OFFICE OF UNDERGRADUATE RESEARCH
IMMERSIVE RESEARCH INTERNSHIP EXPERIENCE

Optimizing Ribozyme Reporters Using RNase J in *E.coli* Based Cell-Free Systems

FIRE: THE FIRST-YEAR INNOVATION & RESEARCH EXPERIENCE
MOLECULAR DIAGNOSTICS

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Intent & Motivation

Objectives: **Optimizing the function of ribozyme reporters** in an *E.coli* based cell-free protein expression system via the addition of a 5′ ribonuclease, RNase J1.

Motivation: If optimized, the ribozyme reporters (biosensors) could be designed to detect a variety of target molecules for **diagnostic and bioanalytical applications.**

Background

Self-Cleaving Ribozymes

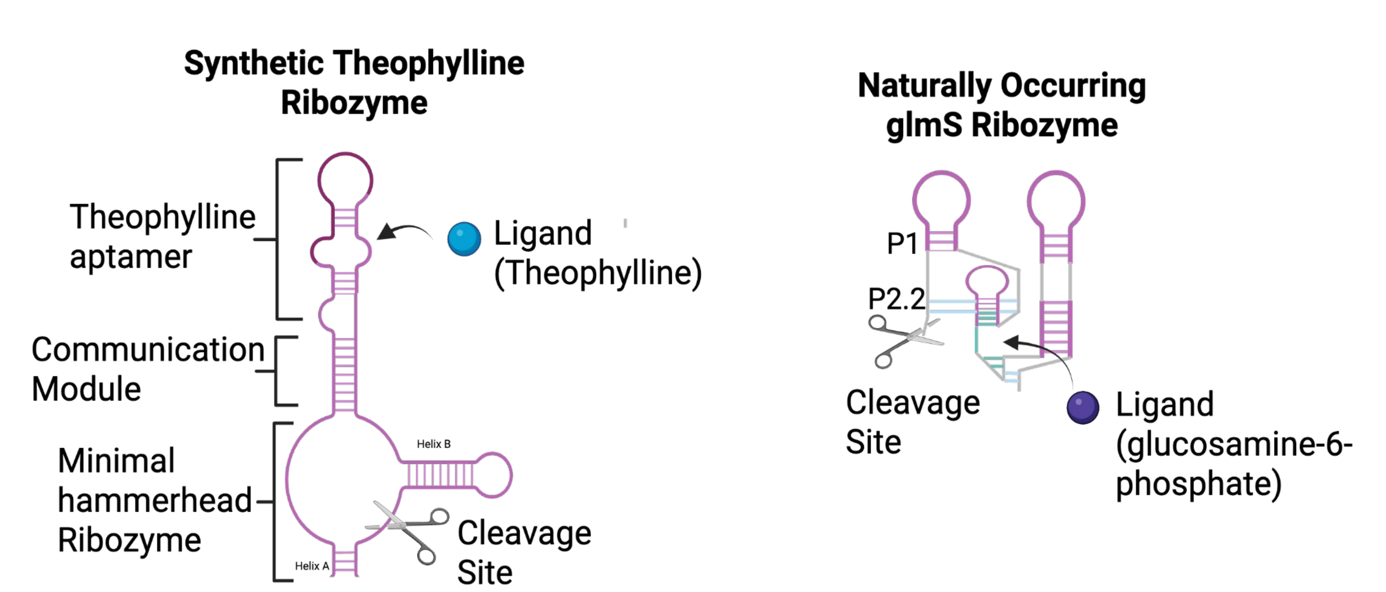

Figure 1. The secondary structure of the synthetic Theophylline Ribozyme[1], along with its respective ligand, theophylline (**A**). The naturally occurring *glmS* ribozyme that originates in Gram-Positive bacteria[2], along with its associated ligand, glucosamine-6-phosphate (**B**). Both ribozymes are catalyzed by the presence of a ligand, which causes them to self-cleave at the cleavage site.

Fluorescent Ribozyme Reporters

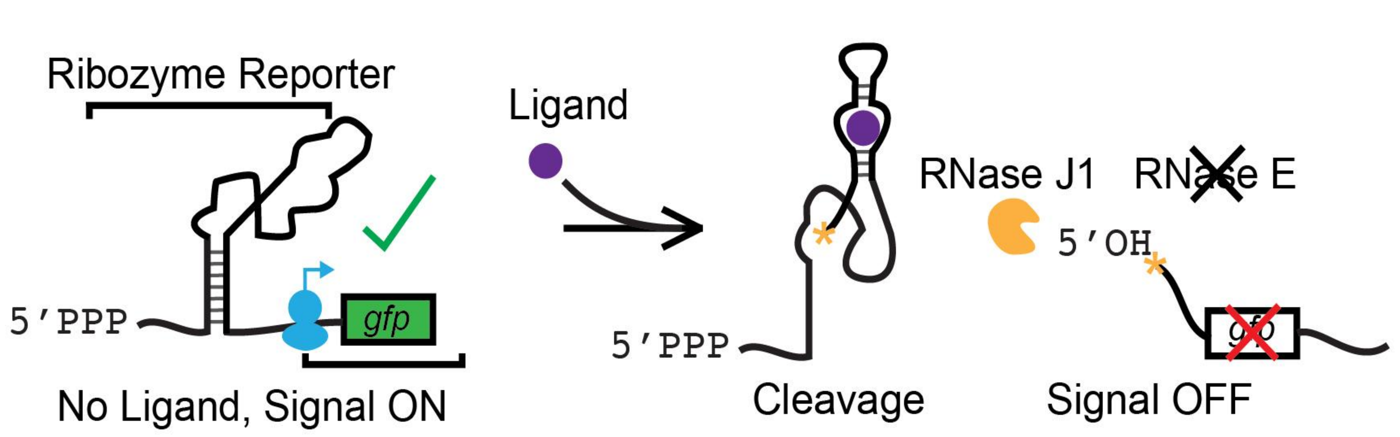

Figure 2. Target ligand binds to the ribozyme reporter, causing self-cleavage. RNase J (unlike RNase E) can degrade the cleaved mRNA segment, inhibiting GFP expression. In the absence of a ligand, the green fluorescent protein (GFP) expression is on.

- Synthetic ribozyme reporters can be tailored to different ligands by changing the aptamer sequence.
- RNase E is the main 5' endonuclease naturally occurring in *E. coli*.[3]
- RNase J1, found in *Bacillus subtilis* [4] is capable of degrading RNA transcripts with a 5' hydroxyl group, which RNase E does poorly.[4]
- RNase J has successfully been used to validate ribozyme reporters in an in vivo environment in *E. coli*.[5].

Methods

PCR Product Preparation

- gBlocks: Pbab GFP, Pbab ribozyme reporter (theophylline & glmS), Pbab RNase J
- Amplification with PCR (DreamTaq), and cleanup with spin columns

Setting up an *E. coli* Cell-Free Reaction (CFR)

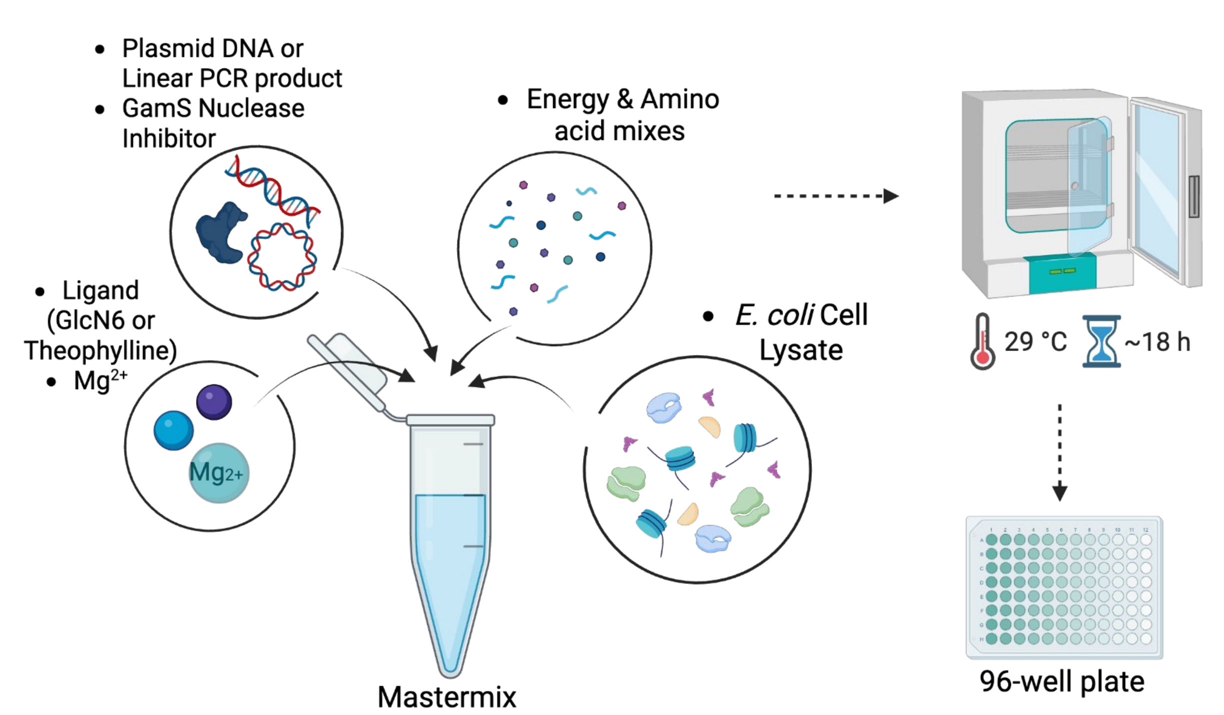

Figure 3. Cell-Free Reaction Master Mix and Setup. Assembled for all reactions tested in a given set of conditions. Added to a total 15 µL CFR of ⅓ lysate*, energy mix, DNA template (PCR product), w/ 0.5µL GamS protein, ±$MgCl_2$,± ligand. For +/- controls added Pbab GFP or water, respectively. *In house or commercial lysate. In house previously prepared from *E. coli* using an adapted Silverman protocol[6] and commercial from myTXTL Pro Cell-Free expression kit[7]

Gibson Assembly of Plasmid DNA

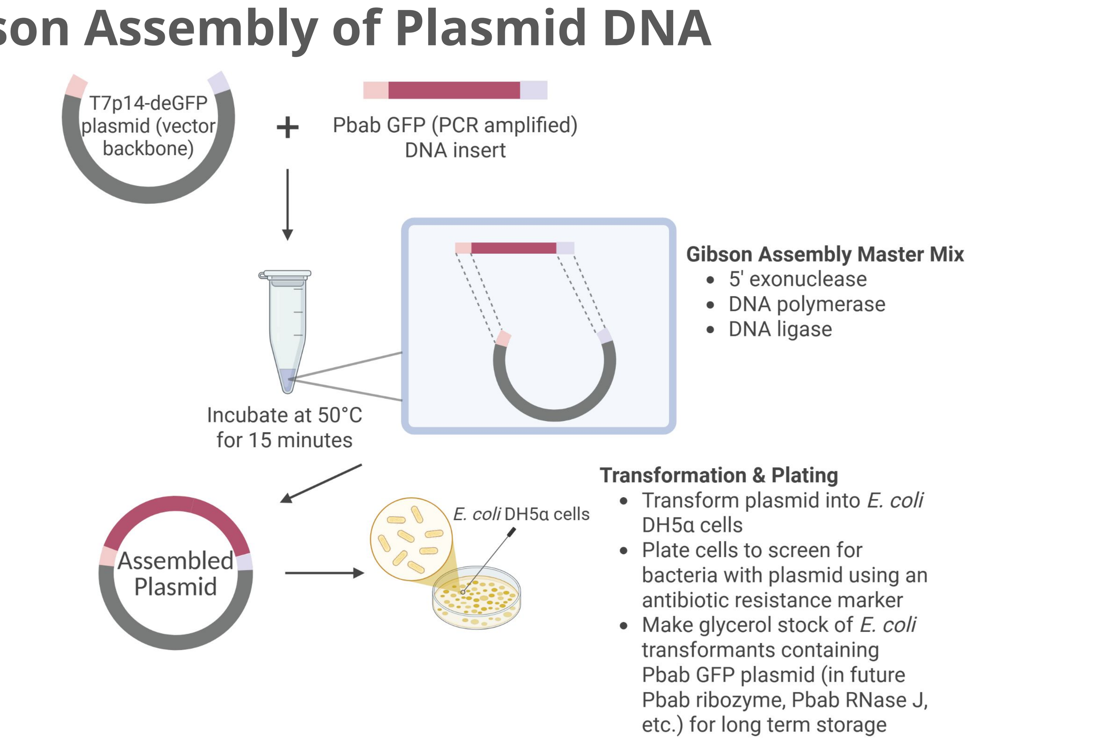

Figure 4. Gibson Assembly and Transformation. Assembled plasmid with T7p14-deGFP vector backbone and PCR-amplified Pbab GFP insert with NEBuilder HiFi DNA Assembly Master Mix. Incubated at 50 °C for 15 minutes. Transformed into *E. coli* DH5α cells via heat shock, recovered in SOC media, and plated on LB agar with carbenicillin. Colonies grown overnight at 37 °C, followed by liquid culture expansion and preparation of 25% glycerol stocks.

Results & Discussion

Optimization of Control Pbab GFP Expression in Cell-Free Lysate

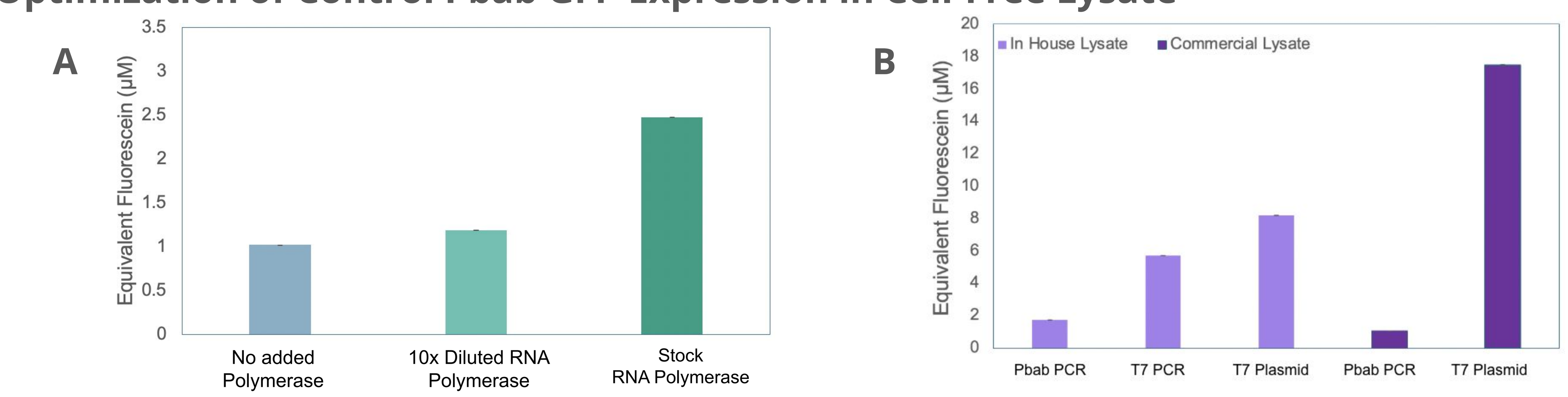

Figure 5. Optimizing GFP Expression. Supplementing *E. coli* RNA polymerase into cell-free reactions with in-house lysate with 10 nM Pbab GFP PCR products. 0.5 µL GamS protein & 3 mM $MgCl_2$ were added per reaction (**A**). Comparison of GFP expression from plasmid and PCR templates using T7 and Pbab promoters upstream of GFP with in-house lysate (w/ GamS protein) and commercial lysate (no GamS) (**B**).

Inhibition of Ribozyme Reporters in Reactions with RNase J1 PCR Products

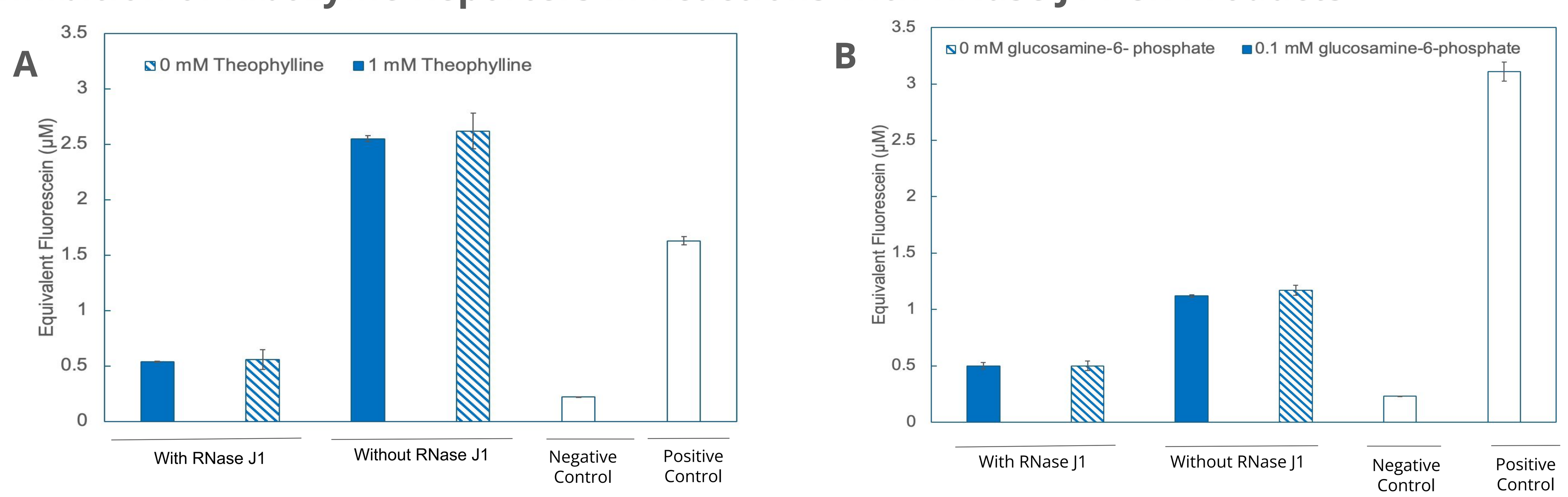

Figure 6. Testing Ribozyme GFP Reporters with 20 nM RNase J PCR Product. Theophylline (**A**) and glmS (**B**) ribozyme GFP reporters were tested under the same conditions: 20 nM ribozyme reporter PCR product, 3 mM $MgCl_2$, 0.5 µL GamS protein, and with and without ligand. Positive control had 20 nM Pbab GFP PCR product and negative control had water.

Optimizing Ribozyme Reporter Expression With RNase J1

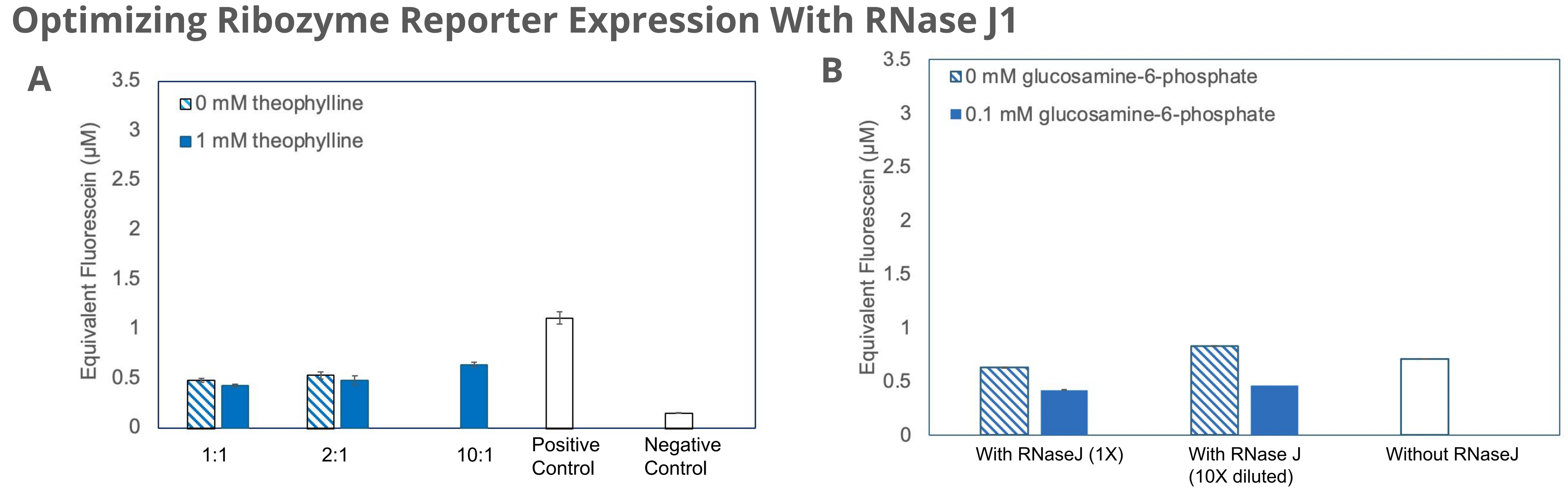

Figure 7. Ratio Experiment: 10 nM, 5 nM, and 1 nM concentrations of RNase J and 10 nM ribozyme reporter (theo apt) PCR products were tested. 0.5 µL GamS protein & 3 mM $MgCl_2$ were added per reaction (**A**). **Two Step Reaction:** Different concentrations of RNase J produced in a prior cell-free reaction were titrated into each reaction with a two step process, with and without 100 µM glucosamine-6-phosphate. Each reaction contained in house lysate, 10 nM glmS GFP ribozyme PCR product, and 0.5 µL GamS protein (**B**).

Acknowledgements & References

Scan for References

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Future Work

- Gibson assembly of all plasmids and confirm via Sanger Sequencing.
- Express and purify RNAse J1 for use in two step cell-free reactions.
- Optimize performance in commercial cell-free expression system.
- Explore use of RppH protein inhibitors to protect against degradation of ribozyme.
- Use mutant inactive ribozymes as a comparison point for active ribozyme fluorescent activity.
- Use PAGE gels to analyze ribozyme cleavage [2].