High-throughput Screening of RNA Stability Through Differential Scanning Fluorimetry

Melanie Castro, Nolan Blackford, Robert Silvers PhD
Florida State University, Department of Chemistry and Biochemistry

Abstract

The use of RNA has become prevalent in the development of new drugs. Current research has been done on using RNA molecules as drugs themselves. Additionally, RNA that contain riboswitches have shown potential as drug targets [1].

The stability of the RNA plays a role in gene expression and is related to its specific function [2]. The current approaches of analyzing RNA stability through techniques like ultraviolet-visible spectroscopy (UV-Vis spectroscopy) are not efficient as they can only take a certain number of samples and require a large volume for each sample [5]. Therefore, there is a need for a high throughput technique that analyzes RNA more efficiently.

The purpose of this research is to establish an alternative technique that accurately measures RNA stability. We have established differential scanning fluorimetry (DSF) as a competent high throughput screening method for analyzing RNA stability.

Introduction

- UV-Vis spectroscopy uses a light source to measure absorbance at specific wavelengths. In this experiment, UV-Vis did not use dye. The RNA itself absorbed the light at 260nm [4].
- DSF is typically used to analyze protein stability [3]. In this experiment, It works by monitoring the destabilization of RNA using fluorescent dyes. Dyes that show specificity towards double or single stranded RNA make this possible. The quantum yield of the dye increases when it binds to the RNA which results in an increase in fluorescence.

Methodology

1. Samples of lyophilized RNA were resuspended in 50mM Tris pH 7.5, 200mM NaCl and 3mM EDTA. Varying concentrations of RiboGreen™ dye and ethidium bromide were prepared with serial dilutions using DMSO as the dilutant such that the DMSO concentration was consistent for every sample. All samples were prepared in microcentrifuge tubes. They were vortexed and centrifuged to evenly distribute the dye. The samples were pipetted into a 96-well plate with 20µL in each well. Three redundant conditions were tested simultaneously to obtain averages. The well plate was centrifuged and loaded into a Quant Studio™ 7 Flex Real-Time PCR machine for analysis.
2. The same 400x RiboGreen™ condition was done on a UV-Vis spectrometer using the melt curve setting.
3. The steps above were replicated with varying urea concentrations.
4. The raw data was processed in Excel. The results of the three redundant runs were averaged and the first derivative was calculated. Data smoothing was done by taking the derivative across more points. Plots of average fluorescence vs temperature and average fluorescence vs the derivative were created. The maximum of the first derivative was used to determine the melting temperature.
5. The urea concentration and its melting temperature were compared and plotted on a graph using Excel.

Results

Test Number	UV-Vis (-) RiboGreen (°C)	UV Vis (+) RiboGreen (°C)	DSF (+) RiboGreen (°C)
1	55.12	54.20	54.73
2	54.47	54.47	54.67
3	55.02	55.17	54.62
4	54.72	55.12	-
5	54.39	55.17	-
Average	54.74	54.83	54.67
Standard Deviation	0.323	0.459	0.055

Fig. 1: 5 µM 10-mer A+B in 50 mM phosphate, 1 mM EDTA, 5 mM NaCl and 2% DMSO/Dye (DMSO added to (-) samples to keep the concentration the same. Dye concentration is 400 times diluted from stock labeled as 10,000x)

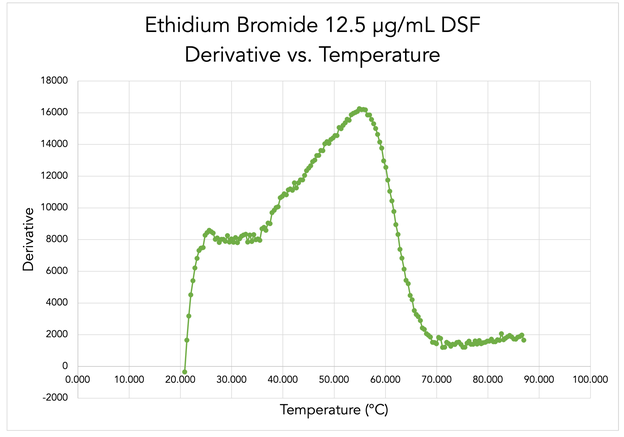

Fig. 2: 1 µM 10-mer A+B, 50mM Tris pH 7.5, 200mM NaCl, 3mM EDTA (2% DMSO comes from dye for DSF)

Ethidium Bromide Intercalating Dye Effects	
Concentration (µg/mL)	Melting Temperature
100.	65.6
50.0	61.7
25.0	58.1
12.5	55.3
6.25	54.1
3.13	53.3
1.56	52.1

Fig. 3: Comparison of melting temperatures at varying ethidium bromide concentrations

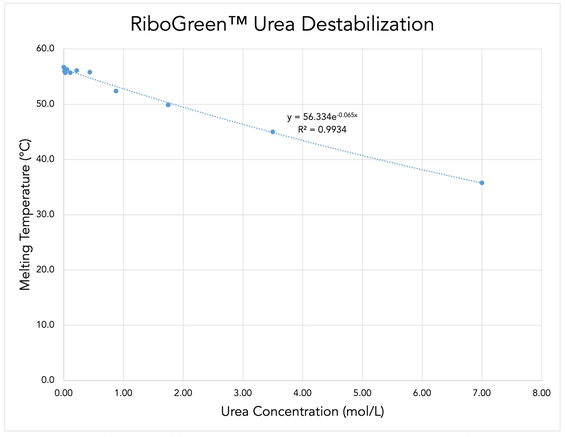

Fig. 4: RiboGreen™ Urea Destabilization: The known melting temperature for this RNA is around 55-56°C. Urea destabilizes RNA and lowers the melting temperature by hydrogen binding to the base pairs.

Urea Destabilization Effects	
Urea Concentration (M)	Melting Temperature (°C)
7.00	35.8
3.50	45.0
1.75	49.9
0.88	52.4
0.44	55.8
0.22	56.1
0.11	55.7
0.05	56.3
0.03	55.7
0.01	56.0
0.00	56.7

Fig. 5: Comparison of melting temperatures at varying urea concentrations and consistent dye concentrations

Conclusion

- We found that the DSF technique was able to create similar results to UV-Vis spectroscopy, thus confirming this method is accurate (Fig. 1).
- For the DSF run, lower concentrations of ethidium bromide had a destabilizing effect, while higher concentrations had a stabilizing effect (Fig. 2 & 3).
- Ethidium bromide is an intercalating dye that forms pi bonds with the RNA's bases. Ethidium bromide has a destabilizing effect and therefore intercalating dyes do no work well for DSF. RiboGreen™ had no significant effect on interfering with RNA stability using DSF.
- Urea was used because it destabilizes RNA secondary structure by solubilizing nitrogenous bases. Hence, lowering the melting temperature of the RNA (Fig. 4 & 5). In this experiment, urea simulated a drug binding to the RNA to destabilize it.
- We were able to identify what concentration of dye is useful for this specific 10-mer double stranded RNA.
- We do not know if these concentrations hold true for all types of RNA sequences. More research needs to be done on different RNA sequences and specific RNA that contain riboswitches.
- We plan to see if this technique can detect changes to RNA stability caused by drug binding.

RNA Sequences

10-mer A Sequence:
5-AGCUCGCAUG-3

10-mer B Sequence:
5-CAUGCGAGCU-3

References

[1] Ai-Ming Yu, Young Hee Choi and Mei-Juan Tu Pharmacological Reviews October 1, 2020, 72 (4) 862-898; DOI: https://doi.org/10.1124/pr.120.019554

[2] Boo, S.H., Kim, Y.K. The emerging role of RNA modifications in the regulation of mRNA stability. Exp Mol Med 52, 400–408 (2020). https://doi.org/10.1038/s12276-020-0407-z

[3] Niesen, F., Berglund, H. & Vedadi, M. The use of differential scanning fluorimetry to detect ligand interactions that promote protein stability. Nat Protoc 2, 2212–2221 (2007). https://doi.org/10.1038/nprot.2007.321

[4] Okamoto T, Okabe S. Ultraviolet absorbance at 260 and 280 nm in RNA measurement is dependent on measurement solution. Int J Mol Med. 2000 Jun;5(6):657-9. doi: 10.3892/ijmm.5.6.657. PMID: 10812018.

[5] Silvers, Robert Et Al. "Differential Scanning Fluorimetry For Monitoring RNA Stability." Chembiochem : A European Journal Of Chemical Biology 16.7 (2015): 1109–1114. Web.

Acknowledgements

Florida State University Undergraduate Research Opportunity Program (UROP)