the DNA is amplified via recombinase polymerase amplification21, and then additional target mRNA copies are generated via transcription of the DNA sequence using a T7 RNA Polymerase. Importantly, because the T7 RNA Polymerase is already a part of our system, an orthogonal polymerase such as T3 RNA Polymerase would need to be used either to perform the initial amplification of target mRNA or to create Cas13a transcripts. The resulting solution containing the amplified target would then be mixed with the proposed detection system. The downside of this step is the additional two-hour incubation it requires, which more than doubles the overall time of the assay.

Once the system has been validated for the attomolar detection of ampicillin resistance mRNA, I would seek to test it against resistance mRNA targets that represent a larger threat to society, such as the carbapenem resistance highlighted above10. Two potential gene targets in this area are *Klebsiella pneumoniae* carbapenemase (KPC) and New Delhi metallo-beta-lactamase-1 (NDM-1)22,23. Rational design of toehold sequences allows easy generation of toehold switches complementary to any target mRNA24. I would then attempt to titrate down detection of these mRNAs from low nanomolar down to attomolar concentrations. With sensitive tests for each of these two genes, I would attempt to detect both at the same time. Detection of multiple targets at once reduces the number of tests that need to be run, providing a more comprehensive profile of a sample. Cross-talk between the two toehold switches should not be an issue as more than 10 orthogonal toehold switches have been identified16. I would accomplish this via the implementation of AND gate logic for the production of Cas13a. Rather than coding for T7 polymerase, I would have each of the toehold switches for the different mRNA targets code for one half of the cooperative *sicA/inF* transcriptional activator, and I would replace the T7 promoter upstream of the Cas13a gene with a sicA/invF-responsive promoter25. The final goal would be to test the system on environmental and patient samples, which are often complex matrices with components that can interfere with detection of already low abundance target molecules. Since the Cas13a detection system was found to be nonfunctional at serum concentrations greater than 2%, dilution of samples in more complex matrices would be required17. If it is found that this unacceptably lowers the sensitivity of the system, I will attempt to utilize an engineered opsonin mannose-binding ligand hybrid system to enrich the amount of target in a sample. These devices are capable of removing pathogens from whole blood as it flows through an extracorporeal circuit via interactions with mannose-binding lectins attached the surface of magnetic beads. After capture of pathogens, a magnetic field can be applied to isolate the beads from the blood26. Testing the detection output of my system at increasing levels of purification using this device is a potential way to rescue this sensitivity.

**Summary**

Antibiotics have been an effective weapon against infection for the better part of a century, but the widespread emergence of resistance to these drugs has rendered many previously benign infections more difficult to treat. While the development of new antibiotics is essential, strategies for combating the increase and the spread of resistance genotypes will allow maximization of the effectiveness of existing tools. Current systems for detection of resistance genotypes in clinical settings can be expensive, slow, or require expertise to use. Cell-free systems for resistance mRNA detection are cheap and easy to use, but do not have the sensitivity necessary for diagnostic applications. The cell-free paper-based system that I propose seeks to combine the signal amplification techniques of previous cell-free systems to achieve increased sensitivity, making it a potentially attractive option for the scalable detection of resistance genotypes.

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