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RNA thermometers for the PURExpress system

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Abstract

Cell-free synthetic biology approaches enable engineering of biomolecular systems exhibiting complex, cell-like behaviors in the absence of living entities. Often essential to these systems are user-controllable mechanisms to regulate gene expression. Here we describe synthetic RNA thermometers that enable temperature-dependent translation in the PURExpress *in vitro* protein synthesis system. Previously described cellular thermometers lie wholly in the 5' untranslated region and do not retain their intended function in PURExpress. By contrast, we designed hairpins between the Shine-Dalgarno sequence and complementary sequences within the gene of interest. The resulting thermometers enable high-yield, cell-free protein expression in an inducible temperature range compatible with *in vitro* translation systems (30–37°C). Moreover, expression efficiency and switching behavior are tunable via small variations to the coding sequence. Our approach and resulting thermometers provide new tools for exploiting temperature as a rapid, external trigger for *in vitro* gene regulation.

Graphical Abstract

Keywords

RNA thermometers; cell-free synthetic biology; RNA folding; PURE system; *in vitro* transcription and translation; temperature sensing

RNA thermometers enable inline control of gene expression based on temperature changes¹. The control element of the thermometer relies on conformational changes in the mRNA secondary structure that occlude the ribosome binding site (RBS) at one temperature and

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Supplementary Information

Supplemental methods; supplementary figures and tables.

free it at another, assuming two-state RNA folding². By relying on nascent, *cis*-acting mRNA to block translation initiation, gene regulation via RNA thermometers is both elegant and immediate³. As such, naturally occurring RNA thermometers are key viral and bacterial signal processing elements⁴, regulating gene circuitry ranging from virulence^{5–9} to temperature shock^{10,11}.

To capture this type of gene regulation synthetically, *de novo* heat-inducible RNA thermometers have been designed, consisting of a single hairpin formed between the Shine-Dalgarno (SD) sequence and a complementary sequence upstream of the Shine-Dalgarno sequence (anti-Shine Dalgarno, or aSD sequence)¹². In practice, these thermometers exhibited a range of temperature-switching behavior in terms of both the sensitivity of the response and the efficiency of translation¹³. Synthetic RNA thermometers have since expanded in terms of function¹⁴ and diversity¹⁵. While RNA thermometers have been previously prototyped for *in vivo* use, their utility in a cell-free context has been limited due to modest expression levels¹⁵. High-yielding, highly sensitive synthetic thermometers would be useful cell-free synthetic biology tools to achieve chemical-free gene regulation, with applications ranging from RNA-ordered gene expression dynamics *in vitro*¹⁶ to tightly controlled modularization of *in vitro* genetic cascades¹⁷. We therefore sought to create robust RNA thermometers for use in a cell-free context.

Using a PURExpress reporter assay with superfolder GFP (sfGFP)¹⁸, we first exploited an existing synthetic thermometer, U9, which is reported to express efficiently in the cellular context of *Escherichia coli* at a translation-permissive temperature of 37°C and repress expression at a translation-restrictive temperature of 30°C¹³. In the context of PURExpress IVTT, however, we found overall *in vitro* expression of U9-sfGFP was near-baseline at 37°C, making any switching behavior practically irrelevant (Supplementary Figure S1). Based on mFold results of the first 100 bp of U9-sfGFP (Supplementary Figure S2), we hypothesized that RNA folding between the SD and the open reading frame (ORF) could be inhibiting translation initiation in tandem with the designed aSD in the 5' UTR, resulting in significant repression even at the reportedly permissive temperature of 37°C. To mitigate bi-directional folding around the SD and favor desired hairpin formation over interactions between the SD and the ORFs, we replaced the U9 5' UTR with a non-folding 5' UTR optimized for *in vitro* protein expression¹⁹ and re-introduced the aSD directly downstream of the start codon, a strategy that has been employed in nature⁸ (Figure 1A). We anticipated that the SD-aSD hairpin formation would be energetically favorable compared to undesired SD-ORF secondary structure based on the proximity of the SD and aSD on the RNA relative to the SD and ORF. As the SD and aSD are closer together, the loop in the SD-aSD hairpin should be smaller and less destabilizing than a loop in a potential SD-ORF hairpin. We initially implemented a minimal, fully complementary SD-aSD hairpin structure with the same aSD sequence as the U9 thermometer, encoding two leucine residues, placed immediately after the start codon (LL thermometer). We then varied this design by engineering the RNA hairpin to feature a variety of different predicted folding strengths based on the length and sequence of the aSD: enlarging the RNA hairpin (SPS, SSL), shifting the aSD to partially bind the SD while maintaining complementarity (SF), introducing mismatches in the aSD to reduce complementarity to the SD (LV), and combining hairpin enlargement and mismatch incorporation (SSS) (Figure 1B). RNA hairpin

folding strengths for the different thermometers were predicted by calculating ΔG° values with mFold²⁰. Although our thermometer design strategy may not be applicable to proteins that are sensitive to N-terminal fusions, it could be implemented with any protein that can accommodate 2–3 relatively inert amino acids at its N-terminus.

Using our PURExpress sfGFP reporter assay, we first characterized the overall expression level of each thermometer construct at 37°C. Unmodified sfGFP with the non-folding 5' UTR (designated wild-type) was used as a comparative control for the designed thermometers. As expected, expression at 37°C decreased as thermometer folding strength increased (ΔG° decreased) (Figure 1C). Of practical importance, several thermometer designs (LV, LL, SSL) showed expression levels within 2-fold of wild-type sfGFP, while a 15-fold decrease was observed for the lowest ΔG° hairpin (SPS). This relationship between ΔG° of the thermometer fold and overall expression has been observed in *in vivo* contexts concerning hairpin formation between SD and aSD in the 5' UTR² or the ORF²¹, and it was also described as a potential source of decreased expression in an *in vitro* expression system²².

Next, we characterized thermometer switching behavior between 37°C and 30°C, two expression temperatures spanning the practical range of high-level expression in the PURExpress system. All thermometers exhibited a greater degree of temperature-dependent switching behavior than the thermometer-free wild-type background, indicating that this enhanced responsiveness resulted from thermometer unfolding and not simply temperature-dependent translation events (Figure 1D). In contrast to the overall expression level at 37°C, the thermometer switching behavior was uncorrelated with predicted ΔG° values, pointing to potential limitations of using this simple calculated metric for predicting the complex temperature-sensitive response. There are other factors in addition to ΔG° that control the switching behavior, ranging from the melting profile of the RNA, where hairpin melting could either be abrupt or gradual between the tested temperatures, to ensembles of higher-energy structures¹⁵. The disconnect between the SSL and LL thermometers, which exhibit the most disparate 37°C/30°C expression ratios of all tested designs despite having similar ΔG° values for their lowest energy states, is a potential example of the relevance of higher-energy structures to temperature switching behavior. The lowest energy structure of SSL only retains a partial SD-aSD hairpin, which completely unravels by the third-lowest energy structure, whereas the five lowest energy structures of LL all contain the entire designed hairpin (Supplementary Figure S3). On the other hand, all predicted secondary structures of the SPS thermometer contained the desired hairpin, potentially leaving too tight of a clamp on translation for effective temperature switching. To more quantitatively evaluate thermometer behaviors dictated by an ensemble of molecular states, we calculated the fraction of unfolded and folded states at both 37°C and 30°C using Boltzmann distributions. We found a correlation between the ratio of sfGFP expression (37°C/30°C) and the ratio of the probability of occupying the unfolded state (37°C/30°C) for each thermometer variation (Supplementary Figure S4).

As incorporation of the LL thermometer into sfGFP resulted in the most sensitive temperature switching behavior while retaining high overall expression at 37°C, we proceeded with the LL design to determine its robustness in the context of different ORFs

downstream of the thermometer. To this end, the LL thermometer-sfGFP construct was N-terminally modified with either a SNAP protein fusion (SNAP-sfGFP)²³ or a His₆ tag (His₆-sfGFP). These two commonly used functional modifications were each placed between the LL thermometer and sfGFP, thus keeping sfGFP as a sensitive expression reporter. Not surprisingly, introducing the LL thermometer into these different ORF contexts affected their overall expression levels to varying degrees (Figure 2A). LL-SNAP-sfGFP had the lowest predicted ΔG° of the three sfGFP constructs, suggesting that hairpin formation still significantly impacts expression efficiency at 37°C (Supplementary Table S1). However, thermometer-dependent temperature switching behavior over a wild-type background was robust regardless of the ORF, further validating our design strategy (Figure 2B). We additionally characterized temperature-dependent expression kinetics of the LL-thermometer encoded in the His₆-sfGFP construct (Figure 2C). We noted a significantly ultrasensitive response to temperature ($n_H = 4.2$) after 60 min of His₆-sfGFP expression, as the majority of the switching occurred in a narrow range, from 32°C to 36°C (Figure 2D).

As an additional demonstration of ORF-independent temperature-switching behavior, we introduced the LL thermometer into mCherry. As codon-optimized mCherry and sfGFP are identical for the first 21 nucleobases²⁴, we introduced a silent mutation into mCherry (C9T, still encoding serine at amino acid position 3) to further diversify the nucleotide sequence.

Compared to wild-type mCherry, this initial LL-mCherry construct showed slightly reduced overall expression (Figure 2E), but more importantly, no significant increase in 37°C/30°C expression ratio, pointing to an ineffective thermometer (Figure 2F). Notably, mCherry contains two internal, in-frame translation start sites (TSSs)²⁵, the first of which is functional and could therefore allow protein synthesis to proceed independent of the LL thermometer hairpin. More importantly, the lowest-energy predicted fold of LL-mCherry contains a hairpin between the second internal TSS and the designed aSD sequence (Supplementary Figure S4), leaving the SD unimpeded and dulling the effect of the thermometer. To rescue temperature-switching behavior, we rationally reduced the number of internal TSSs located in the mCherry ORF to reinforce binding between the actual SD and the designed aSD. We found four silent mutations (G24A/G27A/G48A/G51A) within the internal TSSs that eliminated unwanted base pairing and produced the desired SD-aSD hairpin in the lowest-energy fold (Supplementary Figure S5). Interestingly, inclusion of the four silent mutations into LL-mCherry rescued thermometer switching more efficiently than either completely eliminating the first internal TSS via N-terminal truncation or increasing the spacing between the internal TSSs and the aSD sequence with a His₆ tag (Figure 2F). The success of this rational redesign strategy, based on elimination of competing internal TSSs, underscores the importance of considering the ensemble of RNA folding states in engineering switch-like thermometer behavior.

Here, we have described the design of novel synthetic RNA thermometers that show strong temperature-dependent switching characteristics to effectively control gene expression in cell-free expression systems. Our encoded design requires certain features within a gene of interest, including weak folding between the 5' UTR and the ORF of interest relative to the SD-aSD hairpin as well as a lack of putative folding between the ORF and the aSD sequence. Expression and switching behavior can be manipulated by adjusting the size and

composition of the designed hairpin, as well as by controlling the ensemble of folding states between the SD and the ORF of interest. Additionally, placement of the thermometer into the coding sequence, rather than upstream in the 5' UTR, lengthens the constant region of nucleotides at the start of a given ORF, which could lead to more consistent thermometer behavior across different ORFs (e.g., Figure 2B).

These designed thermometers provide a rapid and non-invasive means to control gene expression in an *in vitro* setting. This type of control mechanism could be particularly useful in applications based on closed reaction systems to control concentration and temporal gene expression gradients. Potential applications range from *in vitro* compartmentalization²⁶, where temperature could be used to modulate the ordering of multiple reactions, to artificial cells²⁷, where temperature could be used as a sensory input for emulsified genetic circuitry²⁸. Furthermore, cell-free synthetic biology systems are often compositionally sensitive, so even if they are not closed, they may benefit from achieving gene regulation with physiological temperature shifts rather than chemical additives. The minimal molecular footprint and robust functionality of our RNA thermometer designs provide useful additions to the repertoire of cell-free synthetic biology tools.

Methods

Thermometer design and in silico validation

Encoded thermometer sequences were created following the design rules of Neupert et al.¹². Hairpin folding behavior and ΔG° values of thermometers were evaluated using the mFold web server²⁰.

Plasmid construction

Thermometer and wild-type constructs were cloned into the PURExpress DHFR control vector backbone following standard cloning procedures (details in Supplementary Information). With the exception of U9 (XbaI/XhoI), all constructs were cloned via NdeI/XhoI restriction sites.

PURExpress reporter assay

In vitro transcription and translation was performed using the PURExpress system (New England Biolabs). We performed 5 μ L reactions using 5 nM plasmid DNA. Reactions were incubated in the Cytation 3 plate reader for 3 hours, with time points taken every 10 minutes. Fluorescence counts obtained were converted to protein concentrations via standard curves using purified sfGFP or mCherry. Overall expression and temperature switching metrics were evaluated at 60 min for sfGFP constructs and at 80 min for mCherry constructs, since mCherry has a ~15 min fluorophore maturation time²⁴, in contrast to the nearly instantaneous maturation time of sfGFP¹⁸.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Design of encoded RNA thermometers. (A) Schematic of predicted LL thermometer structure in sfGFP and its predicted G° value at 37°C . Given a two-state model of RNA folding, the RNA is in an equilibrium between the unfolded state (left) and folded state (right). Increases in temperature shift the balance to the unfolded, translation-permissive state (and vice-versa). (B) Predicted thermometer structures, each encoding a dipeptide or tripeptide at the N-terminus of sfGFP, and their predicted G° values at 37°C . (C) Overall expression at 37°C after 60 min for each thermometer variant versus predicted G° value. Dashed red line indicates wild-type sfGFP expression. (D) Ratio of expression ($37^\circ\text{C}/30^\circ\text{C}$) of each thermometer after 60 min of expression. Dashed red line indicates wild-type sfGFP expression ratio.

Figure 2.

Incorporating the LL thermometer into different ORFs. (A) Expression at 37°C for three different sfGFP ORFs. (B) Ratio of expression (37°C/30°C) used to indicate switching behavior. (C) Heat map of expression level of the LL thermometer encoded into His₆-sfGFP. (D) Temperature-dependent His₆-sfGFP expression of the LL thermometer at 60 min. (E) Expression at 37°C and (F) 37°C/30°C expression ratio for wild-type and LL-thermometer sequences appended to mCherry, $\Delta 12$ mCherry (removes first internal TSS), and His₆-mCherry. 4SM refers to the four silent mutations introduced to rescue thermometer behavior.