**Supplementary Information 2 –** **Free Mg2+ concentration error analysis**

The Metabolome Weakens RNA Thermodynamic Stability and   
Strengthens RNA Chemical Stability

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*Free Mg2+ concentration error analysis*

It is important to understand the error of the free Mg2+ concentration and the impact of such errors on RNA stability to properly assess the results presented in the main text. In this section, we first analyze the precision of the free Mg2+ concentration determination using HQS, then provide a conservative estimate for the true uncertainty of the free Mg2+ in artificial cytoplasm. Lastly, we consider if errors in the free Mg2+ concentration could explain the results presented in the main text.

The biological free Mg2+ concentration range of 0.5 to 3.0 mM is within the linear range of the HQS calibration curve (SI2 Figure 1A). Propagation of the errors from the calibration curve fit used to determine the free Mg2+ concentration indicate that errors are minimized in the biological free Mg2+ concentration range, less than 5% of the calculated value (SI2 Figure 1B). The HQS assay is less precise outside of this range of free Mg2+ concentrations, because large changes in Mg2+ concentrations only lead to small changes in HQS emission. For example, in the absence of free chelators, 200 mM Mg2+ produces the same fluorescence emission as 100 mM Mg2+ because HQS is already almost completely bound at 200 mM Mg2+. Thus, the HQS assay is precise, with an uncertainty less than 5% of measured free Mg2+ concentration, within the biological free Mg2+ range but not outside of it.

It may appear that the free Mg2+ concentration is changing rapidly with the total Mg2+ concentration near 2 mM free Mg2+ in Figure 1E because there is almost no change at lower total concentrations and the linear y-axis/log10 transformed x-axis. However, the actual slope for this region is not large, at 0.08 mM free Mg2+ for each 1 mM increase in total Mg2+. This buffering is evidence that the free Mg2+ is constant near 2 mM given perturbations of the total Mg2+.

The true uncertainty for the free Mg2+ concentration in our experiments is hard to calculate, but 10%, or twice the propagated uncertainty from the fit in the biological free Mg2+ range (SI2 Figure 2B), is a conservative value. At 2 mM, this would be an uncertainty of 0.2 mM. A free Mg2+ concentration error of 0.2 mM would require a total Mg2+ error of 2.5 mM, given the buffering of 0.08 mM free Mg2+ for each 1 mM increase in total Mg2+ by Eco80.

A Mg2+ concentration error of 0.2 mM is unlikely to impact our results. For example, we applied a tightly bound ion (TBI) theoretical model for mixed Na+ and Mg2+ solutions to calculate the change in free energy (ΔΔG°37) from a 380 mM Na+ 2 mM free Mg2+ reference state as a function of the free Mg2+ concentration (SI2 Figure 1C).1 Accordingly, errors in the free Mg2+ concentration are unlikely to cause the free energy changes we observed in Figure 2, notably, the +0.69±0.12 we observed in Eco80. Indeed, a 1 mM free Mg2+ concentration, an error of 50%, would be required to cause even a 0.25 kcal/mol fluctuation in the ΔG°37. A 1 mM free Mg2+ concentration error would require our estimate for the total Mg2+ required to provide 2 mM free Mg2+ in Eco80 to be off by 12.5 mM, given the buffering capacity of Eco80. In summary, given the free Mg2+ buffering capacity of the artificial cytoplasm’s and the precision of the HQS assay in the biological free Mg2+ range, the results of our experiments cannot be explained by errors in the free Mg2+ concentration alone.

Chart

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**SI2 Figure 1** Analysis of free Mg2+ errors in Eco80. **(A)** Figure 1B modified with a x-axis to show that the biological free Mg2+ range is within the linear range of the HQS calibration curve. **(B)** Uncertainty in the free Mg2+ concentration calculated from HQS emission, estimated by propagating uncertainty in the calibration fit coefficients, as a function of the free Mg2+ concentration calculated from HQS emission. **(C)** TBI theoretical model prediction of the ΔΔG°37 as a function of the free Mg2+ concentration for a 8 nucleotide helix. The reference state approximates the 2 mM free Mg2+ condition in this manuscript, 380 mM Na+ and 2 mM Mg2+.

*Supplemental references 2*

(1) Tan, Z.-J.; Chen, S.-J. RNA Helix Stability in Mixed Na+/Mg2+ Solution. *Biophys. J.* **2007**, *92* (10), 3615–3632. https://doi.org/10.1529/biophysj.106.100388.