# Reviewer 1:

*The manuscript by Sieg et al. reports the determination of folding thermodynamic parameters of RNA double helices using the MeltR software which is an open-source package in R programming language. The authors applied the software to fitting melting curves of RNA, and they compared thermodynamic parameters calculated using MeltR to those using the comparable software MeltWin. MeltR provides three analyses of RNA melting curves, and the analyses using MeltR are shown to be as accurate as MeltWin. More importantly, MeltR provides global-fitting analysis, not provided by MeltWin. The authors demonstrate the global-fitting analysis can produce thermodynamic parameters with minimal user specifications. This manuscript concludes that accurate determination of folding thermodynamic parameters can be made by MeltR. I think the study is important to the fields of biophysics. I recommend the publication of this manuscript after the authors consider the following comments.*

We thank the reviewer for the positive review. We have addressed the following comments as suggested, which included adding an additional 19 experimental datasets to our analysis, increasing the overall number of datasets analyzed in this paper from 11 to 30. We believe that this has substantially improved the manuscript. We have described our changes below and highlighted (yellow) changes in the revised manuscript and supplemental file 1.

# Comments:

*(1) I understand the program MeltR may apply to diverse biopolymers other than nucleic acids. However, the manuscript exclusively studies melting curve-fitting of RNA. I suggest the title of this manuscript (facile determination of "biopolymer" thermodynamics) is changed to more specific to nucleic acids.*

We made the requested change to the title.

*(2) All RNA helices that the authors used for the comparison with MeltWin (Table S1) were relatively short (< 10 bp). Did the authors compare thermodynamic parameters of longer RNA helices calculated using MeltR to those using MeltWin? The additional study would demonstrate the advantage and wide application of MeltR.*

We intentionally limited our study to short (<10 bp) RNA to avoid violation of the two-state assumption implicit to fitting data with *MeltR* and *MeltWin*. Even for short nucleic acids, great care must be taken for quantitative-analysis with two-state-models, to ensure that the data are consistent with a two-state melting transition, as we demonstrate in Figure 4. For long nucleic acids, a two-state melting transition is exceptionally unlikely and quantitative analysis by fitting to a two-state model is usually inappropriate. However, we believe it may be appropriate for users to perform a semi-quantitative analysis of melting curves of long nucleic acids with *MeltR*, given that the data is appropriately presented. We also agree with the reviewer that the additional study of long RNA would improve the paper by demonstrating the wide application of *MeltR*.

We added two datasets collected on long RNA, each dataset consisting of eight melting curves spanning a >50 fold concentration range for the CPEB3 ribozyme (68 nt) and a *B. subtilis* guanine riboswitch aptamer (73 nt). We fit the datasets with *meltR.A*, performed automated baseline trimming with the *BLTrimmer*, and compared the results to fits with *MeltWin*. The *MeltR* results were within error of the *MeltWin* results. For example, the *Tm* for the CPEB3 ribozyme was determined to be between 71.7 to 72.0 °C with *MeltR* and to be 71.8 °C with *MeltWin*. Note, the derivative analysis provided by *MeltR* is also useful for such a semi-quantitative analysis. For example, the first derivatives of the melting curves indicate that CPEB3 melts in a single broad transition (Figure S5A) and that the guanine riboswitch aptamer melts in two distinct transitions (Figure S5B). We reported the results for fitting CPEB3 in the new Table S2 but did not report results for the guanine riboswitch because first derivative analysis of the melting curves indicates a non-cooperative transition and fitting to a two-state model is inappropriate.

To address the reviewer’s comments in the text, we added the following paragraph to the discussion:

“So far, we have intentionally limited our study to short (≤13 nt) RNA to avoid violation of the two-state assumption implicit to fitting data with *MeltR* and *MeltWin*. Even for short nucleic acids, great care must be taken for quantitative-analysis with two-state-models, to ensure that the data are consistent with a two-state melting transition, as we demonstrated in Figure 4. For long nucleic acids, a two-state melting transition is unlikely and quantitative analysis by fitting to a two-state model, in the absence of highly cooperative folding, is inappropriate. However, users may want to perform a semi-quantitative analysis of melting curves of long nucleic acids with *MeltR*. To demonstrate this application, we collected two datasets on long RNA, each dataset consisting of eight melting curves spanning a >50 fold concentration range for the cleaved-CPEB3 ribozyme (68 nt) and the *B. subtilis g*uanine riboswitch aptamer (73 nt). We first fit the datasets with *meltR.A* and plotted the first derivative analysis provided by *meltR.A*. The first derivative analysis indicates that CPEB3 melts in a single transition (Figure S5A) and is thus appropriate for semi-quantitative analysis by fitting to a two-state model.However, the first derivative analysis indicates that the guanine riboswitch aptamer melts in two distinct transitions (Figure S5B) and is thus inappropriate for semi-quantitative analysis by fitting to a two-state model. We then performed automated baseline trimming for the CPEB3 data with the *BLTrimmer* and compared the results to fits with *MeltWin* in Table S2. The *MeltR* fits were within error of the *MeltWin* fits, indicating that *MeltR* is accurate in comparison to *MeltWin* for fitting long nucleic acids (Table S2).

*(3) I am very interested in the assessment of RNA melting curves that the upper or lower baseline is not clear in the experimental temperature range. Does MeltR work well for RNA helices with very high and low Tm, in comparison to MeltWin? The choices of baselines are particularly important in analyzing such RNA helices.*

We understand that well established baselines are not always possible for the experimentalist. We, therefore, investigated this situation and found that *MeltR* can work for helices with very high and low melting temperatures, both *meltR.A* and the *BLTrimmer* can fit data. The limiting case is when the data is so truncated that it does not resemble a melting curve and there is no information to fit.

To address this comment, we added datasets collected on the self-complementary 5’-UAUAUAUA-3’ and 5’-AGCCGGCU-3’ sequences, which have *Tm*s near 20 and 70 °C respectively, to our analysis in Figure 3, SI Table 1, and SI Figure 4. Lower-baselines were not clearly defined for the low-temperature melting sequence, and upper-baselines were not clearly defined for the high-temperature melting sequence (Figure R1). In both cases, *MeltR* was able to accurately determine thermodynamic parameters using automated baseline trimming in comparison to *MeltWin* (SI Table 1) and in agreement with the consensus nearest-neighbor model. For example, *MeltR* determined a Δ*G*°37 of -2.17 (-2.36 to -1.92) kcal/molusing method 2 on the low-melting, 5’-UAUAUAUA-3’ dataset, within error of the *MeltWin-*determined Δ*G*°37 of-2.30 (±0.17) kcal/mol. This is within 0.25 and 0.12 kcal/mol for *MeltR* and *MeltWin,* respectively*,* of the -2.42 kcal/mol expectation for the consensus nearest neighbor model.

**A picture containing invertebrate, worm

Description automatically generated**

**Figure R1** Example data sets of a low and high-temperature melting RNA where baselines are not well defined.

To address the reviewer’s comments in the text, we added the following paragraph to the discussion:

“Ideally, users should design experiments so that melting curves exhibit *Tm*s between 35 and 65 °C, providing at least 30 °C to define lower and upper baselines in the standard experimental temperature range of 5 to 95 °C. However, this may not always be possible. In these cases, users may analyze data near this limit, if enough of the sigmoidal shape of the melting curve is defined. For example, our analysis includes datasets collected on the self-complementary oligonucleotides 5′-UAUAUAUA-3′ and 5′-AGCCGGCU-3′, which have *Tm*s near 20 and 70 °C, respectively. Thus, lower baselines were not clearly defined for 5′-UAUAUAUA-3′, and upper baselines were not clearly defined for 5′-AGCCGGCU-3′. In both cases, *MeltR* was able to accurately determine thermodynamic parameters using automated baseline trimming in comparison to *MeltWin* (SI Table 1). For example, *MeltR* determined a Δ*G*°37 of -2.17 (-2.36 to -1.92) kcal/molusing method 2 on the low-melting, 5’-UAUAUAUA-3’ dataset, within error of the *MeltWin-*determined Δ*G*°37 of-2.30 (±0.17) kcal/mol. This is within 0.25 and 0.12 kcal/mol for *MeltR* and *MeltWin,* respectively*,* of the -2.42 kcal/mol expectation for the consensus nearest neighbor model.”

*(4) It is mentioned that MeltR provides "Monomolecular.2State" for self-structured strands. However, the manuscript does not contain the data of self-structured strands like hairpins. Can the authors add the data of monomolecular RNA folding?*

We included data modeled assuming monomolecular folding RNA in SI Figure 4. However, we agree that benchmarking *MeltR* on real, monomolecular RNA folding data will improve the manuscript. We added 15 published datasets collected on triloop, tetraloop, and pentaloop hairpins to our analysis in Figure 4, Figure S4, and Table S1. We also incorporated analysis of the monomolecular folding RNA into the results:

“We next tested *MeltR* by fitting real, experimental datasets for 28 different helices, where each dataset contained melting curves of the same helix at 5-12 different *Ct*s (Table S1). We compiled 15 published datasets for monomolecular, self-structured-hairpin RNA,26–28 7 published datasets for self-complementary RNA,19 and 6 new datasets collected on non-self-complementary RNA. The data were fit with *meltR.A* followed by the *BLTrimmer* (Table S1). For bimolecular helices, the three *MeltR* methods were in good agreement, with an average percent error between methods of 4.4%, 4.9%, 1.5%, and 0.6% for Δ*H*°, Δ*S*°, Δ*G*°37, and *Tm*, respectively. Likewise, for monomolecular helices, the two compatible *MeltR* methods, methods 1 and 3, were in good agreement, with an average percent error between methods of 3.1%, 3.1%, 4.7% (~0.14 kcal/mol), and 0.0% for Δ*H*°, Δ*S*°, Δ*G*°37, and *Tm*, respectively.

To obtain an independent measure of accuracy, we compared thermodynamic parameters calculated using *MeltR* to those using *MeltWin*. For the hairpin RNA and self-complementary duplexes, parameters calculated using *MeltWin* were from published sources19,26–28 (Table S1), while for the heteroduplexes, parameters calculated using *MeltWin* are presented for the first time (Table S1). Data from *MeltR* method 1 were in good agreement with those from *MeltWin* method 1 for bimolecular datasets, with an average %error in Δ*H*°, Δ*S*°, Δ*G*°37, and *Tm* between programs of 2.7%, 3.2%, 2.4%, and 2.1%, respectively (Figure 3A). Likewise, for method 2, data from *MeltR* were in good agreement with *MeltWin* for bimolecular datasets, with an average %error in Δ*H*°, Δ*S*°, Δ*G*°37, and *Tm* between programs of 2.8%, 3.3%, 2.2%, and 2.2%, respectively (Figure 3A). For monomolecular datasets, the average %errors were slightly larger, with an average error in Δ*H*°, Δ*S*°, Δ*G*°37, and *Tm* between programs of 4.1%, 4.0%, 6.3%, and 2.0% for method 1, respectively (Figure 3A). Even so, the absolute errors between programs for monomolecular and bimolecular structures were small, ~0.17 kcal/mol on average in terms of the Δ*G*°37. Interestingly, the unique *MeltR* method 3 of global fitting reproduces both *MeltWin* methods 1 and 2 for bimolecular and monomolecular datasets (Figure 3B-E).

We next tested *MeltR* parameters generated from fitting the real data for agreement with the predicted values from the consensus nearest neighbor model. Folding parameters for each of the 28 helices in the real datasets were calculated using published Watson-Crick nearest neighbor parameters.7,19,26–28 Errors in Δ*G*°37 between the nearest neighbor model and *MeltR*/*MeltWin*-calculated values were 0.34-0.39 kcal/mol on average and smaller than 1 kcal/mol for all helices (Figure S4E). Lastly, there is no significant difference in errors in comparison to the consensus nearest neighbor model between any of the methods from *MeltR* and *MeltWin*, meaning that errors produced by *MeltR* are not statistically different from errors produced by *MeltWin* (Figure S4E). In conclusion, *MeltR* accurately determines folding parameters.”

*(5) To make the manuscript more easy reading, I recommend enlarging the letters in Supplementary Figures (the letters "Frequency" in the vertical axis of Figure S2 are hard to read, as an example).*

We increased the font size in the supplementary figures, including doubling the font size for the automatically generated plots shown in Figure S1 and Figure S2.