Supporting Information

Development of a High-Throughput Biochemical Assay to Screen for Inhibitors of Aerobactin Synthetase IucA

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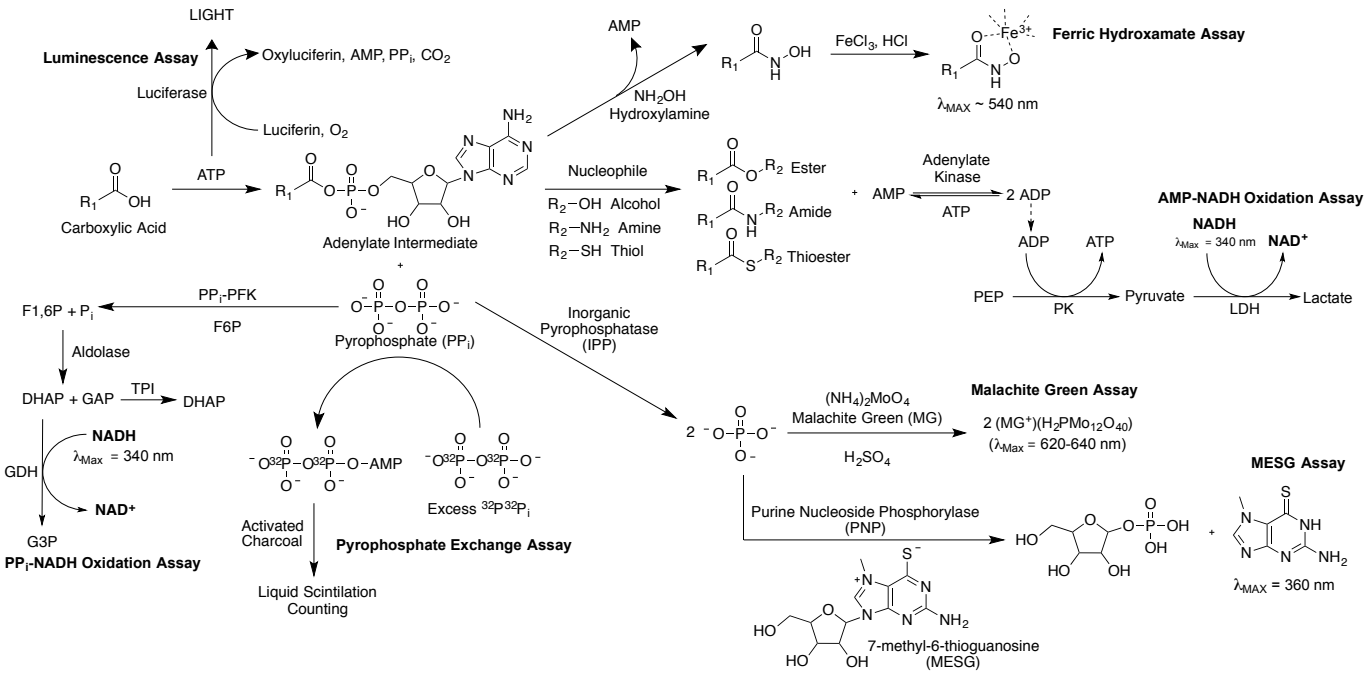

FIGURE S1. Selected assays for monitoring the activity of adenylate-forming enzymes. Synthetases such as the NIS synthetase lucA catalyze two partial reactions. In the first, a carboxylate-containing substrate is converted to an activated acyl-adenylate intermediate. In the second, the adenylate intermediate is attacked by an alcohol, amine, or thiol-containing substrate to form an ester, amide, or thioester-containing product, respectively. Seven selected assays were considered for monitoring lucA activity based on detecting either AMP, PP_i, or the ligation product are outlined above. The ferric hydroxamate assay was initially considered for the primary screening assay, but was found to have insufficient sensitivity.[1] The malachite green (MG) assay was determined to be much more sensitive and was employed as the primary screening assay.[2] The luminescence assay was employed as a secondary orthogonal assay to evaluate purchased compounds.[3] The coupling enzymes from the AMP-NADH oxidation assay were utilized to evaluate the promiscuity of the most potent inhibitors.[4] The MESG assay is a continuous assay based on phosphate detection that closely complements the MG assay.[5,6] The PP_i-NADH oxidation assay parallels the PP_i-NADH oxidation assay, but by virtue of being coupled to PP_i production, the assay can specifically monitor the adenylation partial reaction.[7] Finally, the PP_i exchange assay is a radiometric technique that specifically measures the reverse of the adenylate-forming partial reaction.[8]

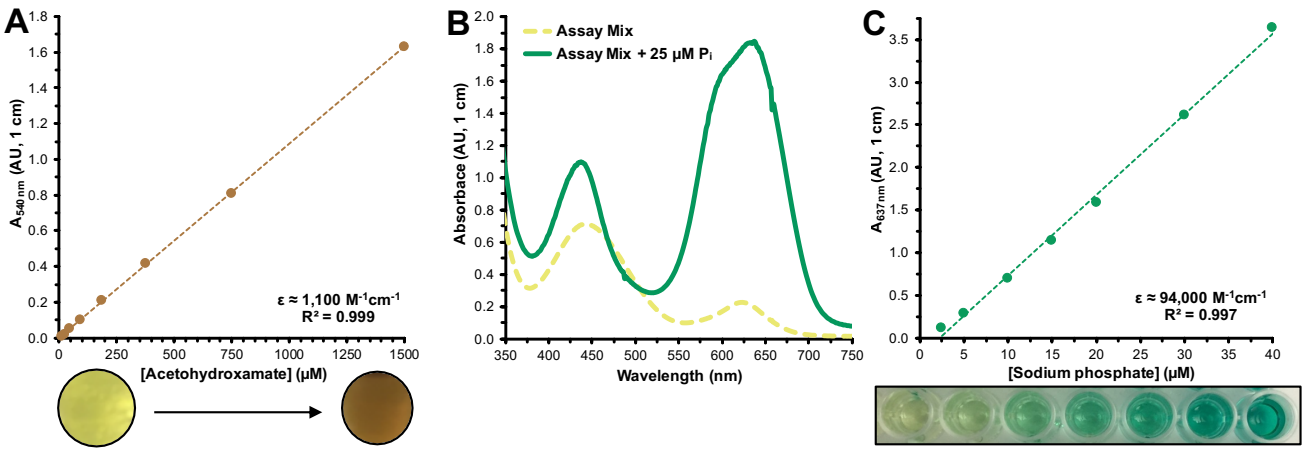

FIGURE S2. Evaluating reporter assays. (**A**) Standard curve demonstrating the sensitivity of the ferric hydroxamate assay employing acetohydroxamate:Fe^{3+} as a proxy analyte. Below, photographs of negative (no lucA) and positive control reactions illustrating the colorimetric readout. (**B**) The absorbance spectra of MG assay solutions with and without the addition of sodium phosphate highlighting an optimal detection wavelength of 620-640 nm. (**C**) Standard curve revealing the sensitivity of the MG assay using sodium phosphate standards. Below, photograph of the developed standards illustrating the colorimetric readout.

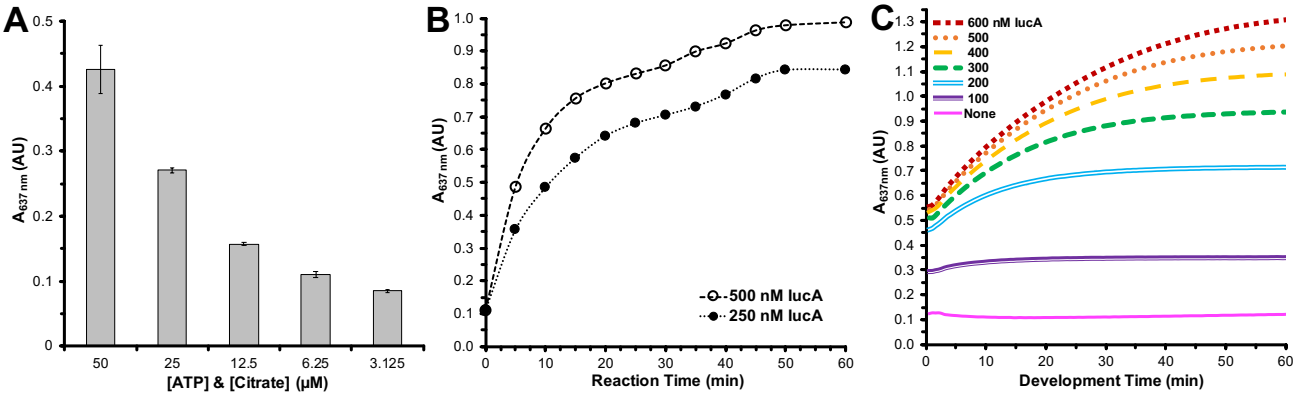

FIGURE S3. Optimizing MG assay parameters. (**A**) Comparison of absorbance signal obtained after carrying out the lucA-catalyzed reaction with various concentrations of substrates ATP and citrate. Plotted is mean of triplicate reactions ± SD (**B**) The reaction progress curves at two enzyme concentrations. (**C**) Monitoring the assay absorbance signal following addition of development solution for reactions catalyzed by various concentrations of lucA.

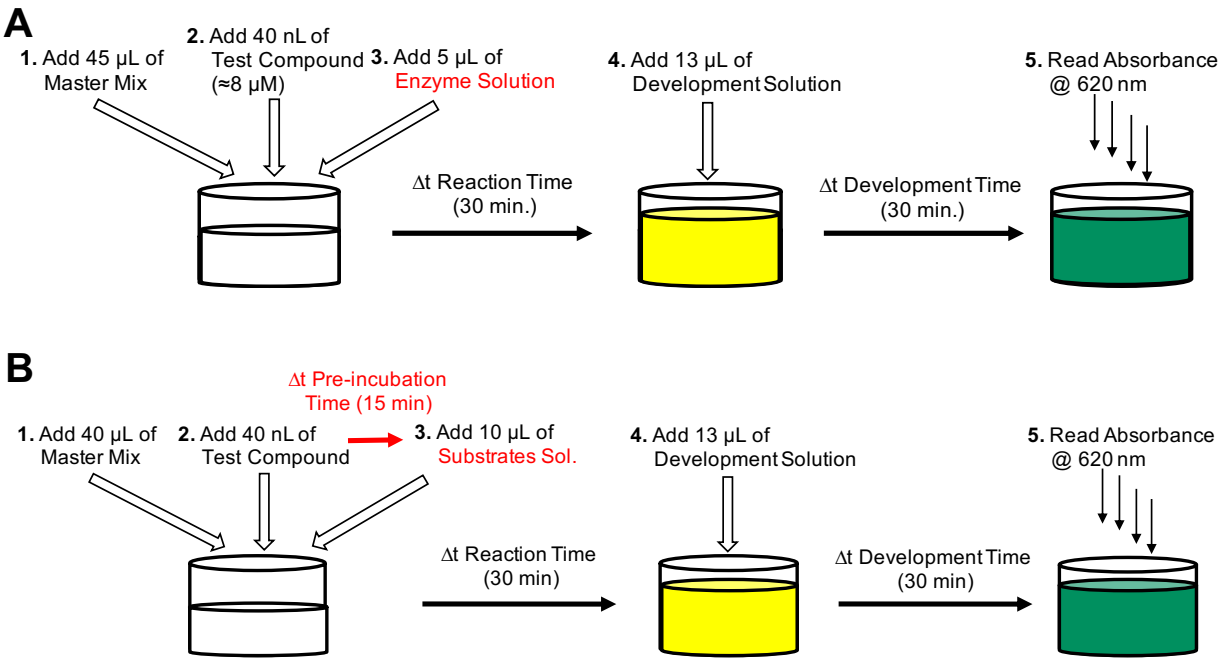

FIGURE S4. High-throughput screening protocols using the MG assay. (**A**) "Enzyme-initiated" protocol in which test compounds were added to a master mix solution containing substrates prior to reaction initiation with a solution of lucA. (**B**) "Substrate-initiated" protocol in which test compounds were pre-incubated with a master mix solution containing the target enzyme lucA for 15 min prior to reaction initiation with a solution containing the three substrates ATP, citrate, and hydroxylamine.

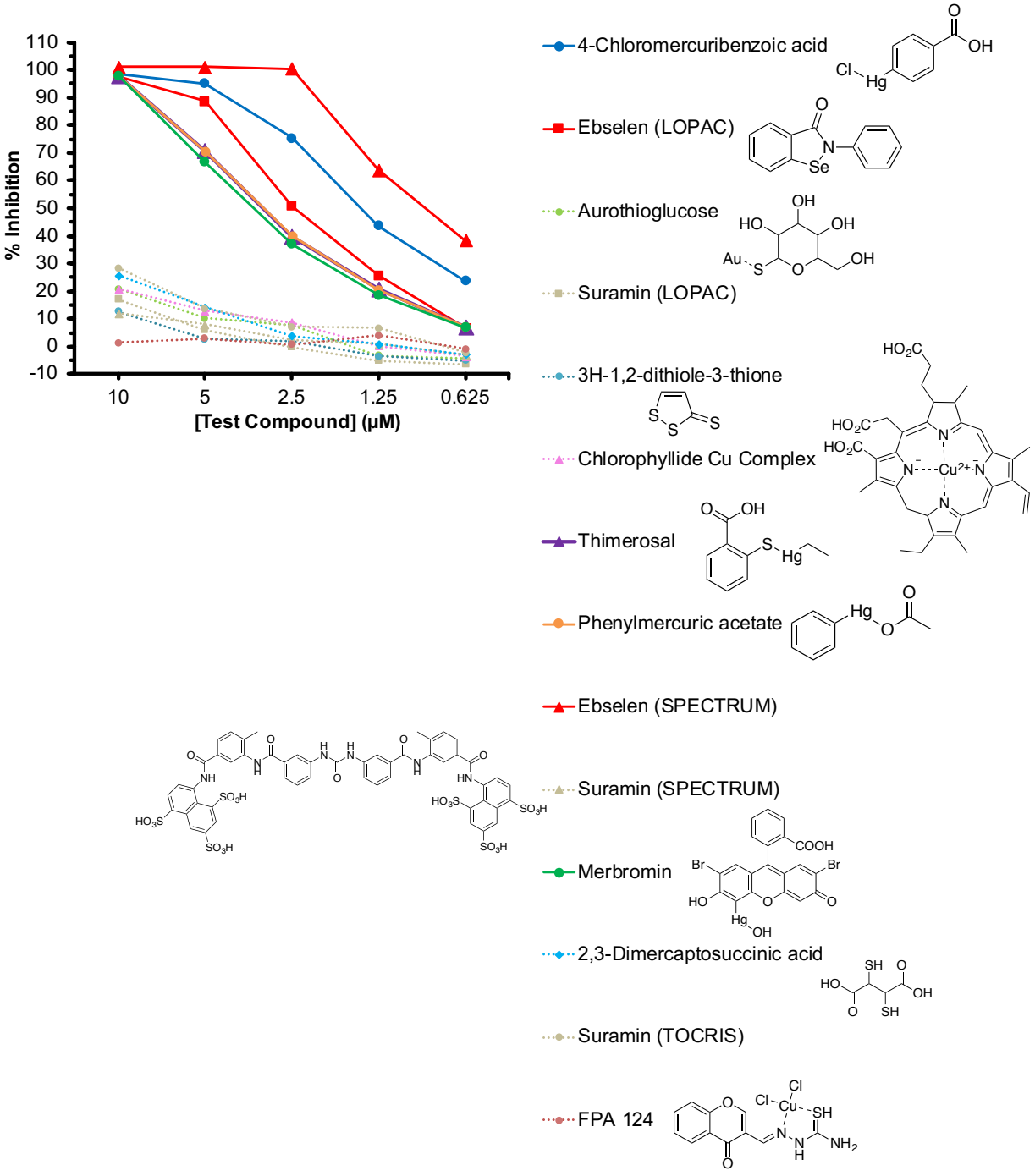

FIGURE S5. Follow-up replication of 14 hits in the 4,400-compound pilot screen. Plotted are the mean of duplicate measurements. Chemical structures are listed near chemical names. Some compounds were identified as hits in multiple collections.

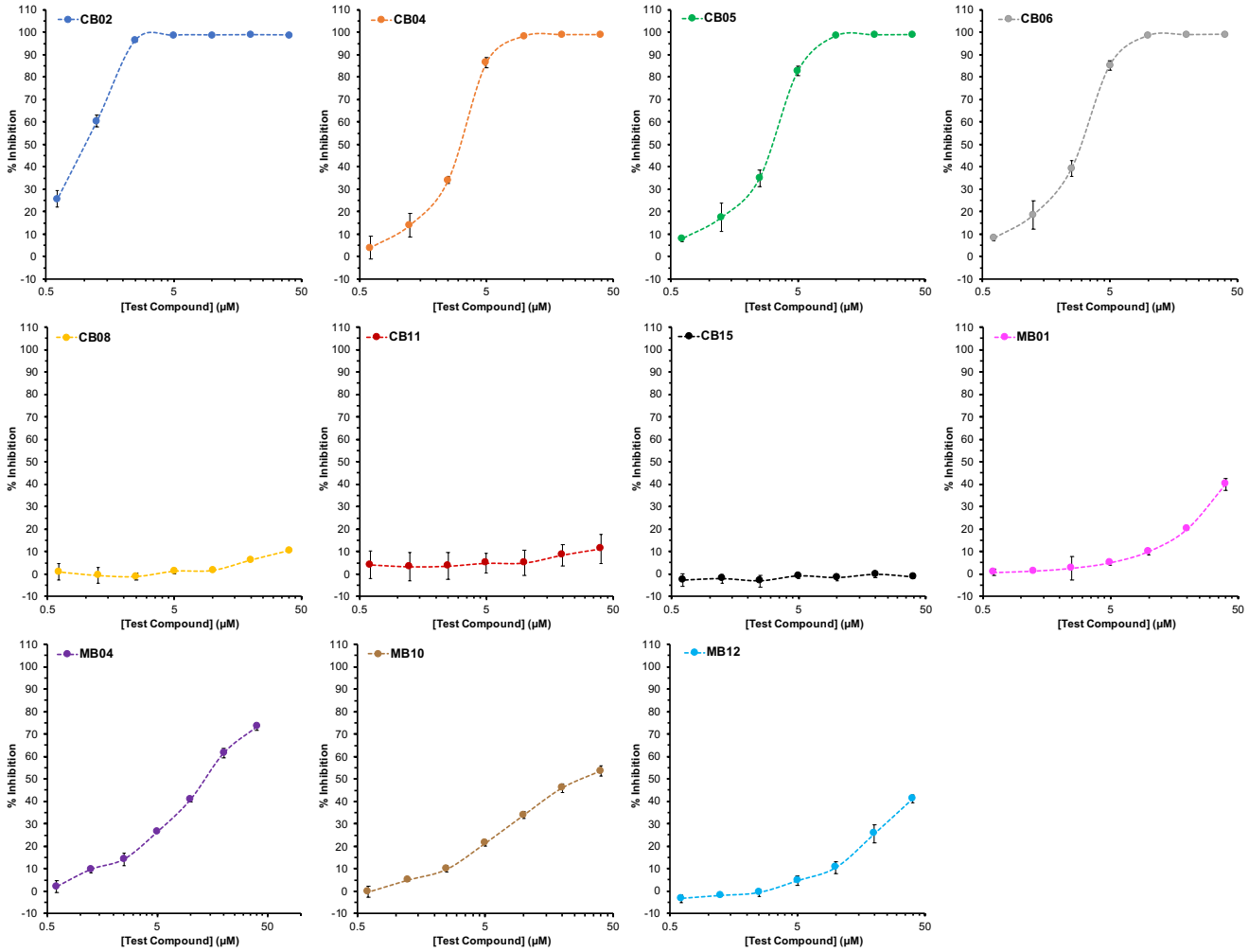

FIGURE S6. Dose-response of 11 purchased compounds using the primary MG assay. Data points are connected with smooth dashed lines and represent the mean of triplicate measurements ± SD.

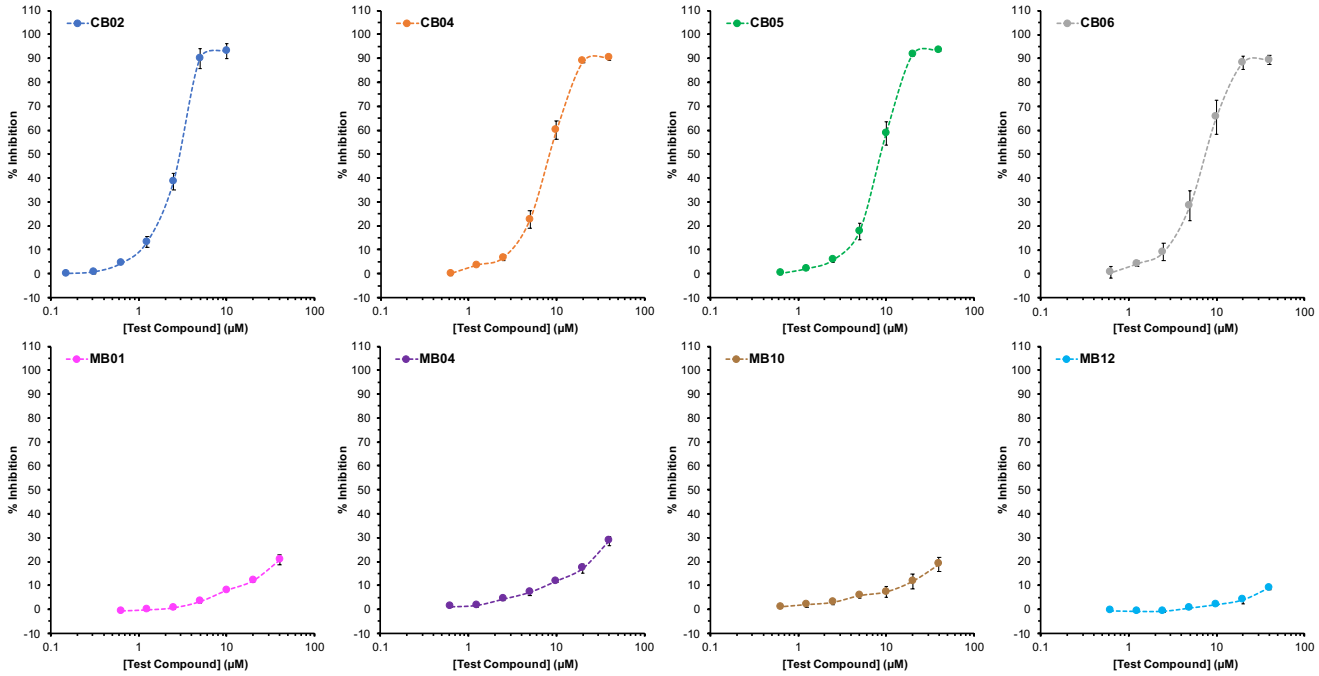

FIGURE S7. Dose-response of eight purchased compounds evaluated using the secondary luminescence assay. Compounds that did not display any appreciable activity in the primary assay (CB08, CB11, and CB15) were not evaluated with the orthogonal luminescence assay. Data points are connected with smooth dashed lines and represent the mean of triplicate measurements ± SD.

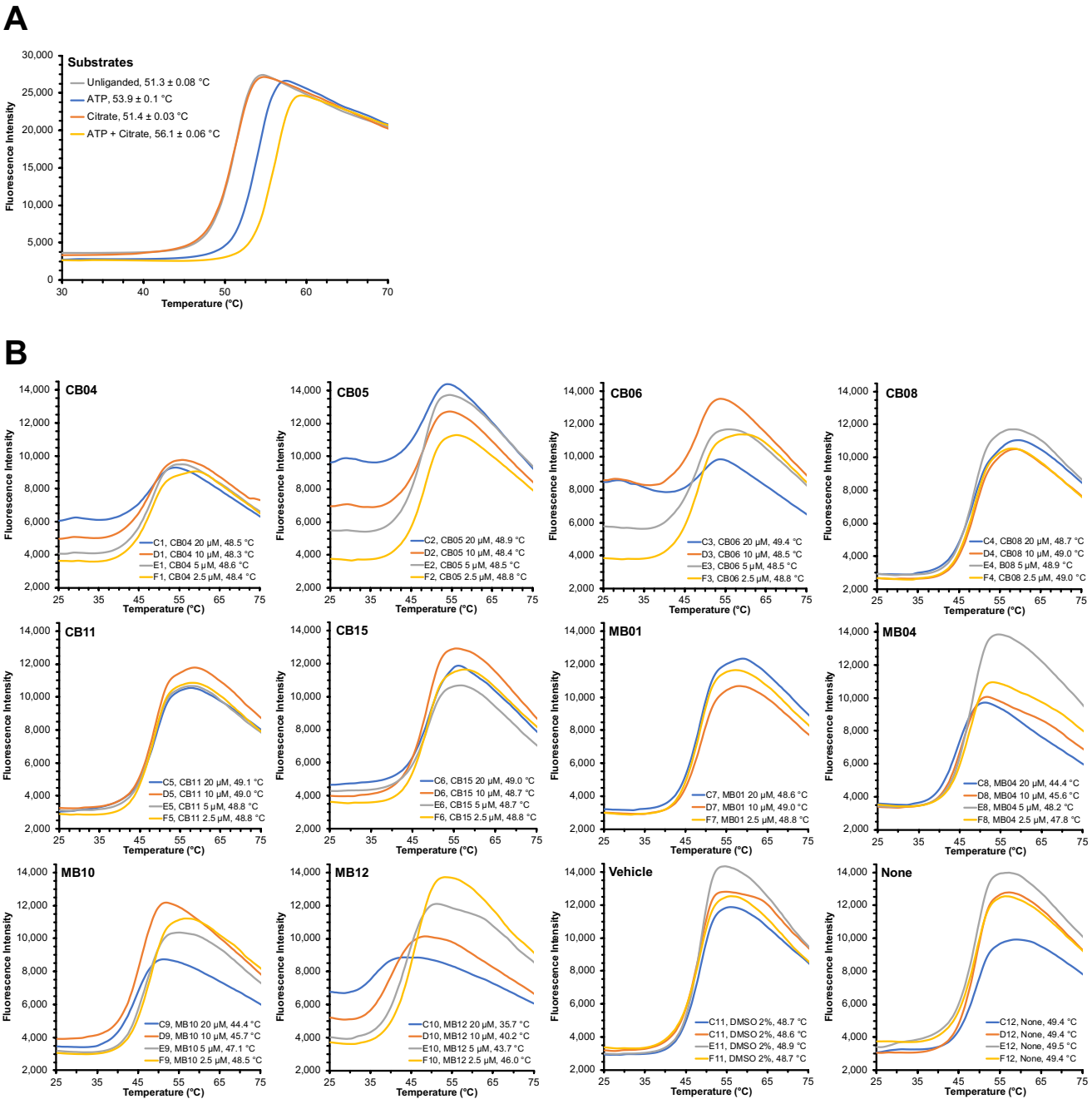

FIGURE S8. (**A**) Representative melting curves of lucA with substrates ATP (1.5 mM), citrate (1.5 mM), and the combination of ATP + citrate. Each substrate was evaluated in triplicate and the mean melting temperature (T_M) ± SD is listed in the legend. A positive thermal shift is observed for lucA in the presence of ATP and ATP + citrate, but not with citrate alone. (**B**) Melting curves of lucA with four concentrations (20, 10, 5, & 2.5 µM) of purchased test compounds by fluorescence-based thermal shift assay. Listed in each graph legend is the calculated T_M for each curve.

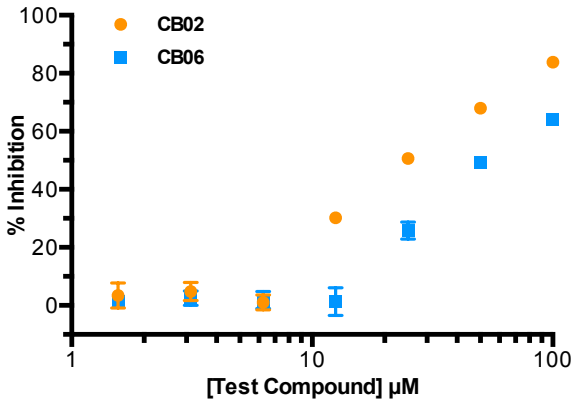

FIGURE S9. Evaluating the off-target potential of CB02 and CB06 employing myokinase, pyruvate kinase, and lactate dehydrogenase from the AMP-NADH oxidation assay. Data points represent the mean of triplicate reactions ± SD.

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