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# Design of a Toolbox of RNA Thermometers

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**Abstract.** RNA thermometers mediate responses to temperature changes in various natural circuits, and have been developed in a synthetic context as well. However, a toolbox of RNA thermometers with different sensitivities to temperature is lacking. Here, we address this issue using a combination of computational and experimental methodologies. We analysed a set of available synthetic RNA thermometers through a quantification of their activity as a function of temperatures in a cell-free expression molecular breadboard system as well as through computation of their melt profiles. Based on this, we computed melt profiles of a library of RNA thermometers and found that the library contained RNA thermometers with a range of sensitivities and thresholds in their response to temperature. We constructed this library and found, through preliminary measurements, a wide range of responses to temperature, which in some cases matched the computational predictions. The constructed library represents a toolbox of RNA thermometers with different sensitivities and is foundational work towards synthetic biology applications such as efficient control of large volume chemical

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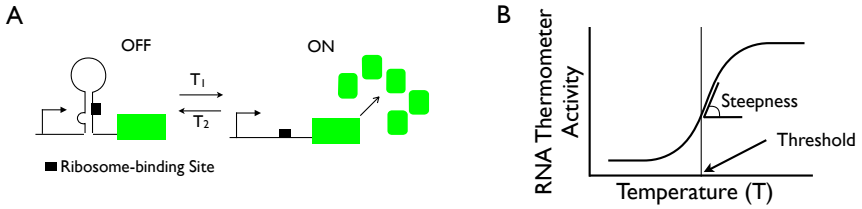


Figure 1: Steepness and threshold in the response to temperature of a RNA thermometer. A. Illustration of the functioning of a simple RNA thermometer. B. Black solid line represents the activity of the RNA thermometer as a function of temperature. The steepness and threshold characteristics of this response are emphasised in the figure.

reactors, precise spatiotemporal control of gene expression as well as tools to engineer robustness to temperature in biomolecular circuits.

# 1 Introduction

The design and development of biomolecular circuits and components, often based on their natural counterparts, is a key requirement for synthetic biology applications, such as in metabolic engineering and medicine [8]. RNA offers an attractive substrate for such an objective, both due to its substantial regulatory and sensing potential as well as availability of computational tools to estimate its behaviour. Indeed, RNA molecules can be designed to sense extremely specific signals such as metabolites or other RNAs [2] as well as more global signals such as temperature [3].

Temperature-sensing RNA molecules, also called RNA thermometers, are present in diverse cellular contexts and can mediate behaviours such as heat shock response and pathogenesis. At its simplest, a RNA thermometer consists of an RNA sequence containing the Ribosome Binding Site (Fig. 1A). At a permissive temperature, the Ribosome can access this Ribosome Binding Site and translation proceeds efficiently. At a restrictive temperature, the RNA sequence folds in such a manner so as to prevent this access and inhibit translation. Depending on whether the transition between the folded and unfolded structures occurs gradually or abruptly as temperature changes, these have been categorized as zippers and switches, respectively [1]. Examples include the *rpoH* gene mediating heat-shock response in *E. coli* and the *cIII* gene regulating the life cycle of phage $\lambda$. While these natural examples have a complicated secondary structure with multiple stems, hairpins, and bulges, RNA thermometers have been designed with simpler secondary structures, with only a single stem-loop protecting the Ribosome Binding Site [5]. Broadly, these designs were based on shifting the melting temperature through changing the strength of the secondary structure [4, 5]. In particular, factors such as increasing the length of the stem, the reduction in base pair mismatches in the stem, or a reduction in the size of the hairpin loop, resulted in a more stronger secondary structure, and hence an increase in the melting temperature. These results are important early work towards an elucidation of mechanisms underlying RNA thermometer operation, modulation of their response to temperature, and towards prediction of their behaviour.

There are three particularly striking aspects about RNA thermometers. First, for example, is the startling complexity in the secondary structure of the naturally occurring RNA thermometers in comparison to the synthetic ones, which seem to give a similar response to temperature. Second, is the different secondary structures, of possibly varying stabilities, that an RNA thermometer can fold into, at a given temperature. The mapping from the ability of these secondary structures to efficient translation determines the RNA thermometer activity. Third, is the different kinds of responses to temperature that are possible among the published synthetic RNA thermometers [5]. These

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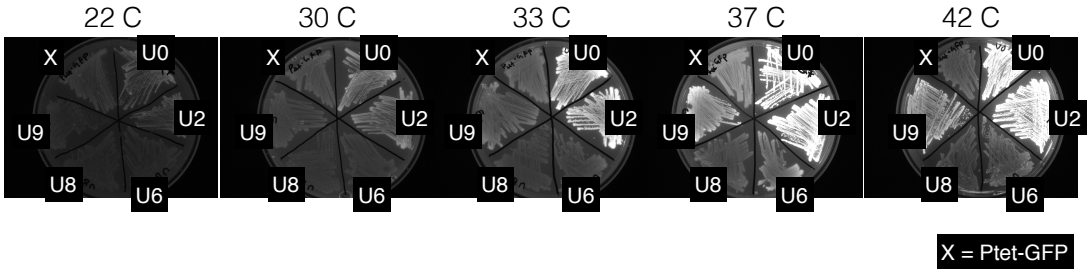


Figure 2: Initial determination of RNA thermometer activity using fluorescence of colonies. Intensity is proportional to the expression of green fluorescent protein under the control of an RNA thermometer. RNA thermometers used are indicated. X represents a $P_{tet}$-GFP construct.

include both switch-like responses, where responses seem to abruptly increase around a threshold temperature, as well as linear-like responses, where responses seem to increase in a more gradual manner as temperature increases. In fact, even a change by one base pair appears to shift the response from linear-like to switch-like (U2 to U9 in [5]). It is generally unclear how to obtain a set of RNA thermometers with varying steepnesses and thresholds (Fig. 1B).

Here, our objective is to generate a toolbox of RNA thermometers with different steepnesses and thresholds. We approach this using a combination of experimental measurements in a cell-free molecular breadboard framework and computations of RNA melt profiles. We study a set of existing synthetic RNA thermometer constructs by experimentally quantifying their response to temperature and computing their computed melt profiles. Next, we systematically investigate sequence variations in the neighbourhood of a particularly strong RNA thermometer, finding that a range of steepnesses and thresholds can be generated. Finally, we construct this library and present preliminary experimental measurements suggesting that a variety of steepnesses can be obtained, which match the predictions in some cases. These results should help in developing a toolbox of temperature regulatory biomolecular circuit components for synthetic biology applications.

# 2 Results

## 2.1 Approach

As starting point for our investigation, we studied a set of five previously published synthetic RNA thermometers (U0, U2, U6, U8 and U9 from [5]). This set was chosen to include both switch-like and linear-like responses, as assessed from the gel blots [5].

To get an initial estimate of the RNA thermometer activity as a function of temperature, these plasmids were transformed into *E. coli* (One Shot®Top10F' chemically competent cells, Invitrogen, Karlsruhe, Germany). These cells were then streaked onto multiple LB agar plates, and incubated overnight at different temperatures. Subsequently, these colonies were imaged under UV light in a fluorescence imager (Fig. 2) We found that the fluorescence of the colonies had an increasing trend as a function of temperature. Further, there were discernible differences within the RNA thermometers regarding the trend of increase. For example, the increase in both U0 and U2 was at a more gradual pace relative to temperature than in U9, which had a relatively abrupt increase between $33°C$ and $37°C$. This exercise confirms the temperature dependent activity of the RNA thermometers.

For a more quantitative estimate of RNA thermometer activity, we used the *E. coli* cell-free expression molecular breadboard (TX-TL, [6]). Primary advantage of this was to offer a fast and quantitative assessment. All TX-TL reactions were performed in black transparent-bottomed 384-well plates (Perkin Elmer) and GFP measurements (excitation - 485nm, emission - 515nm, gain - 61)

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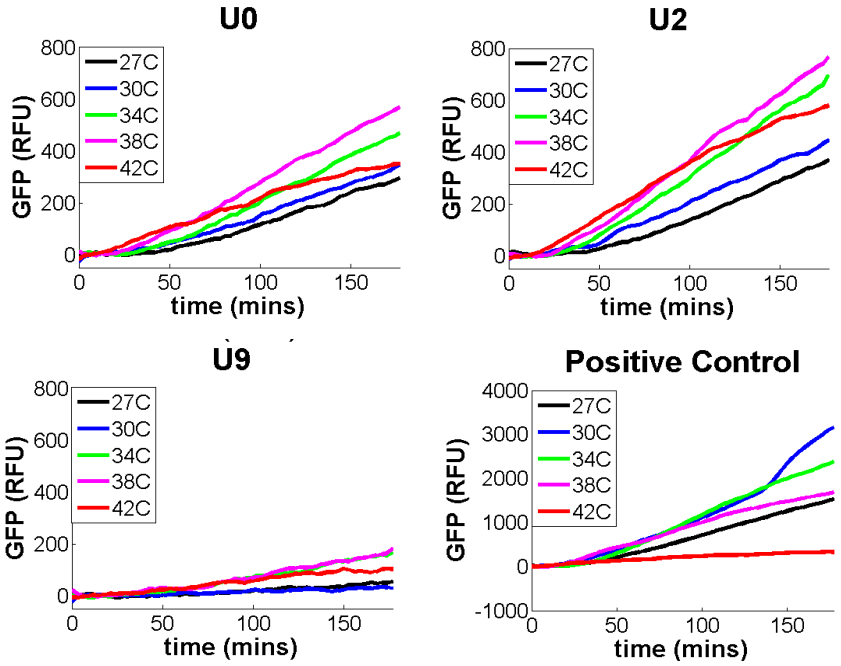


Figure 3: Quantitative kinetic measurements of RNA thermometer activity at different temperatures in a cell-free expression system. Different colors represent different temperatures.

were taken in Synergy BioTek I plate reader. TX-TL reactions require a crude cell extract obtained from *E. coli* cells and a buffer, calibrated to the cell-like conditions provided by that extract. Buffer calibration involves Mg-glutamate and K-glutamate optimization. The amount of plasmid DNA to be used was also calibrated to provide an acceptable signal while not saturating the TX-TL machinery. During calibration it was observed that U0, U2, and U9 RNA thermometers provided an easily measurable fluorescence signal.

To evaluate the temperature dependence of these RNA thermometers, TX-TL reactions were performed at $27°C$, $30°C$, $34°C$, $38°C$ and $42°C$ in the platereader, with measurements taken every 3 minutes for a total duration of 180 minutes (Fig. 3). We found that, in contrast to a constitutively expressed promoter expressing a green fluorescent protein (deGFP-T500) used as a positive control for the TX-TL reactions, which peaked at $30°C$, the green fluorescent protein (GFP-Trps16) expressed by the RNA thermometer constructs continued to increase till $38°C$. Further, there were differences in the activities among the RNA thermometers. For example, while both RNA thermometers U0 and U2 showed expression at all temperatures, RNA thermometer U9 had minimal expression for temperatures lower than $34°C$, after which it started to increase, indicating a threshold-like effect. We also performed the TX-TL reactions of U0, U2, and U9 in PCR blocks and measured fluorescence in a platereader at $t = 150$ minutes (Fig. 4). Consistent with measurements presented above, expression of these RNA thermometers increased with temperature. Activity of RNA thermometers U0 and U2 increased steadily, while that of RNA thermometer U9 stayed low initially and then increased at $\sim 35°C$. We conclude that these behaviours, especially among the RNA thermometers, are consistent with what is expected.

In addition to the temperature dependent change in the secondary structure of the RNA thermometer, there might be other aspects of the measurement process that might be temperature dependent. Indeed, when the RNA thermometer U2 expresses a different green fluorescent protein, deGFP-T500 instead of GFP-Trps16, the response to temperature, as measured by a time point at $t = 150$ minutes, is different (Fig. **??**). This suggests that the two green fluorescent proteins have different properties relative to temperature. In our investigations, we focus on differences in the

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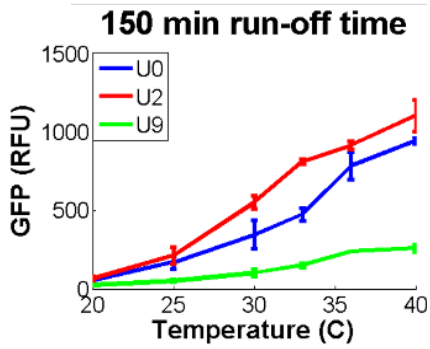


Figure 4: Quantitative end-point measurements of RNA thermometer activity at different temperatures in a cell-free expression system. Different colors represent different RNA thermometers.

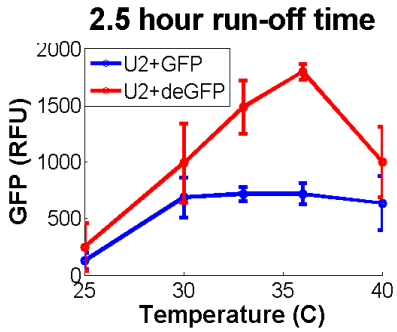


Figure 5: Comparison of the effect of reporter protein on RNA thermometer activity determination.

temperature responses to RNA thermometer activity, keeping other factors such as reporter protein and measurement context as identical as possible.

To complete our study, we computationally analysed the melt profiles of these RNA thermometers using the web server Nupack [9]. For a given RNA thermometer sequence, this can provide the fraction of unpaired base pairs as a function of temperature. As the RNA thermometer activity is proportional to the extent of its melting, we assumed that the fraction of unpaired base pairs as a measure of its activity. We computed the melt profiles of the RNA thermometers U0, U2, and U9 as well as the derivatives of these melt profiles with respect to temperature (Fig. 6). The significance of computing the derivative is that it clearly brings across the differences between switch-like responses and linear-like responses. For example, for a perfectly linear response to temperature, the derivative is flat. In contrast, for an abrupt switch at a particular threshold temperature, the derivative has a really high value in the vicinity of the threshold temperature. For RNA thermometer U9, the lower peak in the derivative of the melt profile was closer to a switch-like ideal than that of the RNA thermometer U2, suggesting that U9 has a more switch-like behaviour than U2. Further, the derivative of melt profile of RNA thermometer U2 starts increasing at lower temperatures than that of RNA thermometer U9, suggesting that there should be more activity at lower temperatures of U2 relative to that of U9. Finally, the derivative of the melt profile of RNA thermometer U0 is closes to the linear-like ideal, suggesting that this should be the most gradual increase among the considered thermometers. All of these indications from the melt profile calculations are consistent with experimental measurements. Therefore, these melt profiles could be used as way of estimating both steepness and threshold of an RNA thermometer's response to temperature.

Together, these results have analysed a set of synthetic RNA thermometers, providing a quantification of their activity, which was found to be consistent with previous measurements, as well as

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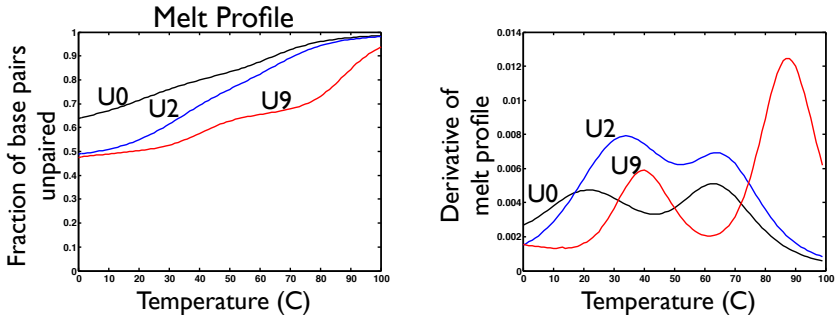


Figure 6: Computational analysis of melt profiles.

connections to computational melt profiles.

## 2.2 Computational Characterization of a RNA Thermometer Library

Given the correlation between computations and experimental measurements described above, we decided to use computationally obtained melt profiles as a way to characterised a possible library of RNA thermometers to see if different responses are possible, and also if they have different steepnesses and thresholds in their response to temperature. We also noted that the difference between RNA thermometers U2 and U9 contrasted above was only one base pair. This guided our strategy of designing a library by exploring the space of all sequences which are different from a starting sequence by one base pair. As a starting sequence, we chose the RNA thermometer U2, which has a relatively bright response at all temperatures. Given that this sequence has 43 base pairs, the size of the library should be $4 \times 43 = 172$, as each base pair may be replaced by the other three base pairs or deleted altogether. To reduce the size of the library, we chose to focus on those bases that are paired in the most favoured secondary structure (Fig. 7A). There are 18 of these, giving rise to $4 \times 18 = 72$ variants (Supplementary Information).

For each of these 72 sequences, we computed the melt profiles and compared with the corresponding melt profiles of the RNA thermometer U2 (Fig. 7B–C, individual profiles in Supplementary Information). Based on this comparison, we categorized the sequences into two categories. In the first category, we placed sequences whose melt profiles were similar to that of the RNA thermometer U2. Accordingly, we expect these to have a temperature response that is similar to that of U2. In the second category, we placed sequences whose melt profiles were different than that of the RNA thermometer U2. We also noted where the principle difference lay. For example, if the melt profile had a larger peak than U2, it was more switch-like. In contrast, if it was more flatter, then it was linear-like. We also noted here, whether the melt profile shifted along the temperature axis in comparison to that of U2.

These computations suggest what may be expected from an actual construction of these different RNA thermometers. In particular, these computations show that a variety of different responses to temperature could be obtained. Further, they provide classifications of the RNA thermometers in terms of their expected temperature responses.

## 2.3 Preliminary Experiments of Construction and Characterisation of a Library of RNA Thermometers

Towards generating a toolbox of RNA thermometers of different steepnesses and thresholds and to check agains the expectations presented in the previous section, we constructed a library of RNA thermometers according to the strategy outlined above. The cell-free expression platform is able

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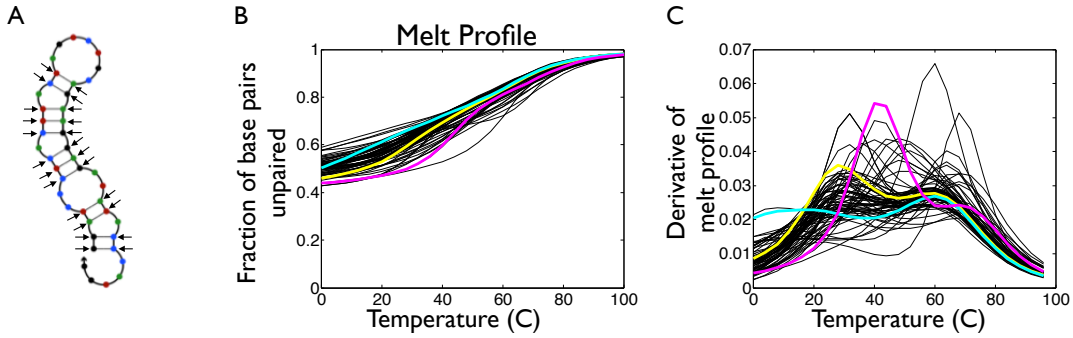


Figure 7: Computationally obtained melt profiles of library. A. Secondary structure of RNA thermometer U2, with arrows indicating bases that are mutated to all other base pairs or deleted, one at a time. B. Black lines represent the melt profiles of all RNA thermometers in the library. Yellow line represents the melt profile of U2. Cyan line represents a melt profile which is more linear-like than U2. Magenta line represents a melt profile which is more switch-like than U2. C. Derivatives of the melt profiles computed through a first difference.

to function with linear DNA, a feature that enables a faster design cycle. Therefore, the RNA thermometer library was first assembled as a linear DNA.

Each linear piece of DNA contained a promoter, an RNA thermometer region containing the Ribosome Binding Site, a green fluorescent protein (deGFP-T500), and a terminator, together with 250bp of junk DNA on either side to prevent(?) degradation [7]. These constructs were constructed using an overlapping PCR technique. One set of primers was used to amplify the region containing the RNA thermometer, with the required mutation present on one of the primers. Another set of primers was used to amplify deGFP, with the required mutation present on one of the primers. These two fragments were then fused using PCR with the outer set of primers. These reactions were carried out using Phusion Hot Start Flex 2X Master Mix (NEB) and primers were ordered from IDT Inc. (San Diego, California, USA). Sequences of the primers used are listed in the Supplementary Information file. A mixture of all PCR products, when run on a gel, showed the right size of the product (also in Supplementary Information).

We used these linear pieces of DNA as template for the TX-TL reactions at both $30°C$ and $37°C$. Measurement at $30°C$ was on a Synergy BioTek I platereader and at $37°C$ was on a Perkin Elmer Victor X3 platereader. In both cases, measurements are taken every 3 minutes for a total duration of 480 minutes. Subsequently, these readings were normalised by the GFP$\rightarrow$ nM of each platereader as well as by the nM of DNA used in each reaction. We plotted these measurements at $t = 270$ minutes of each of the RNA thermometers from the library as well as the starting RNA thermometer U2 (Fig. 8A). We also plotted these after normalisation by the expression profile of RNA thermometer U2 at each temperature (Fig. 8B).

An immediate observation from these measurements is the diversity in profiles that are generated, consistent with our expectations. Next, we compared the profile of each RNA thermometer with the classification expected from a computational melt profile. Overall, we found some cases where the expectation was met ($\sim$ 30%), some where it was reasonably met ($\sim$ 11%), and others where they were not met ($\sim$ 46%). These different cases are illustrated in the Figure through representative examples. These results provide an initial comparison of measured RNA thermometer responses with expectations. More importantly, they provide a toolbox of RNA thermometer elements with different temperature profiles.

For eventual applications, it is desirable to place these RNA thermometers inside cells and measure their responses to temperature. For this, the linear DNA pieces corresponding to different RNA

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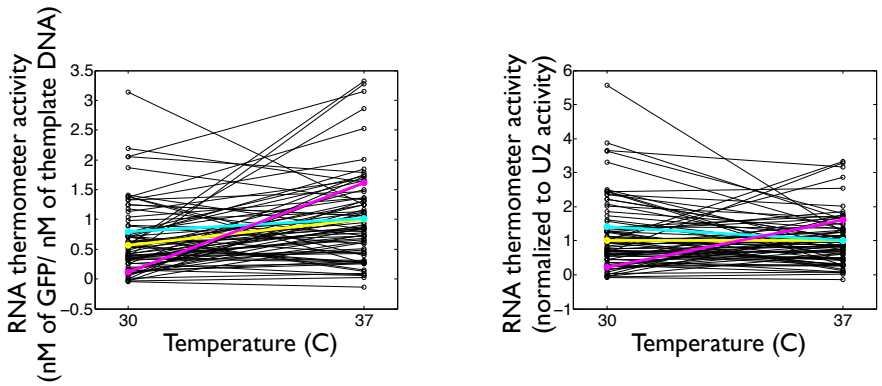


Figure 8: Preliminary experimental results of library synthesis and characterisation. A. Black lines connect measurements of RNA thermometer activity in the library expressed in terms of nM of GFP per nM of template DNA. Yellow line represent such a measurement for RNA thermometer U2. Cyan and magenta lines represent the same for RNA thermometer with a lower and higher steepness, respectively. B. Same plots as in A, except that the activities are normalized by the activity of RNA thermometer U2.

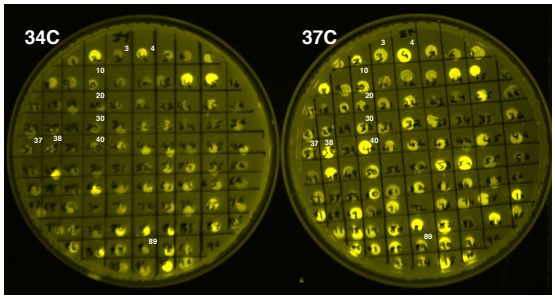


Figure 9: *E. coli* colonies from a screen for plasmids with different RNA thermometers at the indicated temperatures.

thermometers was mixed and then cloned into a vector. A screen for successfully cloned colonies should have a mixture of RNA thermometers. These were incubated overnight at both $30°C$ and $37°C$ and imaged with a fluorescence imager (Fig. 9). Again, the images underline the diversity of responses that could be obtained.

# 3 Discussion

RNA offers an attractive substrate for the important goal of developing components for biomolecular circuit design. Using a combination of computational and experimental methodologies, we report the following three results for the goal of designing RNA-based temperature sensors, which are referred to as RNA thermometers. First, we quantify the activity of a set of existing RNA thermometers as a function of temperature using a cell-free expression system and correlate the responses with their computational obtained melt profiles. Second, based on these, we systematically explored the computational melt profiles in the immediate neighbourhood of a RNA thermometer, finding the potential for a variety of responses to temperature, including with different steepnesses and thresholds. Third, we present preliminary measurements of a library of RNA thermometers constructed in this fashion, which give rise to a variety of temperature dependences, and, in some cases, match the responses expected from the computational analysis. This library can be used in the development of circuits that need to interface with temperature.

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It is interesting that even a one base pair change in the sequence of an RNA thermometer can generate a drastic change in the response to temperature. We have observed this in our results both computationally and through our preliminary experimental measurements. In fact, our results can be viewed as a sensitivity analysis of the map from a given RNA sequence to its response to temperature.

Results presented here are encouraging for the development of RNA thermometers and naturally lead to several concrete directions of future work. A first set of tasks has to do with refining the measurements of the library and through a verification of the constructs used through sequencing. Refinement to the measurements include considering a range of temperatures, analyzing the entire time profiles, and through measurements inside cells. Based on these, we may reevaluate the match to the expected computational profiles. A second set of tasks is to demonstrate functional applications of the RNA thermometers developed here. An example could be using these as the input stage of a pulse generating incoherent feedforward loop circuit, thereby generating a pulse that is triggered by temperature.

We have presented experimental and computational results for RNA-based temperature sensors. These have a wide variety of applications for analysis and design of biomolecular circuits. These could serve in a variety of places where heat is used as an induction mechanism, including genetic recombining as well as in large-scale bioreactors. At a finer level, these could also help in precise spatiotemporal control of protein expression deep inside biological media such as tissues. Finally, these could also serve as components that could be used to compensate for temperature dependent effects in biomolecular circuit designs, thereby making these more robust.

**Acknowledgements.** The authors thank Prof. Dr. Ralph Bock and Dr. Juliane Neupert from Max-Planck-Institut fr Molekulare Pflanzenphysiologie, Potsdam, Germany, for kindly gifting us the plasmids pBSU0, pBSU2, pBSU6, pBSU8 and pBSU9. We gratefully acknowledge H. M. T. Choi, C. Hayes, J. Kim, and A. Thubagere for their guidance and help in both experimental measurements and computational analysis.

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