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IMMERSIVE RESEARCH INTERNSHIP EXPERIENCE
MOLECULAR DIAGNOSTICS

Utilizing RNase J1 to Degrade Ribozyme Reporter Signal in Cell-Free Systems

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Intent & Motivation

Objective: Determine whether the enzyme RNase J1 from *Bacillus subtilis* can be used to degrade the fluorescence signal of Green Fluorescent Protein (GFP) from the Pistol ribozyme reporter in *E.coli*-based **cell-free protein expression systems.**

Motivation: If successful, this system could be adapted to **ligand-responsive** ribozyme reporters, able to detect a variety of target molecules.

Background

Cell Free Systems

- Cell-free biosensor systems use protein synthesis machinery **outside of the cell**, eliminating barriers of cell-based systems such as the need for cell maintenance.[1]

The Pistol Ribozyme

- Ribozymes are **RNA-based** molecules that can catalyze reactions, such as **self-cleavage.**[2]
- Ribozymes can be used to control the expression of downstream fluorescent reporter proteins. Using this, **reporter protein activity** has been used to assess ribozyme activity in vivo.[3]
- The **Pistol ribozyme** undergoes self-cleavage in the presence of Mg^{2+}, yielding to distinct RNA products: (1) the upstream fragment beginning with a 2'3'-cyclic phosphate, and (2) the downstream fragment with a **5' hydroxyl group.**[4]

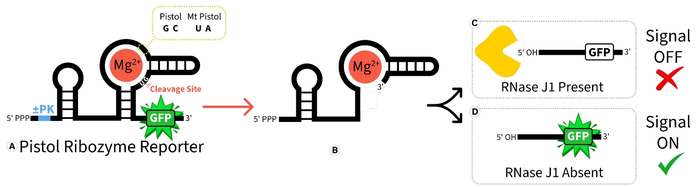

Figure 1. ***Pistol Ribozyme Self-Cleavage Mechanism.*** **(A)** The Pistol Ribozyme Reporter consists of a Pistol upstream of the GFP sequence. Pistol constructs can self-cleave, while Mt. Pistol constructs cannot. Some constructs contain an upstream pseudoknot (PK) for protection from degradation. **(B)** Self-cleavage occurs when Mg^{2+} is bound to the ribozyme reporter. **(C)** Exogenous RNase J1 can degrade the 3' cleaved mRNA transcript, decreasing GFP expression. **(D)** In the absence of RNase J1, GFP is expressed.

Role of RNase J1

- Native *E.coli* RNases **cannot** efficiently degrade 5' hydroxyl RNA transcripts.
- RNase J1 is a 5' to 3' ribonuclease from *Bacillus subtilis* that **preferentially degrades** RNA transcripts with 5' hydroxyl ends.[5]
  - Prior research has demonstrated in vivo that RNase J1 can selectively degrade transcripts following ribozyme self-cleavage.[3]

Methodology

Preparation of Ribozyme Reporters

- **PCR amplification** of Pistol ribozyme reporter gBlocks.
  - All under control of the PBAB (bacterial) promoter.
- Cleaned PCR products and **quantified** using Invitrogen Qubit Fluorometer.
- PCR Cloning used to assemble plasmid with Ribozyme Reporters.

RNase J1 Expression

- **Expressed RNase J1** from plasmid in *E.coli*-based cell-free lysate (myTXTL).
- **Evaluated** RNase J1 expression using SDS-PAGE gel and Western blot.

Pistol Ribozyme & RNase J1 in *E.coli*-Based Cell-Free System

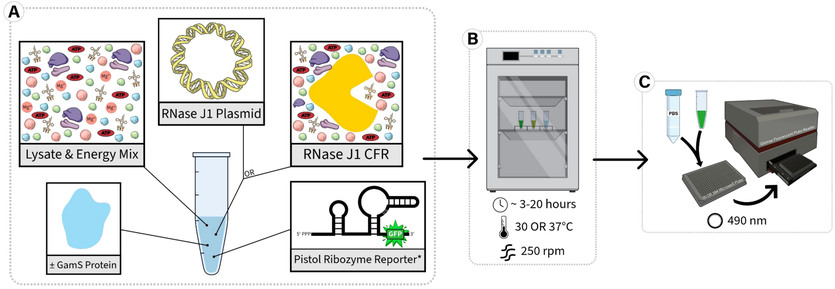

Figure 2. ***E.coli-Based Cell Free Reaction (CFR) Preparation and Measurement.*** **(A)** CFRs were assembled using either 15 µL in-house reactions (5 µL lysate, 5 µL PANOX energy mix, 0.5 µL gamS protein, and reaction-specific components) or 12 µL myTXTL reactions (9 µL myTXTL Pro Master Mix and reaction-specific components). Reaction-specific components included PCR-amplified Pistol constructs or GFP control plasmids*, RNase J1 CFR or plasmid (with 1% xylose or 1 mM IPTG), and nuclease free water. **(B)** CFRs were incubated at either 30°C or 37°C, shaking at 250 rpm for 3-20 hours. **(C)** CFRs were diluted with Phosphate-Buffered Saline (PBS) and fluorescence was measured in the Glomax Fluorescent Plate Reader.

- **One-Pot Setup:** Titration of RNase J1 plasmid.
- **Two-Pot Setup:** Titration of RNase J1 Cell-Free Reaction.
- Measured GFP Fluorescence with Glomax, normalized to Fluorescein standard curve, and **compared** across experimental conditions (Table 1).

Table 1. ***Experimental Conditions and Predicted Fluorescence for CFRs***

Ribozyme Reporter	RNase J1 present?	Self-Cleavage?	Predicted Fluorescence
Pistol GFP (± PK)	-	✓	High
Pistol GFP (± PK)	+	✓	Low
Mt. Pistol GFP (± PK)	+/-	✗	High
None: GFP	+/-	✗	High
None: Water	-	✗	Low

Results & Discussion

RNase J1 Expression: SDS-Page Gel and Western Blot

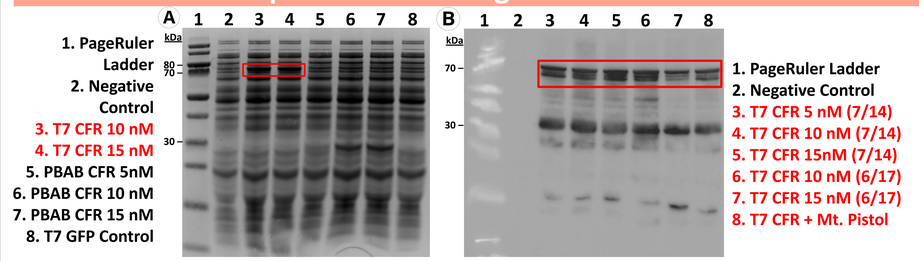

Figure 3. ***Analysis of T7 and PBAB RNase J1 Expression in myTXTL Cell-Free Lysate.*** myTXTL CFRs run with various concentrations of T7 RNase J1 plasmid (with a 10xHis-SUMO tag) & PBAB RNase J1 plasmid, incubating for 18 hours at 27°C. Negative control was CFR with water. Expected molecular weight of T7 RNase J1 is ~72 kDa & PBAB RNase J1 is ~62 kDa. **(A)** Proteins separated on 10% Bis-Tris MES SDS-PAGE gel and visualized using a Coomassie Blue stain. Red box over lanes 3 & 4 highlight possible RNase J1 expression. **(B)** Proteins transferred to PVDF membrane for Western blot analysis with anti-His antibody. Red box over lanes 3-8 provide evidence of T7 RNase J1 presence in CFRs conducted on 7/14 & 6/17. Negative control shows no bands.

- Initial plasmid DNA concentration **did not** appear to affect expression.
- Evidence of **10xHis-SUMO RNase J1 production** (protein bands ~70 kDa) observed in T7 RNase J1 CFRs. No band corresponding to Pbab RNase J1 protein observed.
- **Degradation products observed** in Western Blot at **~35 kDa**.
- Two-pot CFRs used **10xHis-SUMO (T7) RNase J1 CFRs**.

Pistol Ribozyme & RNase J1 in *E.coli*-Based Cell-Free System

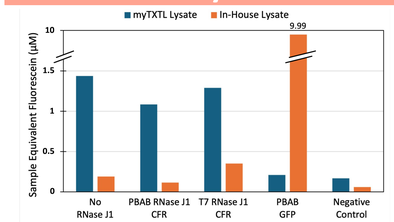

Figure 4. ***Comparison of Two Pot Setup Between Commercial (myTXTL) and In-House E.Cell-Free Lysates.*** CFRs were constructed using PK Pistol PCR product, and tested two-pot setup with prior RNase J1 CFR. CFRs conducted in myTXTL lysate & in-house lysate; incubated at 30°C for 18 hours before fluorescence measured (no replicates).

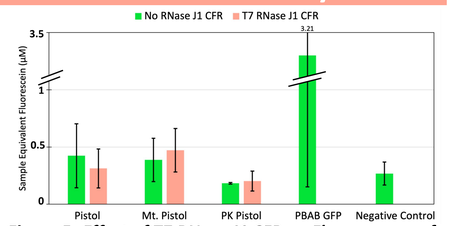

Figure 5. ***Effect of T7 RNase J1 CFR on Fluorescence of Pistol Ribozyme Reporter Constructs.*** Sample Equivalent Fluorescein was compared in reactions containing no RNase J1 CFR or T7 RNase J1 CFR. Reactions performed using in-house lysate, incubated at 37°C overnight before fluorescence measured. Mean ± standard error reported.

- **High variability** observed between experiments.
- All Pistol constructs produced fluorescence levels close to the negative control, making potential decreases due to RNase J1 **difficult to distinguish.**
- RNase J1 **did not** consistently reduce fluorescence in the One-Pot or Two-Pot Setups.
- Native RNases in lysate may also be **degrading** cleaved ribozyme reporter transcripts, decreasing GFP signal regardless of the presence of RNase J1.

Acknowledgements & References

This research was supported by the OUR *Immersive Research Internship Experience* (IRIE) Program. We sincerely thank Dr. Spirito for her mentorship and guidance, Defne Ustundag for her support as peer mentor, and the Winkler Laboratory for their collaboration. Figures created in Adobe Express and Excel.

References

Future Work

- **Reduce protein degradation** in RNase J1 expression reactions.
- Repeat trials to determine the **reproducibility** of data.
- Explore ways to **boost GFP** expression, such as adding additional Mg^{2+}.
- **Optimize** CFRs with PK Pistol, PK Mt. Pistol, and PK GFP variants.
- Research the **interactions** of other native RNases in cell-free lysate on Pistol activity.