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Technical Note

Assessment of Colorimetric Reporter Enzymes in the PURE System

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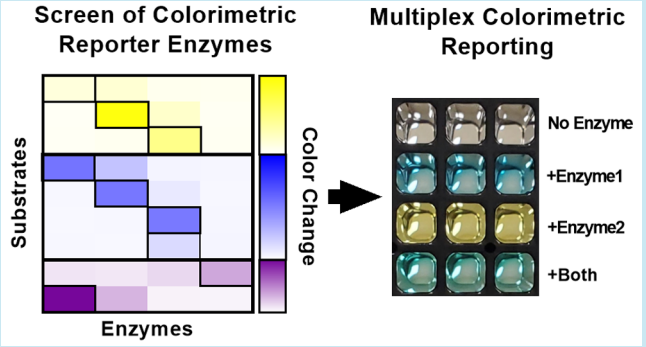
ABSTRACT: Colorimetric reporter enzymes are useful for generating eye-readable biosensor readouts that do not require a device to interpret, an attractive property for applications in remote or developing parts of the world. The use of cell-free gene expression further facilitates such applications *via* amenability to lyophilization and incorporation into materials like paper. Currently, detection of multiple analytes simultaneously with these systems requires multiple reactions or a device. Here we evaluate seven enzymes and 15 corresponding substrates for functionality in a particular cell-free expression system known as PURE. We report eight enzyme/substrate pairs spanning four enzymes that are compatible with PURE. Of the four enzymes, three pairings exhibit no cross-reactivity. We finally show that at least one pairing can be used to create a third color when both are present, highlighting the potential use of these reporters for multiplex sensing.



KEYWORDS: *cell-free systems, colorimetrics, reporter enzymes, PURE system*

INTRODUCTION

Eye-readable colorimetric reporters are highly attractive for sensing applications in resource-limited environments, such as developing economies or remote locations. In recent years, cell-free gene expression (CFE) systems[1] expressing such reporters have been leveraged for this reason while also offering shelf stability,[2,3] low cost, incorporation into paper and other materials,[4−8] and application-relevant sensitivities and specificities. Recent studies have demonstrated the detection of a variety of analytes, including pathogens,[4,9−11] biomarkers,[5,12,13] explosive precursors,[14] vital nutrients,[15] and water contaminants.[16−18]

Several advances have been made to improve the interpretation of CFE sensors, including sample-specific visual calibration,[15] portable readers,[17,19] electrochemical multiplexing,[20] and interfacing with a glucose meter.[11] Ultimately, elimination of any device in favor of eye-readable assays is attractive for addressing cost, weight, and ease-of-use limitations. To date, nearly all colorimetric CFE sensors have relied on LacZ as the reporter enzyme,[4] with some recent work using catechol 2,3-dioxygenase.[18] Expanding the range of enzymes and substrates that can be used for CFE sensors offers an expanded palette of colors, the potential for improved performance, and multiplexing of multiple sensors in a single reaction with unique outputs. We therefore set out to identify a set of colorimetric reporters that function in CFE. We focused on performance in the PURE system[21] because of concerns over background signal from homologous genes in host lysates, which we later confirmed. From seven enzymes and 15 substrates, we present four enzymes and eight substrates that are compatible with PURE, including at least one combination that is viable for multiplexing.

RESULTS AND DISCUSSION

We identified seven candidate colorimetric reporter enzymes from the literature (Tables 1, S1, and S2). Each enzyme has one to three substrates, all commercially available, offering one to three color options per enzyme. We evaluated each substrate for background signal in PURE reactions and for "decoupled" activity where the enzyme was expressed in PURE and then the substrate was added. Of the 15 enzyme/substrate pairs, 10 showed activity spanning five enzymes, and one additional pair was eliminated for high background, leaving four enzymes and nine substrates for further testing. Figure 1A depicts the colors observed for the remaining pairs (see Supplementary Results and Figures S1−S3 for additional details about these screens). We confirmed that six of the nine substrates showed high background activity in lysate-based CFE, presumably due to endogenous enzymes (Figure

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Table 1. Summary of Enzymes and Substrates Used in This Study[a]

Gene	Substrate Name	Pre-Color	Observed Post-Color	Absorbance Wavelength (nm)	Decoupled Performance	Coupled Performance
LacZ	ONPG	Colorless	Yellow	405	Works but fades	Works but fades
	X-gal	Colorless	Blue	615	Works	Works
	CPRG	Yellow	Purple	585[b]	Works	Works
GusA	pNPG	Colorless	Yellow	405	Works	Works
	X-GlcA	Colorless	Blue	615	Works	Works
	Mag-GlcA	Colorless	Blue[c]	585[b]	Works	No signal
CelB	pNP-Glc	Colorless	Yellow	405	Works	Works
	X-Glc	Colorless	Blue	615	Works	Works
AES	pNP- butyr	Colorless	Yellow	405	No signal	n/a
	Mag-butyr	Colorless	Purple[c]	615[b]	Works but cloudy	Works but cloudy
NagZ	pNP-GlcNAc	Colorless	Yellow	405	No signal	n/a
	X-GlcNAc	Colorless	Blue	615	No signal	n/a
PhoA	pNPP	Colorless	Yellow	405	No signal	n/a
	BCIP + NBT	Colorless	Blue	615	No signal above high background	n/a
Est2	pNP-hexanoate	Colorless	Yellow	405	High background	n/a

[a]All substrates were added at a final concentration of 5 mM within 5 μL CFE reactions. [b]These substrates required adjusted wavelengths to account for unexpected color changes or instrument overflows. See the Supplementary Results and Figure S3. [c]These substrates did not appear with the expected color by eye when tested.

S4). We then tested the cross-reactivity of the enzymes and found no crosstalk between LacZ/GusA, LacZ/AES, or CelB/AES (Figures 1B and S5).

We next evaluated the reporter/substrate pairs "coupled", *i.e.*, with the substrate added at the beginning of the reaction. We found that higher concentrations of substrate did not decrease the observed signal over the 0−5 mM range tested (Figure 1C), despite inhibition of control reactions (Supplementary Results and Figures S6−S9); however, in some cases higher concentrations slowed the signal as quantified by the time when the trajectory was different from the no-substrate control by the t test ($p < 0.005$, first 10 min ignored; Figure 1D). We note that speed is a critical factor in some sensing applications. Only GusA/Mag-GlcA did not work in the coupled case. Finally, we tested combinations of reporters that can be used together. After screening several combinations (Supplementary Results and Figures S10 and S11), we showed that at least one pair of these reporter/substrate combinations can produce four

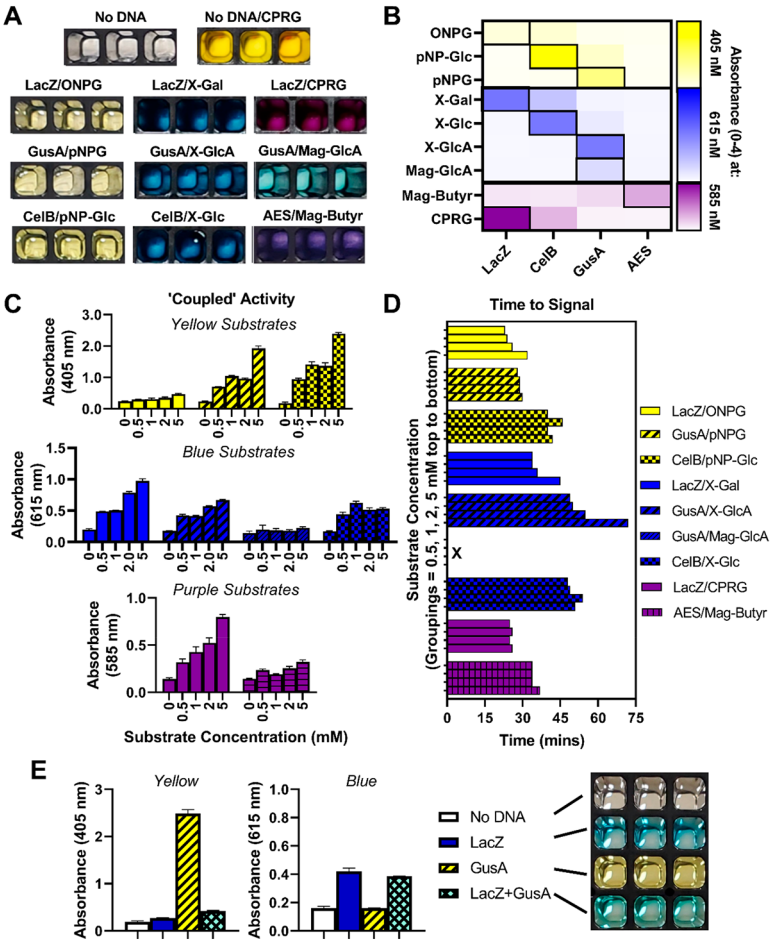

Figure 1. Colorimetric reporters in PURE reactions. (A) Images of plate wells exhibiting the visual color change for each enzyme/substrate pair in triplicate. (B) Cross-reactivity of the enzymes. Absorbance values shown here are after incubation for 1 h. Time-course trajectories and end-point images are available in Figure S5. (C) End-point measurements of "coupled" reactions at various substrate concentrations. Bar colors and patterns correspond to the legend in (D). (D) Time to detectable signal for "coupled" reactions. Each group of four columns represents substrate concentrations of 0.5, 1, 2, and 5 mM from top to bottom. The time to detectable signal was determined by statistical test of each trajectory (Figure S9) compared to the no-substrate control. The "X" indicates that no signal above background was detected for any concentration of GusA/Mag-GlcA. (E) Color changes observed after 3 h for "coupled" reactions with DNA encoding for LacZ, GusA, both, or neither with 5 mM pNPG and 5 mM X-Gal at 405 nm (yellow, left) or 615 nm (blue, middle) or by image (right). Each row in the image represents triplicate reactions.

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distinct colors from two enzymes, suggesting utility for multiplexing of sensors in a single reaction (Supplementary Results and Figure 1E).

CONCLUSION

We offer here a panel of colorimetric reporters for synthetic biology. While we focused on the PURE CFE system, the same enzyme/substrate pairs could be useful in cell-based or lysate-based CFE applications depending on endogenous enzyme activity. We note that optimization of the expression conditions could improve the performance of some these pairs; as an example, recent work showed expression of active alkaline phosphatase (PhoA) in PURE by addition of a disulfide bond enhancer and zinc.[22] For applications in sensing, our results show that simply optimizing the concentration of the substrate used can play a significant role in signal strength and response time. The demonstration of potential multiplexing of CFE sensors with an eye-readable signal increases the range of field-forward applications possible with CFE. In general, our work expands the synthetic biology toolkit for the development of sensors.

METHODS

Construct Assembly. Linear fragments of reporter genes were synthesized (Integrated DNA Technologies, Inc.) with ends overlapping the insertion site of the pY71 vector. Reporters were cloned into the vector using the In-Fusion Seamless Cloning Kit (Clontech). Sequence-verified circular templates were amplified in *Escherichia coli* DH5α cells, isolated using a Miniprep Kit (Qiagen), quantified using a spectrophotometer (Thermo Scientic NanoDrop 1000), diluted to 40 nM, and stored at −20 °C until use.

PURE Reactions. Reactions used the PURExpress *In Vitro* Protein Synthesis Kit (New England Biolabs) prepared according to the manufacturer's protocol at volumes of 5 μL with final concentrations of 0 or 2 nM DNA and 0−5 mM substrate. Substrates were resuspended in the solvent indicated in Table S1 to a stock concentration of 100 mM. Reactions were loaded into 384-well black microplates with clear bottoms (Corning), covered with a plate sealer (Thermo Scientific, cat. no. 3501), centrifuged at 1500*g* for 1 min, and incubated at 37 °C for 2 h. For decoupled reactions, the reaction mixtures were incubated, and then the substrate was added to a final concentration of 5 mM, bringing the total volume to 5 μL. To track colorimetric or fluorescence changes, plates were incubated with shaking in a Biotek Synergy H1 microplate reader at 37 °C for 1−2 h with reads every 1−5 min. For absorbance, read settings are given in Table 1; for fluorescence, measurements were made with 485 nm excitation and 528 nm emission. Images were taken with a Samsung Galaxy smartphone. The images in Figure 1A were cropped from Figure S5.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.1c00360.

Supplementary Results, Figures S1−S11, and Tables S1 and S2 (PDF)

Sequence files for plasmids used (ZIP)

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Author Contributions

C.E.S., J.B.M., and S.M.B. designed experiments, performed experiments, and analyzed data. G.E.M. performed experiments. M.W.L. obtained funding, directed experiments, analyzed data, and wrote the manuscript.

Notes

The authors declare no competing financial interest.

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