Polyamine/Nucleotide Coacervates Provide Strong Compartmentalization of Mg^{2+}, Nucleotides, and RNA

Supplemental Information

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MATERIALS AND METHODS:

Preparation of coacervates. Coacervate samples were prepared from nucleotides (5' Adenosine monophosphate (AMP), 5' Adenosine diphosphate (ADP) and 5' Adenosine triphosphate (ATP)) and poly(allylamine) hydrochloride 15kDa (PAH) purchased at the highest purity from Sigma-Aldrich and were not further purified before use. Solutions were prepared with Nanopure® 18.2 MΩ-cm water and were buffered with a final concentration of 2 mM (2-(*N*-morpholino)ethanesulfonic acid (MES) pH 5, 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) pH 7, *N*-Cyclohexyl-2-aminoethanesulfonic acid (CHES) pH 9). Low buffer concentrations were used in order to limit the amount of interactions these zwitterionic molecules had in coacervate formation. The pH of all solutions were checked by pH paper before and after measurements to make sure the pH remained constant. In most samples, a 1 mM stock of PAH was added to water and buffer to give a final concentration of 38.5 μM, or 10 mM positive charge moieties (~15,000 g/mol ÷ 58 g/mol (monomer) ≈ 260 monomer units, 260 * 38.5 μM = 10 mM). A 50 mM $MgCl_2$ stock solution was added to samples to give final concentrations between 0 and 25 mM. A 50 mM nucleotide stock concentration was added to samples to give final concentrations of 2.5 mM ATP, 3.3 mM ADP, and 5 mM AMP for charge-balancing, or 10 mM in each nucleotide for concentration-balancing. To promote mixing of polyelectrolytes, samples were vortexed after addition of all reagents and allowed to equilibrate for at least 2 h. Optical microscopy images indicated the formation of coacervates or aggregation of particles. Microdroplets were confirmed to be fairly homogeneous in size and shape, typically along the order of 3-5 μm.

Coacervate Formation Conditions. To determine conditions under which phase separation occurs, a combination of turbidity measurements and optical microscopy methods were used.

Sample turbidity was assessed by changes in transmittance at 600 nm using a Beckman UV-Visible Spectrometer, as described.[1] All samples were prepared immediately before analysis and vortexed to resuspend any phase droplets that may have settled to the bottom of the conical tube prior to analysis. Absorbance readings were converted to turbidity using the equation 100-%T, where %T is the percent transmittance. To convert absorbance (A) to %T, we used the equation $\%T = 10^{2-A}$. We fit the turbidity plots to the following equation for ½ values and relative steepness:

$$\%T = T_{min} + (T_{max} - T_{min}) \frac{\left({}^{[x]}/_{c_{1/2}}\right)^n}{1+\left({}^{[x]}/_{c_{1/2}}\right)^n} \quad (1)$$

Where T_{min} and T_{max} represent the minimum and maximum turbidity for a given set of samples, variable [x] represents the nucleotide or poly(allylamine) concentration, $c_{½}$ represents the concentration of either nucleotide or poly(allylamine) to achieve half the maximum turbidity, and n represents the steepness coefficient. Fits from this equation can be found in SI Figures 1 and 2. Relative $c_{½}$ and n values for SI Figures 1 and 2 are found in SI Tables 5 and 6.

Although coacervate formation is with associated with an increase in solution turbidity,[2-4] so is aggregation. We confirmed the type of phase formation by white-light optical microscopy using a Nikon Inverted microscope with a 20x objective, and qualitatively identified each sample as single phase, coacervate, or aggregates. A simple check of the phase formation was also completed on all samples by means of centrifugation methods. Upon centrifugation at 13,300 *g* for 10 min, samples that contained aggregates formed a film along the side of a conical tube, whereas samples that contained coacervates had a very small pellet at the bottom of the tube.

Dynamic Light Scattering and Zeta Potential Measurements. The droplet surface electrical charge (Zeta potential) was determined as a function of $MgCl_2$ concentration, pH and nucleotide

at 25 ± 1 °C using a Zetasizer Nano (Malvern Instruments, Westborough, MA). Samples of 1 mL were prepared using the same procedure as stated above. Before measurements, each set of samples was briefly vortexed to resuspend any droplets that had settled to the bottom of the tube. The final values were calculated based on three separately prepared samples, each of which were measured in triplicates. Each measurement had an average of 10 readings with a 30 second delay in between readings.

Estimation of Coacervate Phase Volume. A set of volume standards were prepared in order to better estimate the volume of the coacervate phase for each set of nucleotide solutions varying in pH and Mg^{2+} concentration. A known volume of a solution containing xylene cyanol and bromophenol blue was pipetted into a 1.7 mL conical tube and centrifuged to ensure each droplet was at bottom of the tube. Solutions with 1 mL of PAH, nucleotides and Mg^{2+} at varying pH were vortexed and allowed to come to equilibrium (~2 h). Samples were then centrifuged at 13,300 g for 15 min to coalesce all coacervate droplets to the bottom of a 1.7 mL conical tube. Each sample was compared against the known standards by visual inspection. An example of how this was done can be visualized in SI Figure 5. Given that the volumes of the coacervate were considerably smaller than the dilute phase, several large solutions were also prepared with a total volume of 10 mL. Under these conditions, similar volume standards were prepared in a 15 mL tube. Coacervate phases of ~5 μL resulted, which could be more easily determined, giving more confidence in the estimated volumes.

Determination of Nucleotide Concentration. Partitioning of nucleotides into the coacervate phase was determined through UV absorption (ε_{260} = 15,400 $M^{-1}cm^{-1}$ for 5' ATP, 5' ADP and 5' AMP).[5] Samples were prepared as stated above, noting the total concentration of the nucleotides in each case. Solutions were allowed to equilibrate for 2 h, followed by centrifugation at 13,300

g for 10 min. The upper half of the supernatant for each sample, which should not contain any coacervates, was removed and placed into a separate conical tube and diluted up to 400-fold with distilled, deionized water. Absorbance values pertaining to the diluted supernatants were converted to nucleotide concentration using Beer's law. This concentration was subtracted from the total concentration of nucleotides added and then multiplied by the total volume to determine the moles of nucleotides in the coacervate phase. The moles of nucleotides left were divided by the estimated volume of the coacervate phase.

RNA partitioning determination. Partitioning experiments were completed by scintillation counting through radiolabeling. All partitioning experiments were completed at 25°C. In terms of radiolabeling, heterogeneous, random sequence RNA consisting of 54 nucleotides (N_{54}), polyA, HH16 S or HDV E were radiolabeled (γ-ATP (Perkin Elmer, Waltham, MA), T4 Polynucleotide Kinase (NEB, Ipswich, MA)) and gel purified on a 10% acrylamide, 8.3 M urea denaturing gel. Purified, radiolabeled RNA (~1 μL) was added to solutions containing nucleotides and poly(allylamine), varying $MgCl_2$ concentration (0-25 mM) and pH (2 mM MES pH 5, 2 mM HEPES pH 7, 2 mM CHES pH 9). Samples were vortexed, then centrifuged at 13,300 *g* to collect the droplets. The upper half of the supernatant volume, which contained the dilute phase was removed from the coacervate phase, and placed into a separate tube. The two tubes, one with just the dilute phase and one containing both phases were scintillation counted separately. The partitioning of radiolabeled RNA was determined by first subtracting the counts from the supernatant volume alone from the vial containing supernatant and coacervate phase. In addition, the counts from the supernatant, or continuous phase, were doubled. The counts in the continuous phase and coacervate phase were divided by their respective volumes (shown in Supplemental Table 5) to obtain the concentration of counts in both phases. The volumes of the

continuous phase and coacervate phase were previously determined to be approximately 99.95-90 μL and 0.05-10 μL, respectively. The partitioning constant was calculated based upon the equation listed below, which compares the concentration of RNA in the continuous phase against the concentration of RNA in the coacervate phase. The partitioning constant is reported as the negative $\log_{10}$ value, so a $K_{partitioning} = 10^{-6}$ would be reported as a $-\log_{10}K_{partitioning} = pK_{partitioning} = 6$.

$$K_{partitioning} = \frac{C_{continuous}}{C_{coacervate}}$$

UV Melt of RNA oligonucleotides. A solution containing either 0.5 μM of hammerhead 16 (HH16) ribozyme substrate strand[6] or the Human Hepatitis Delta Virus (HDV)[7] ribozyme enzyme strand were prepared in the presence of 2 mM HEPES Buffer pH 7. Samples were heated to 90ºC for two minutes, then allowed to cool down to room temperature for 10 minutes. A final concentration of 5 mM Mg^{2+} was added to both samples, and allowed to equilibrate for 10 minutes. Samples were spun down to remove any particulates or gas bubbles, and melted at 260 nm from 5-95ºC with a 1ºC/min step. Sequences for the HH16 and HDV RNA are listed below:

Hammerhead 16 Substrate: 5' GGG AAC GUC GUC GUC GC 3' (17 nt)

Human Hepatitis Delta Virus Enzyme: 5' GGU CCC AGC CUC CUC GCG GCG CAA GCU GGG CAA CAU UCC GAA AGG UAA UGG CGA AUG GGA CC 3' (62 nt)

Ion partitioning determination. Atomic absorption spectrophotometry was used to determine Mg^{2+} partitioning. For each experiment, two identical samples were prepared using the method stated above. One of these samples was subsequently centrifuged at 13,300 *g* for 10 min and half of the continuous phase was removed and diluted in 10 mL of distilled water. The identical solution did not undergo centrifugation, but was diluted to 10 mL with distilled water. This sample contained both the coacervate and dilute phase. Absence of centrifugation ensured that we measured all of the coacervate phase. (Due to the small size of the coacervate phase, it would

have been impossible to determine whether all of the coacervate phase was re-suspended in solution.) Both solutions were analyzed with a Shimadzu Flame Atomic Absorption Spectrometer 7000 (Columbia, MD). To determine the partitioning of the Mg^{2+} within the continuous phase versus the coacervate phase, the concentration of Mg^{2+} in (mg/L) was converted concentration using the same methods above.

SUPPLEMENTAL TABLES AND FIGURES:

Supplemental Table 1: $c_{1/2}$ values and steepness coefficients for turbidity plots as a function of nucleotide concentration

	ATP			**ADP**			**AMP**		
pH:	5	7	9	5	7	9	5	7	9
c ½ (mM)	0.5 ± 0.1	1.2 ± 0.1	0.9 ± 0.1	1.8 ± 0.3	1.4 ± 0.1	1.2 ± 0.1	2.5 ± 0.2	2.7 ± 0.1	2.3 ± 0.1
n	1.9 ± 0.2	11.9 ± 2.1	8.1 ± 3.5	7.9 ± 4.7	7.4 ± 2.1	10.6 ± 3.9	4.2 ± 0.6	5.1 ± 0.5	14.6 ± 5.1

Data from turbidity plots displayed in SI Figure 1 were fitted using the equation found in SI material and methods. The $c_{½}$ values and n coefficients from fits are listed in the table above.

Supplemental Table 2: $c_{1/2}$ values and steepness coefficients for turbidity plots as a function of poly(allylamine) concentration

	ATP			**ADP**			**AMP**		
pH	5	7	9	5	7	9	5	7	9
c ½ (mM)	108.8 ± 44.2	87.5 ± 4.1	82.8 ± 4.9	90.6 ± 8.1	68.9 ± 3.5	90.4 ± 1.3	31.4 ± 4.1	47.6 ± 1.7	54.8 ± 1.1
n	16.0 ± 69.6	15.6 ± 5.2	19.9±10.0	3.9 ± 0.9	8.5 ± 3.1	14.0 ± 1.5	6.7 ± 1.9	6.6 ± 4.2	3.7± 0.2

Data from turbidity plots displayed in SI Figure 2 were fitted using the equation found in SI material and methods. The $c_{½}$ values and n coefficients from fits are listed in the table above.

Supplemental Table 3. Dissociation constants (M) for Mg^{2+} to nucleotide (L) at 25 °C (μM)[a]

	5' ATP	5' ADP	5' AMP
$Mg^{2+} + HL^{y-} = MgHL^{2-y}$	5.75×10^{-3}	1.07×10^{-2}	2.29×10^{-2}
$Mg^{2+} + L^{y-} = MgL^{2-y}$	6.02×10^{-5}	6.76×10^{-4}	1.29×10^{-2}

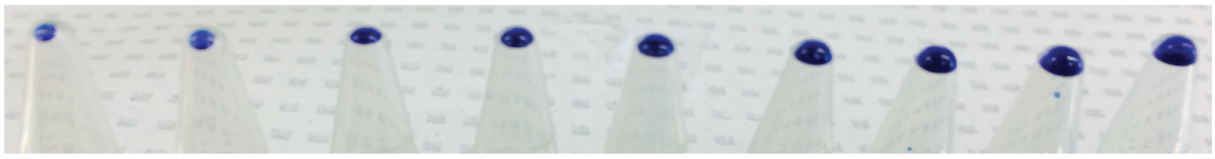
Dissociation constants were adapted from Izatt, et. al.[8], which cites logK of binding of ions to nucleotides under various temperatures and pH conditions. L^{y-} represents a nucleotide in either its protonated (HL) or deprotonated (L) form. 5' ATP exhibits the tightest binding to Mg^{2+}, exhibiting a K_d in the micromolar range. The presence of the proton on the γ-phosphate significantly weakens binding by two orders of magnitude. This trend is seen in 5' ADP samples as well. 5' AMP, which only has two available oxygen species along the phosphate for Mg^{2+} binding, exhibits the weakest K_d.

Supplemental Table 4: Phase chart for solutions with different pH and nucleotides and $MgCl_2$ concentrations

		Total [$MgCl_2$] (mM)						Total [$MgCl_2$] (mM)			
Nucleotide	pH	0	0.5	5	25	Nucleotide	pH	0	0.5	5	25
2.5 mM ATP	5	Coacervates form under all conditions				10 mM ATP	5	Coacervates form under all conditions, higher polydispersity			
	7						7				
	9						9				
3.3 mM ADP	5	Coacervates form under all conditions				10 mM ADP	5	Coacervates form under all conditions, higher polydispersity			
	7						7				
	9						9				
5 mM AMP	5	Coacervates		None		10 mM AMP	5	Aggregates			
	7	Aggregates		Coacervates			7				
	9	Coacervates		Aggregates			9				

Phase chart for solutions containing varying nucleotides, Mg^{2+} concentration and pH environment. Left side shows phase formation for charge-balanced solutions. Right side shows phase formation for concentration-balanced solutions.

Supplemental Table 5: Standardized droplets used to estimate coacervate phase volumes.



		Volume of coacervate phase (µL)					
[$MgCl_2$] (mM):		0	1	5	10	15	25
Nucleotide	**pH**						
2.5mM ATP	5	3.0	3.0	2.5	2.0	2.0	1.0
	7	3.0	3.0	2.5	2.0	2.0	1.0
	9	3.0	3.0	2.5	2.0	2.0	1.0
3.3mM ADP	5	0.5	0.5	0.5	0.5	0.5	0.5
	7	1.0	1.0	1.0	0.5	0.5	0.5
	9	1.0	1.0	1.0	0.5	0.5	0.5
5.0mM AMP	5	10	10	9.0	8.0	7.0	7.0
	7	10	10	9.0	8.0	6.0	5.0
	9	10	10	8.0	5.0	5.0	5.0

		Volume of coacervate phase (µL)					
[$MgCl_2$] (mM):		0	1	5	10	15	25
Nucleotide	**pH**						
10mM ATP	5	3.0	3.0	2.5	2.0	2.0	1.0
	7	3.0	3.0	2.5	2.0	2.0	1.0
	9	3.0	3.0	2.5	2.0	2.0	1.0
10mM ADP	5	2.0	1.5	1.0	0.5	0.5	0.5
	7	2.0	1.5	1.0	0.5	0.5	0.5
	9	1.5	1.5	1.0	1.0	0.5	0.5
10mM AMP	5	15	15	15	13	12	11
	7	13	13	13	12	11	10
	9	11	11	11	11	9.0	9.0

Volumes of phases estimated from standard volumes of xylene cyanol dye. Volumes were found to change depending on salt concentration, pH and nucleotide identity. The total volume used for these estimations was 1000 µL. In addition, larger solutions were prepared with a volume of 10 mL for select samples to validate these values. The volumes of the coacervate were found to scale with the increased volume of the solution (as described in the Materials and Methods).

Supplemental Table 6: -logK partitioning of N54 in coacervate phase determined by scintillation counting

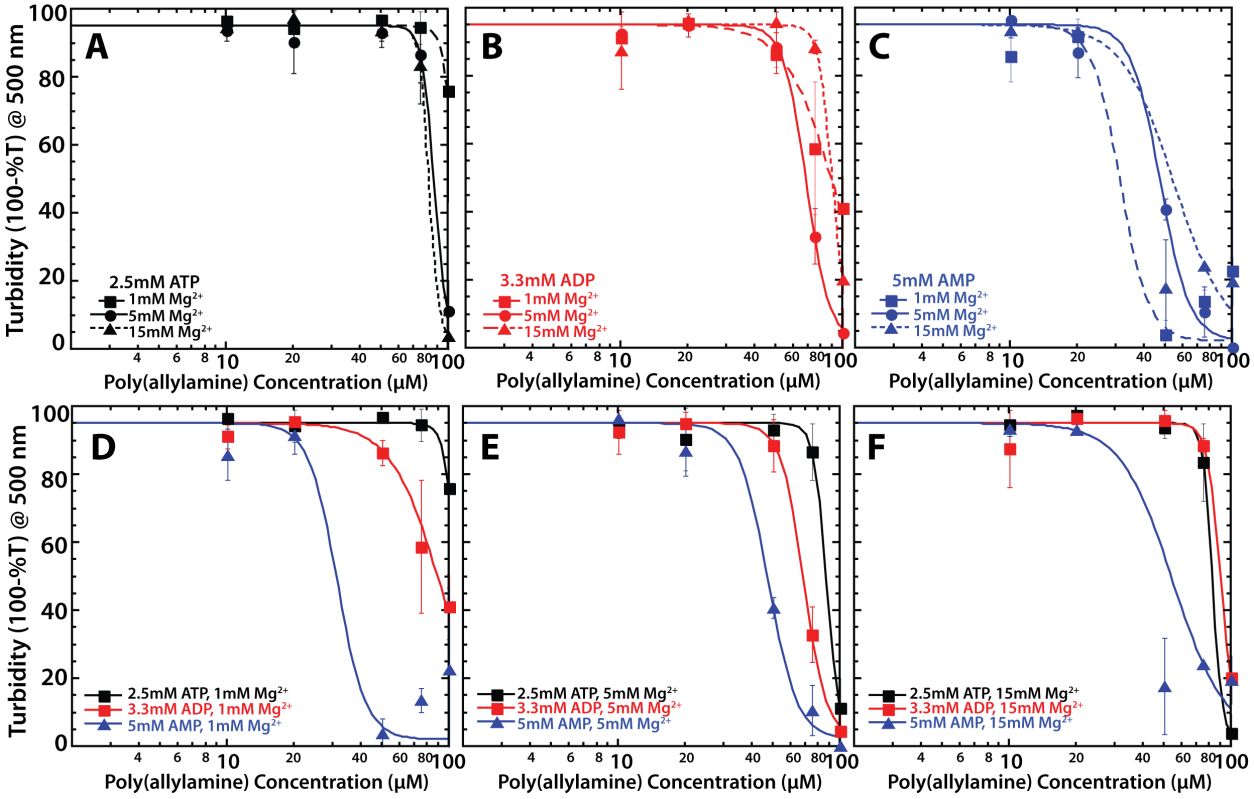
Equal Charge Concentration

[$MgCl_2$] (mM):		0	0.5	1	5	10	15	25
Nuc.	**pH**							
ATP	**5**	4.704 ± 0.503	5.126 ± 0.212	4.366 ± 0.478	4.830 ± 0.599	4.424 ± 0.630	4.386 ± 0.937	5.301 ± 0.239
	7	4.625 ± 0.543	5.257 ± 0.205	4.983 ± 0.346	5.146 ± 0.331	5.475 ± 0.136	4.913 ± 0.437	5.463 ± 0.360
	9	4.627 ± 0.510	4.935 ± 0.400	4.923 ± 0.573	4.959 ± 0.502	5.034 ± 0.272	5.081 ± 0.461	5.455 ± 0.461
ADP	**5**	3.173 ± 0.543	3.424 ± 0.244	3.495 ± 0.421	3.362 ± 0.603	3.926 ± 0.597	4.200 ± 0.241	4.173 ± 0.278
	7	3.565 ± 0.472	3.895 ± 0.472	3.380 ± 0.684	4.195 ± 0.525	3.797 ± 0.718	3.948 ± 0.420	3.767 ± 0.332
	9	4.049 ± 0.189	4.126 ± 0.661	3.961 ± 0.717	3.873 ± 0.541	3.661 ± 0.651	3.943 ± 0.369	4.140 ± 0.366
AMP	**5**	2.654 ± 0.941	4.151 ± 0.245	4.306 ± 0.296	4.242 ± 0.498	4.203 ± 0.093	4.049 ± 0.116	3.694 ± 0.316
	7	4.312 ± 0.216	3.213 ± 0.834	3.854 ± 0.913	3.901 ± 0.665	3.813 ± 0.423	4.297 ± 0.227	4.067 ± 0.076
	9	4.314 ± 0.211	4.329 ± 0.255	4.283 ± 0.515	4.370 ± 0.023	4.446 ± 0.332	4.339 ± 0.081	4.035 ± 0.193

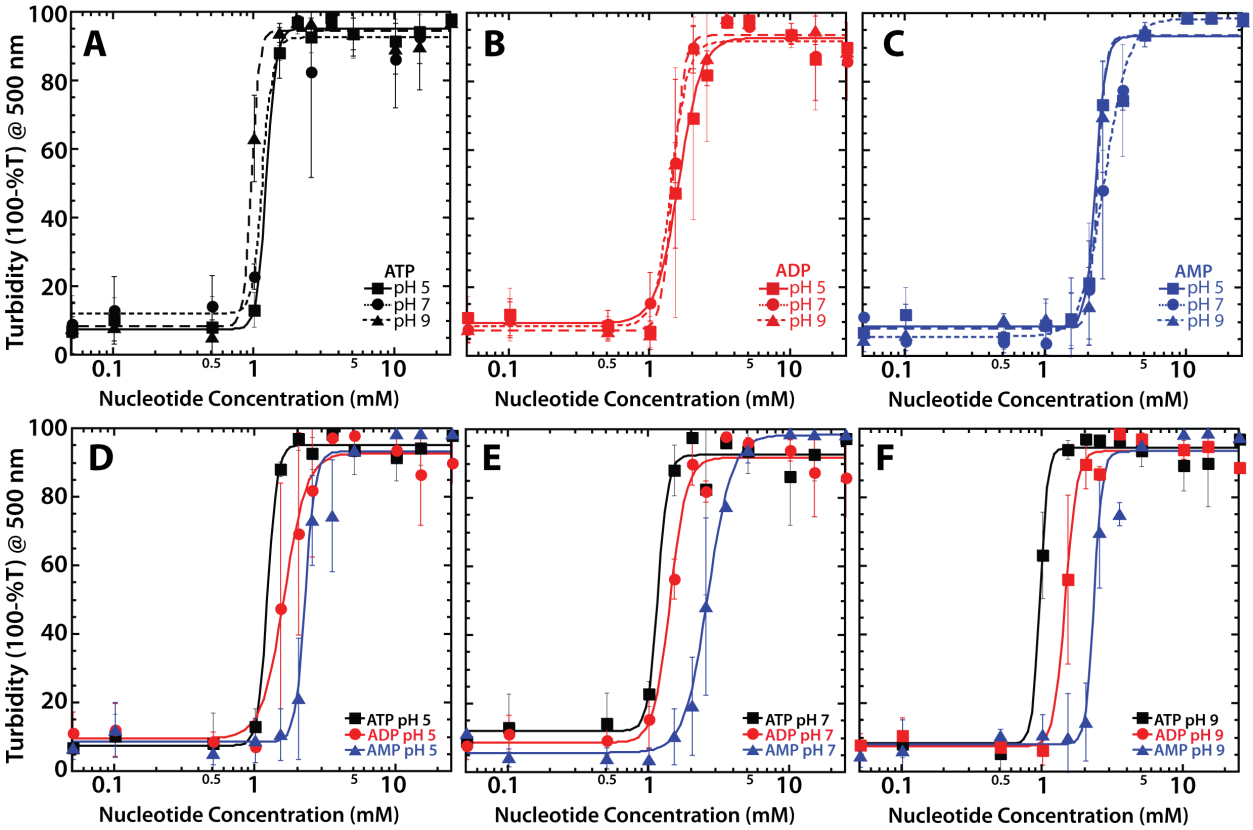
Equal Nucleotide Concentration

[$MgCl_2$] (mM):		0	0.5	1	5	10	15	25
Nuc.	**pH**							
ATP	**5**	2.104 ± 0.281	2.098 ± 0.303	1.982 ± 0.351	1.811 ± 0.494	2.100 ± 0.557	2.197 ± 0.402	2.519 ± 0.273
	7	1.511 ± 0.561	2.077 ± 0.263	1.709 ± 0.612	1.939 ± 0.399	2.289 ± 0.231	1.873 ± 0.620	2.308 ± 0.566
	9	2.073 ± 0.320	1.928 ± 0.407	1.212 ± 0.895	0.033 ± 1.598	1.988 ± 0.501	2.454 ± 0.240	2.304 ± 0.479
ADP	**5**	3.944 ± 0.563	3.447 ± 0.251	3.355 ± 0.849	3.178 ± 0.898	3.762 ± 0.708	3.604 ± 0.822	3.560 ± 0.294
	7	2.017 ± 1.287	2.073 ± 1.391	3.771 ± 0.136	3.407 ± 0.543	4.280 ± 0.441	4.011 ± 0.264	4.100 ± 0.283
	9	4.056 ± 0.090	4.154 ± 0.224	3.801 ± 0.408	3.349 ± 0.510	4.199 ± 0.255	4.129 ± 0.361	3.946 ± 0.138
AMP	**5**	3.251 ± 0.357	3.086 ± 0.352	2.888 ± 0.757	3.118 ± 0.549	3.224 ± 0.288	2.920 ± 0.554	2.801 ± 0.405
	7	3.303 ± 0.345	3.175 ± 0.233	3.292 ± 0.679	3.152 ± 0.387	3.080 ± 0.480	2.705 ± 0.589	2.477 ± 0.255
	9	2.730 ± 0.864	2.833 ± 0.480	2.883 ± 0.627	2.965 ± 0.295	3.096 ± 0.195	2.735 ± 0.825	2.587 ± 0.308

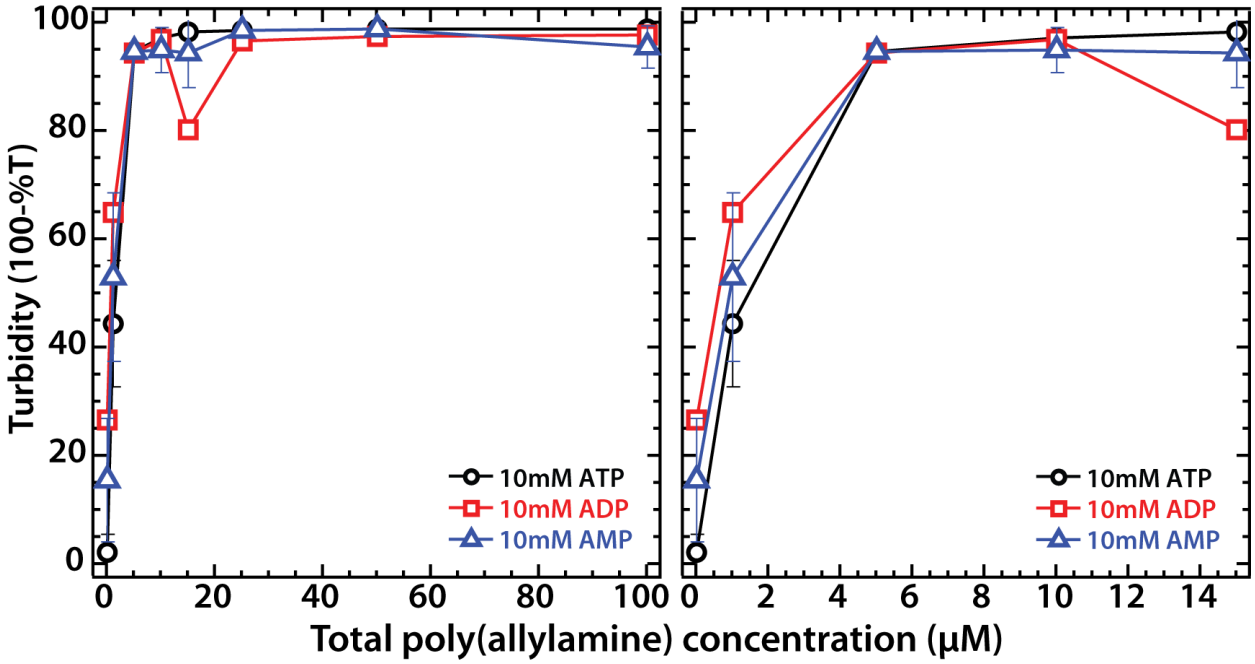
As the values of –log K increase, partitioning of the RNA into the coacervate phase strengthens. Partitioning is compared against pH (down a column) and total Mg^{2+} concentration (across a row). Top and bottom panel shows partitioning values of N_{54} into coacervates containing different nucleotides present at equal charge concentration and equal nucleotide concentrations, respectively. All standard deviations were calculated from at least triplicate measurements.



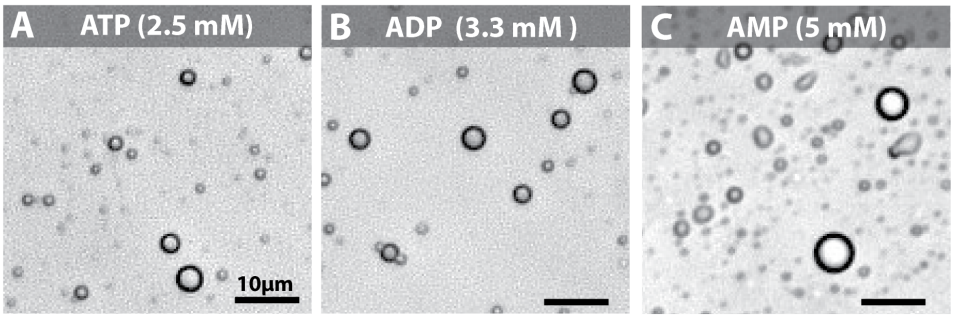
Supplemental Figure 1: Turbidity as a function of PAH concentration for ATP, ADP and AMP (**A-C**) parametric in Mg^{2+} concentration at fixed nucleotide concentration and identity. (**D-F**) parametric in nucleotide identity at fixed Mg^{2+} concentrations. Samples in all panels are in 2 mM HEPES Buffer pH 7.



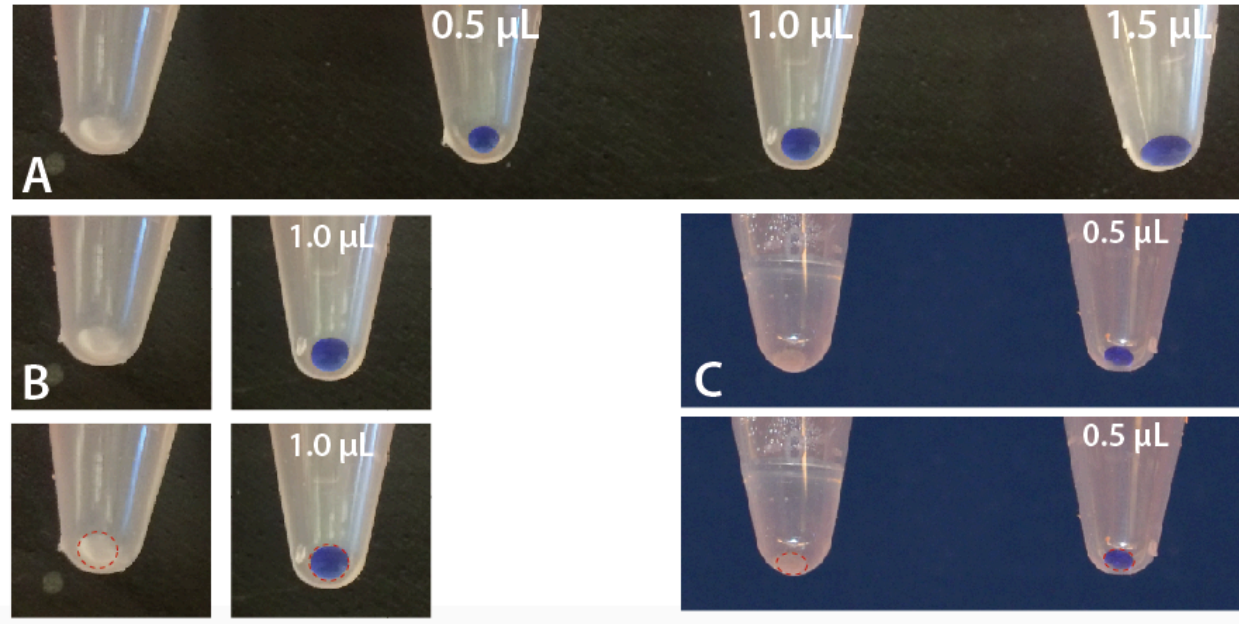
Supplemental Figure 2: Turbidity as a function of nucleotide concentration for ATP (black), ADP (red) and AMP (blue). (A-C) parametric in pH at fixed nucleotide and (D-F) parametric in nucleotide identity at fixed pH. Panel E is also featured in the main text (provided in Figure 1A). Samples in all panels are in 5 mM $MgCl_2$ and 2 mM Buffer (MES pH 5, HEPES pH 7 or CHES pH 9).



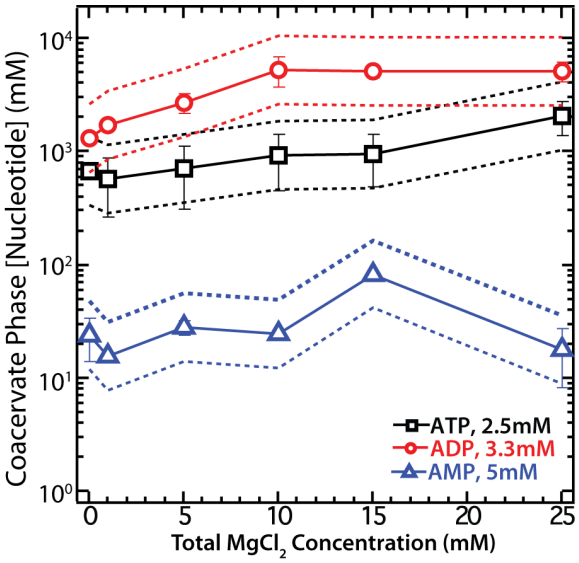
Supplemental Figure 3: Turbidity of solutions containing equal concentrations of nucleotides as a function of poly(allylamine) concentration. The left panel shows poly(allylamine) concentrations from 0-100 μM), and the right panel shows PAH concentrations out to 15 μM. Samples containing ATP are shown in black traces, ADP are shown in red, AMP shown in blue. All turbidity measurements were completed at in 2 mM HEPES pH 7 and 5 mM Mg^{2+} for these experiments.



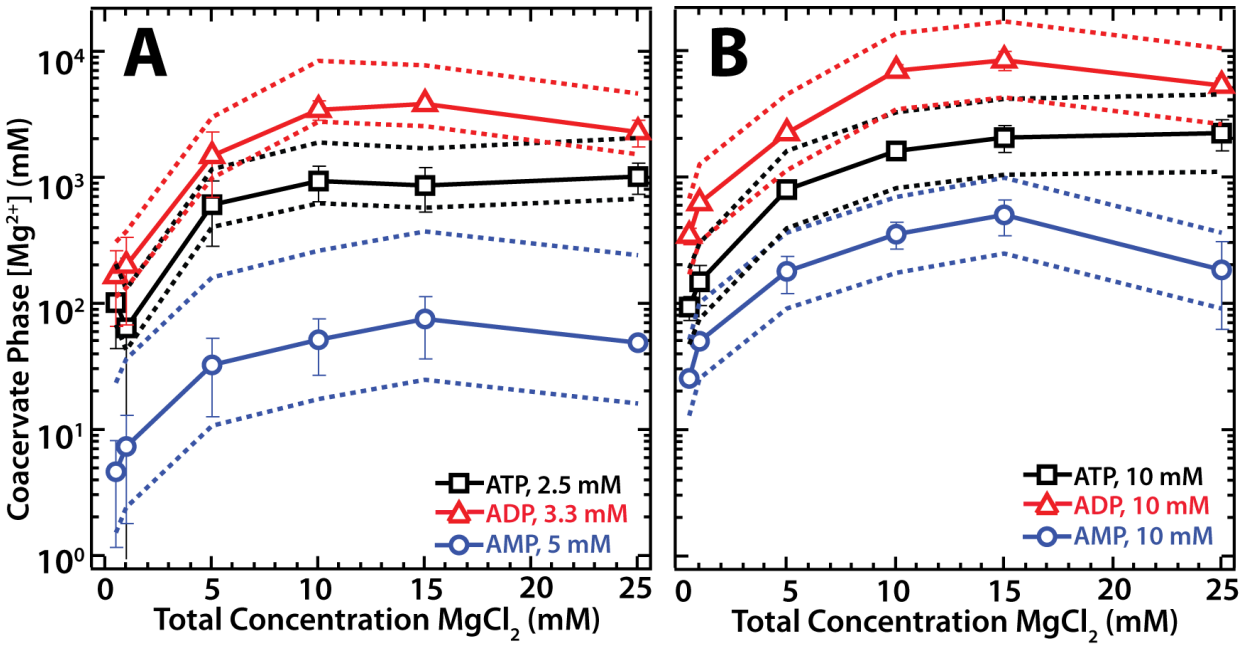
Supplemental Figure 4: Optical microscopy images of PAH/nucleotide coacervate droplets at greater magnification. All solutions were prepared with 38.5 µM PAH, 2 mM HEPES pH 7 and 5 mM Mg^{2+}. **(A)** 2.5 mM ATP, **(B)** 3.3 mM ADP, and **(C)** 5.0 mM AMP.



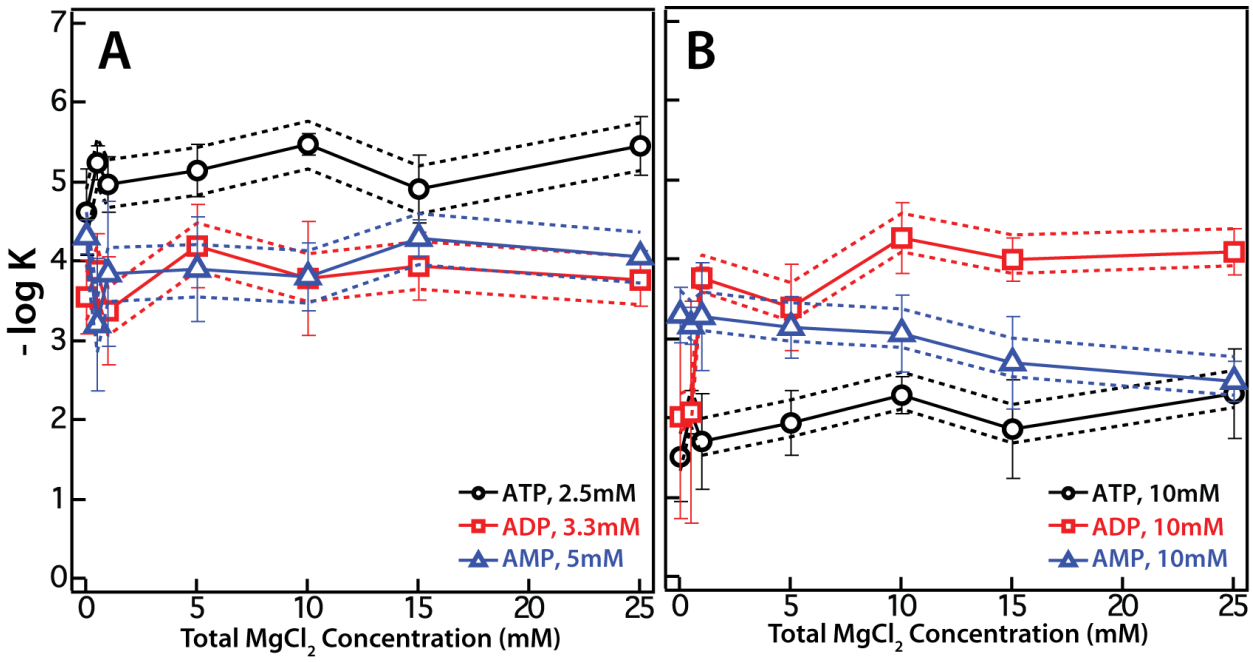
Supplemental Figure 5: Unknown volumes of coacervate phases were compared against known volumes of organic dye. (A) Solution containing 3.3 mM ADP, 38.5 µM PAH and 5 mM Mg^{2+} at pH 7 was compared against organic dye with known volumes of 0.5 µL, 1.0 µL and 1.5 µL from left to right. (B) Coacervate phase most closely resembles the same volume as the 1.0 µL standard volume. Top panel shows phases alone, while the bottom panel displays red guidelines that are the same size superimposed on both the coacervate phase (left) and dye (right). These images are taken from panel A. (C) Coacervate solution that was prepared with 3.3 mM ADP, 38.5 µM PAH and 10 mM Mg^{2+} at pH 7 and was spun down has a coacervate phase that most closely resembles the same volume as the 0.5 µL standard volume. Top panel shows phases alone, while the bottom panel displays red guidelines that are the same size superimposed on both the coacervate phase (left) and dye (right).



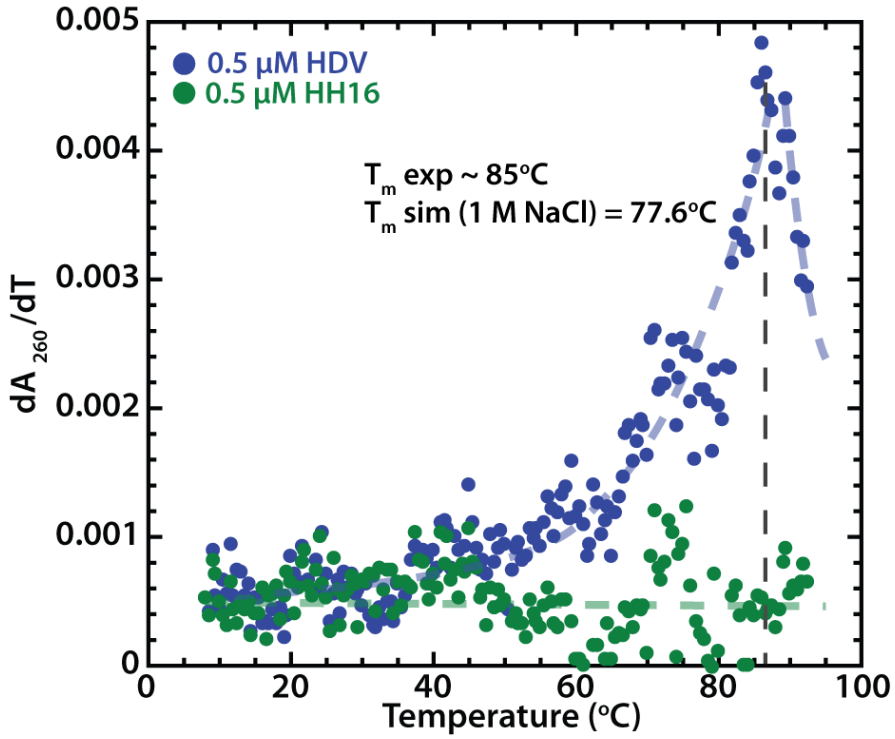
Supplemental Figure 6: Effect of uncertainty in coacervate volume on the resulting nucleotide concentration in the coacervate phase as a function of Mg^{2+} concentration at pH 7. Concentration of nucleotides in the coacervate phase was calculated with the estimated volume (symbols and solid lines), two-fold higher volume (lower dashed lines), or two-fold lower volume (higher dashed lines) for 2.5 mM ATP (black), 3.3 mM ADP (red) and 5 mM AMP (blue). Error bars represent the standard deviation from at least three measurements.



Supplemental Figure 7: Effect of uncertainty in coacervate volume on the resulting total Mg^{2+} concentration in the coacervate phases. In all panels, concentration of total Mg^{2+} was calculated with the estimated volume (symbols and smooth lines), two-fold higher volume (lower dashed lines), or two-fold lower volume (higher dashed lines) of the coacervate phase for ATP (black), ADP (red) and AMP (blue). All Mg^{2+} concentrations were calculated at pH 7. Concentration of Mg^{2+} in coacervate phase of solutions containing charge balanced and concentration-balanced systems, respectively. Figure 5 displays the concentration of magnesium in both the coacervate phase and the dilute phase.



Supplemental Figure 8: Effect of uncertainty in coacervate volume on the resulting N_{54} RNA partitioning into the coacervate phase of solutions that were (**A**) charge-balanced and (**B**) concentration-balanced, at pH 7 as measured by scintillation counting. In both panels, the RNA partitioning was calculated with the estimated volume (symbols and solid lines), two-fold higher volume (lower dashed lines), or two-fold lower volume (higher dashed lines). Partitioning values of RNA in solutions containing ATP (black), ADP (red) and AMP (blue) at **(A)** equal charge concentration and **(B)** equal nucleotide concentration.



Supplemental Figure 9: Derivative plot of a UV melt at 260 nm of RNA oligomers as an indicator of structure. Both RNAs had a final concentration of 0.5 µM and were melted in the presence of 5 mM Mg^{2+}. The HH16 substrate strand (green circles) did not show any significant melting, indicating little to no structure. The HDV enzyme strand (blue circles), showed a melting temperature of 85ºC indicative of significant secondary structure. Dashed blue line follows the trend of the dotted points. Experimental melting temperature of HDV E matches well with simulated T_m based on nearest neighbor parameters = 77.6ºC in 1 M NaCl.

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