# RNA Duplex Strand Separation

Investigation of strand separation conditions in the context of ribozyme-catalysed RNA replication

2019 Summer Internship Report

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***Abstract*—Multiple cycles of ribozyme-catalysed RNA replication can only be achieved by denaturing the duplex formed after every cycle. However, acid denaturation in the presence of high $Mg^{2+}$ concentration leads to problems associated with decreased ribozyme activity and ribozyme aggregation. This project hence aims to investigate these two issues.**

**Less harmful strand separation conditions such as using $K^+$, silica, and high temperatures at pH 4, as well as optimal extension conditions such as using K10, lowering the temperature and increasing the triplet concentration, and using suitable templates were found to better tackle the problem regarding the decreased ribozyme activity.**

**Additionally, to address the issue of ribozyme aggregation, the concentration of $Mg^{2+}$ which causes ribozyme aggregation was discovered. 4 mM $Mg^{2+}$ leads to partial aggregation while 20 mM leads to complete aggregation. Aggregation was found to occur in acid.**

**Using the information gained, an attempt was made to achieve multiple rounds of replication of template II using three different denaturation methods.**

## I. Introduction

### A. RNA World Hypothesis

According to principles of basic molecular biology, nucleic acid replication is catalysed by protein enzymes and protein enzyme synthesis is directed by nucleic acids. The 'RNA World Hypothesis' posits that the origin of life began with RNA molecules that catalysed their own synthesis [1]. It arose to resolve the dilemma regarding the origin of life as to whether proteins or nucleic acids originated first. Watson Crick base-pairing allows for an RNA template to direct the synthesis of its complementary strand, which allows for the encoding and propagation of genetic information. Ribozymes are RNA molecules that can take on catalytic roles [2]. Together, these two capabilities enable the generation of more complex molecules with improved function through natural selection.

### B. Main problems with strand separation

RNA replication of structured templates can be achieved by using trinucleotide triphosphates as substrates and a triplet polymerase ribozyme as a catalyst [3]. However, one main problem stands in the way of multiple rounds of RNA replication. The replication of a single-stranded template ends in a duplex and separation of this duplex is necessary for the strands to act as templates in the next round of replication. Hence, repeated separation is needed for multiple cycles of replication. However, thermal denaturation requires high temperatures to separate long RNA strands. At high temperatures, both the RNA template and the ribozyme are susceptible to degradation pathways which are accelerated by high magnesium ion concentrations required for the functioning of the ribozyme [4]. This decreases the extension activity of the ribozyme.

Another problem arises upon acid denaturation of the RNA duplex. In the presence of high magnesium concentrations and low pH, the ribozyme aggregates, which may affect extension activity.

Hence, the aim is to explore RNA strand separation conditions that are compatible with multiple rounds of ribozyme-catalysed RNA replication. In particular, conditions should not overly decrease ribozyme extension activity or cause ribozyme aggregation.

To address the issue regarding the decrease in ribozyme activity, three approaches were explored. The first was to find less harmful strand separation conditions, the second was to better understand the effect of acid denaturation, and the third was to find optimal extension conditions.

To address the issue regarding ribozyme aggregation, three other approaches were explored. The first was to find out how much $Mg^{2+}$ causes aggregation, the second was to understand the nature of aggregation to better target solutions, and the third was to explore additives that decrease aggregation.

Using all this information, an attempt at multiple cycles of RNA replication was taken.

## II. Materials and Methods

### A. Finding less harmful strand separation conditions by varying $Mg^{2+}$ and $K^+$ concentrations

2.5 pmol of mutualistic heterodimer of type 5 and type 1 triplet polymerase ribozymes [3] were annealed at 80 °C for 2 minutes and at 17 °C for 10 minutes.

The annealed ribozymes (0.5 µl) were then added into 2 µl of denaturation solution, yielding a 2.5 µl solution containing 0.1% tween-20, 5 mM HCl, and varying concentrations of $K^+$ (0 to 2 M) and $Mg^{2+}$ (0 to 1 M). The mixture was then incubated at room temperature for 10 minutes and subsequently neutralised with 0.5 µl of 1 M Tris pH 8.3.

2 µl of extension solution containing 0.2% tween-20, 1 µM F10 primer, 1 µM LT8GAA template, 10 µM GAA triplets, and varying concentrations of $Mg^{2+}$ was added to each sample to yield a final $Mg^{2+}$ concentration of around 200 mM.

The resultant 5 µl of reaction mixture was then placed in dry ice for 5 minutes, then incubated at -7 °C for one hour to allow for extension.

Finally, 2 µl of the incubated reaction mixture was added to 1 µl of 0.5 M EDTA, 1 µl of 20 µM competing oligonucleotide, and 6 µl of 10M urea with bromophenol blue. This mixture was then denatured at 94 °C for 5 minutes and cooled to room temperature. It was then loaded onto a denaturing gel containing 20% acrylamide, 8 M urea, and 1x TBE buffer. The gel was run at 25W for 1 hour 15 minutes and visualized on GE Amersham Typhoon.

*B. Finding less harmful strand separation conditions by varying $K^+$ and ribozyme concentrations*

Methodology similar to that in section II.A. was utilised, the only differences being the ribozyme concentration which varied from 0.025 µM to 0.5 µM, and the presence or absence of 5 mM HCl and 200 mM KCl. $Mg^{2+}$ concentration was kept constant.

*C. Finding less harmful strand separation conditions by using silica as a surface*

In order to characterise the effect of pH on silica-catalysed strand separation, formate buffers at pH 2.5 and pH 4.0, an imidazole buffer at pH 6.6, and tris buffers at pH 7.4 and pH 8.3 were prepared. A 10 mM buffer solution with 100 mM NaCl was prepared at each pH. 300 µl of each resultant solution was used to pre-wash a Qiaquick PCR purification filter.

To each filter, 1 pmol of an RNA duplex (FQ) containing a fluorophore on one strand and a quencher on the complementary strand was added, along with 1 pmol of the same RNA duplex without the fluorophore and quencher (M). This allows strand separation to be quantified through the amount of fluorescence.

30 µl of the 10 mM buffer with 100 mM NaCl was then added to the filter. This was then incubated at room temperature with shaking for one hour. The flow-through was spun down at maximum speed.

15 µl of the flow-through was directly added to 50 µl of 0.5 M Tris pH 8.3 and 35 µl of Millipore water, while the other 15 µl was first denatured by adding 0.3 µl of 1M HCl and incubating for 15 minutes, before being added to 50 µl of 0.5 M Tris pH 8.3 and 35 µl of Millipore water.

To obtain the eluate, 50 µl of TE buffer was added to the filter, incubated for one minute, and spun down at maximum speed. This was repeated another time. 50 µl of the eluate was directly added to 50 µl of 0.5 M Tris pH 8.3, while the other 50 µl was first denatured by adding 0.3 µl of 1M HCl and incubating for 15 minutes, before being added to 50 µl of 0.5 M Tris pH 8.3.

The fluorescence signals were measured using Pherastar with excitation of 485 nm and emission of 520 nm.

*D. Characterising the binding of ribozyme to silica*

For each sample, 10 µl silica beads from glass milk were washed with Millipore water three times, and then pre-washed in 100 mM of buffer with 100 mM NaCl. The buffers used were of pH 2, 2.5, 3, 3.5, 4, 4.5, 5.8, 6.2, 6.7, 7.2, 7.7, 8.2, 9.5. Then, 50 µl of 100 mM buffer with 100 mM NaCl and 2.5 pmol of ribozymes T5 and T1 was added to the beads. The resultant mixture was incubated at room temperature with rotation for one hour.

Subsequently, the beads were spun down at maximum speed and 20 µl of supernatant was added to 50 µl of 0.5 M Tris 8.3 and 20 µl of Millipore water. To the 30 µl of remaining supernatant, 30 µl of 1 M Tris pH 8.3 was added to elute ribozyme bound to the silica, and 40 µl of the resulting solution was added to 10 µl of 0.5 M Trish pH 8.3 and 40 µl of Millipore water.

10 µl of SYBR gold stain was used to stain the ribozyme and the fluorescence was measured using Pherastar with excitation of 485 nm and emission of 520 nm. Comparing the readings of the supernatant not neutralised to the supernatant that was neutralised thus shows the fraction of unbound ribozyme.

*E. Effects of tween-20 on silica-catalysed strand separation*

Methodology similar to that in section II.C. was utilised with the difference being that only pH 4.0 buffer was used and that one sample had 0.1% tween-20 while the other did not have tween-20.

*F. Strand separation in silica with CHES buffer*

The Qiaquick filter was pre-washed in solution made of 50 mM CHES buffer, 200 mM $K^+$, and 10 mM $Mg^{2+}$ adjusted to pH 4. Then, a 20 µl solution of the above composition with 1 pmol of FQ and M was added to the filter. In the collection tube, 20 µl of 0.1% tween-20 solution was used to prevent adherence of RNA to the tube.

After incubation for an hour, the tube was spun at maximum speed. 16 µl of flow-through was placed on one tube and another 16 µl was placed in another tube. The remaining flow-through was discarded.

120 µl of elution mixture containing 10 mM CHES and 0.1% tween-20 was added to the collection tube to elute RNA stuck to the collection tube. 48 µl of the elution mixture was then added to each tube containing 16 µl of flow-through from the previous step. 1.69 µl of 1M HCl was added to one tube to denature all RNA duplexes. Then 14.31 µl of 0.5M CHES was added to for neutralisation. For the other tube, 16 µl of 0.5M CHES was added to for neutralisation.

To elute the RNA adhered to the filter, 20 µl of elution mixture described above was added to the filter and spun down at maximum speed into a collection tube with 20 µl of 0.1% tween-20. This was repeated once with 20 µl of elution mixture, and again with 100 µl. 64 µl of eluate was then added to 16 µl of 0.5 M CHES. 1.96 µl of 1 M HCl was added to another 64 µl of eluate which was then added to 14.04 µl of 0.5 M CHES.

The fluorescence signals were measured using Pherastar with excitation of 485 nm and emission of 520 nm. The ratios of the signals of non-acidified to acidified samples was an indication of the proportion of duplexes denatured.

*G. Finding less harmful strand separation conditions using pH and temperature*

20 µl solutions of 50 mM CHES buffer, 200 mM $K^+$, and 10 mM $Mg^{2+}$ adjusted to pH 4 were incubated for 30 seconds at 40 °C, 50 °C, 60 °C, and 80 °C. This was repeated for solutions adjusted to pH 7. The solutions were neutralised, and the fluorescence signals were measured using Pherastar with excitation of 485 nm and emission of 520 nm. To calculate the proportion of duplexes denatured, the signals were taken as a percentage of the signal generated by completely denaturing the duplexes at pH 2.5.

*H. Effect of tween-20 on strand separation using pH and temperature*

The experiment in section II.G. was repeated, but with 0.1% tween-20 and at temperatures 25 °C, 60 °C, 70 °C, 80 °C, 90 °C, and 98 °C. Additionally, samples were heated for 3 minutes instead of 30 seconds.

*I. Understanding acid denaturation*

2.5 pmol of mutualistic heterodimer of type 5 and type 1 triplet polymerase ribozymes [3] were annealed at 80 °C for 2 minutes and at 17 °C for 10 minutes.

The annealed ribozymes (0.5 µl) were then added into denaturation solution containing 5 mM HCl, 0.1% tween-20, and varying concentrations of $Mg^{2+}$ (0 to 1 M).

One set of samples were denatured for 5 minutes and the other for 60 minutes prior to neutralisation. Loading buffer was added and samples were loaded onto a denaturing gel containing 20% acrylamide, 8M urea, and 1x TBE buffer. The gel was run at 25W for 1 hour 15 minutes and visualized on GE Amersham Typhoon.

*J. Finding conditions optimal for extension: templates*

7 different templates of different GC content were tested to find a template that could be replicated to completion. Each template was extended with either 0.5 µM or 2 µM of ribozymes T5 and T1.

6 µl reaction mixtures were set up. 5 pmol of primer, 5 pmol of template, and 50 pmol of each triplet were annealed (80 °C for 2 minutes, 17 °C for 10 minutes) separately from 2.5 pmol or 10 pmol of ribozymes T5 and T1, which were also annealed under the same conditions. To the annealed ribozymes, 1 µl of 1 M $MgCl_2$, 0.25 µl of Tris pH 8.3, 0.125 µl of 2% tween-20, and 0.125 µl of Millipore water were added. The mixture was incubated on ice for 5 minutes and subsequently added to the primer, template and triplet mixture. The reaction mixture was frozen using dry ice and left to extend for one week.

Samples were run through a denaturing gel under similar conditions as described in section II.A.

Of the 7 different templates, 4 were able to be replicated to completion. Primers for the complementary strands of those templates were then ordered, and the extension reactions were setup similar to above. For templates struc30, repeat, and II, primers were only ordered for one register, whereas for template gamma, primers were ordered for all three registers.

*K. Finding conditions optimal for extension: K10*

K10 is known to boost extension activity in low magnesium frozen extension reactions. To find out if K10 affects refolding of the ribozymes after acid denaturation, different concentrations of K10 (0 to 10 mM) were added to reaction mixtures containing 0.5 µM of ribozymes T5 and T1, 5 pmol of primer, 5 pmol of template, and 50 pmol of triplets in 50 mM CHES buffer at pH 9 with 200 mM KCl and 10 mM $MgCl_2$. This was also repeated with only ribozyme T5.

HCl was added to adjust the pH to 2.5, and after 5 minutes of denaturation, the reaction mixture was neutralised with KOH. The reaction mixtures were frozen with dry ice and left to extend for 2 hours at -7 °C.

Samples were run through a denaturing gel under similar conditions as described in section II.A.

*L. Finding conditions optimal for extension: low temperature and high triplet concentration*

5 pmol of primer, 5 pmol of template, 50 pmol of each triplet were annealed at 80 °C for 2 minutes and at 17 °C for 10 minutes. $MgCl_2$ was added to a final concentration of 200 mM, tris pH 8.3 to 16.6 mM, and tween-20 to 0.1%. The final reaction volume was 10 µl. This was incubated on ice for 5 minutes, then frozen on dry ice, and left at -7 °C for extension. This was repeated, but extension was carried out at -15 °C.

The above was then repeated, but with 100 pmol of triplets instead. The whole setup was done for both templates AUseq30 and II.

*M. Amount of $Mg^{2+}$ which causes aggregation*

To find out how much $Mg^{2+}$ causes aggregation and when aggregation occurs, 2.5 pmol of T1, 2.5 pmol of T5, or 2.5 pmol of T5 and T1 were added to solutions containing varying $Mg^{2+}$ concentrations (0 to 1M) and 0.1% tween-20. 5mM HCl was added and the solutions were incubated at room temperature for 5 minutes. Half of the reaction mixture was then sampled and then tris pH 8.3 was used to neutralise the remaining reaction mixture before also being sampled.

After bromophenol blue was added, the samples were loaded onto a native gel (10% acrylamide, 0.5x TBE, 20 mM $MgCl_2$) and run at 4 °C.

*N. Finding out when aggregation occurs (centrifugation)*

5 pmol of T5 and T1 were annealed at 80 °C for 2 minutes and at 17 °C for 10 minutes. In the 20 µl reaction mixture which contained 0.1% tween-20, varying concentrations of $Mg^{2+}$ were added (0 mM, 20 mM, 1000 mM). 5 mM HCl was added and the samples were incubated at room temperature for 10 minutes.

The samples were then spun at maximum speed for 15 minutes. The top 10 µl of supernatant was sampled, then the bottom half was resuspended and sampled.

The above was repeated but the samples were neutralised before being spun at maximum speed for 15 minutes. All samples were loaded onto a denaturing gel as described in section II.A.

*O. Finding out when aggregation occurs (filtration)*

5 pmol of T5 and T1 were annealed at 80 °C for 2 minutes and at 17 °C for 10 minutes. In the 20 µl reaction mixture which contained 0.1% tween-20, varying concentrations of $Mg^{2+}$ were added (0 mM, 20 mM, 1000 mM). 5 mM HCl was added and the samples were incubated at room temperature for 10 minutes.

The samples were then added onto the filter of a Qiaquick column and spun at 6000 rpm. The filtrate was sampled. 50 µl of Millipore water was added to the filter and incubated at room temperature with shaking for 15 minutes to resuspend any particulates stuck on the filter.

The above was repeated but the samples were neutralised before being added to the filter. All samples were loaded onto a denaturing gel as described in section II.A.

*P. The effect of sequence on aggregation*

Different sequences were tested to see if they were resistant to aggregation when exposed to 5 mM HCl in the presence of $Mg^{2+}$. The amounts of templates were: 5 pmol of F118C19, 5 pmol of gamma duplex, 10 pmol of single-stranded gamma, 37 pmol of tCCC, and 100 pmol of F11C7. Each template had one sample without acid added and one sample with 5 mM HCl added.

Ribozymes T5 and T1 at amounts with the same number of nucleotides as each sample were used as controls.

After neutralisation, bromophenol blue was added to the samples and they were loaded onto a native gel.

*Q. Effect of additives and pre-folding on aggregation*

Additives were tested for their effect on preventing aggregation. The additives tested were: 50 µM of tRNA, 25% formamide, 5 µM triplets, and heparin. Pre-folding was also tested. Each additive had one sample without acid added and one sample with 5 mM HCl added.

After neutralisation, bromophenol blue was added to the samples and they were loaded onto a native gel.

*R. Multiple cycles of extension (controls)*

Controls were first setup for the extension reactions. The total reaction volume was 7.125 µl. 0.6 µl of 1 M $MgCl_2$, 0.75 µl of 0.2% tween-20, and 0.1 pmol of template were mixed together. For each template (gamma, II, or repeat), one sample did not have any template, one sample had the + strand, one sample had the – strand, one sample had the undenatured duplex, and one sample had the denatured duplex (prepared by adding 0.48 µl of 0.05M HCl to the above mixture).

Then, 10 pmol of annealed ribozymes T5 and T1 were mixed together with 0.94 µl of 1 M $MgCl_2$, 0.5 µl 1 M tris pH 8.3, 0.06 µl of 2% tween-20, 0.125 µl of each triplet at 200 µM, and 1.25 µl of 20 µM primers. This mixture was added to the mixture above, left on ice for 5 minutes, frozen with dry ice, then left to extend overnight.

All samples were loaded onto a denaturing gel as described in section II.A.

*S. Multiple cycles of extension*

The actual extension reactions were setup only for II, which was able to be fully extended overnight. The reaction mixture contained 50 mM CHES, 10 mM $Mg^{2+}$, 160 mM $K^{+}$, 10 µM K10, 2 µM of T5 and T1, 5 µM of each primer, 5 µM of each triplet, and 0.1 pmol of the duplex.

Starting with the duplex, three different methods of denaturation were utilised. The first was to adjust the pH to 2.5 at room temperature (buffered using 20mM chloroacetate, 0.1% tween-20), the second was to adjust the pH to 4 and then to heat the sample to 90 °C for 3 minutes (20mM acetate, 0.1% tween-20), and the third was to adjust the pH to 4 and then to heat the sample to 80 °C for 3 minutes (20mM acetate, no tween-20).

For each method of denaturation, one sample was denatured once, and another sample was denatured 3 additional times with 2 days in between each denaturation.

The above was repeated, but this time the whole reaction mixture was diluted 50 times before annealing at 80 °C for 2 minutes and at 17 °C for 10 minutes.

An undenatured duplex sample served as the negative control, along with only the + or – strands serving as the templates in the positive controls. A sample containing primers without the templates served as a comparison.

## III. RESULTS AND DISCUSSION

### A. *Finding less harmful strand separation conditions by varying $Mg^{2+}$ and $K^+$ concentrations*

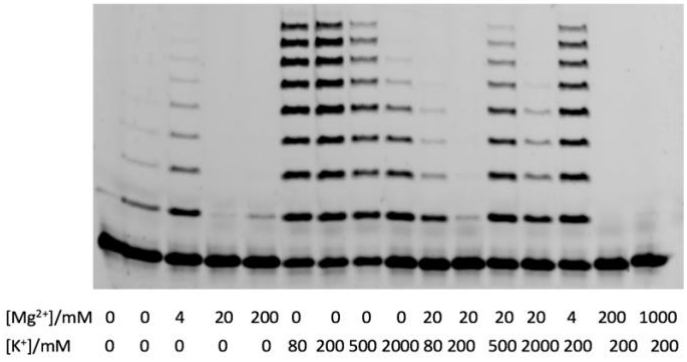


*Fig. 1: Extension activity with varying $Mg^{2+}$ and $K^+$ in acid*

When using a CHES buffer in extension reactions, 10 mM of $Mg^{2+}$ can be used in place of the usual 200 mM. The extension reactions with 4 mM $Mg^{2+}$ and 200 mM $K^+$ along with 20 mM $Mg^{2+}$ and 500 mM $K^+$ showed complete extension, demonstrating that this range of $K^+$ can be added to boost extension activity.

### B. *Finding less harmful strand separation conditions by varying $K^+$ and ribozyme concentrations*

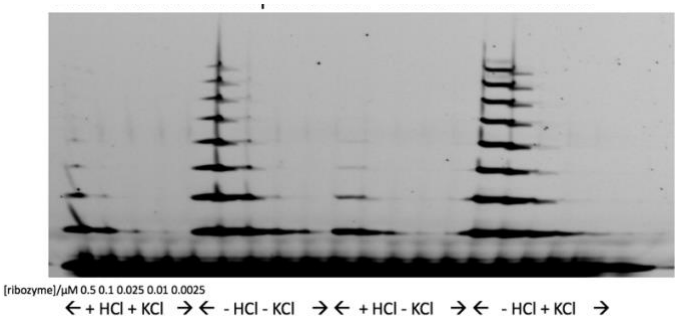


*Fig. 2: Extension activity with or without KCl and HCl*

Experimental results further demonstrate that KCl aids in ribozyme extension activity.

### C. *Finding less harmful strand separation conditions by using silica as a surface*

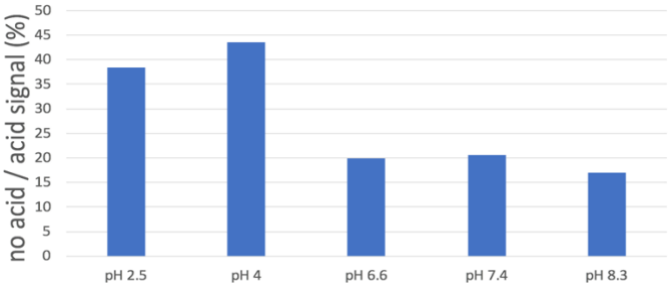


*Fig. 3: Percentage of duplex denatured at each pH (%)*

Silica-catalysed strand separation is optimal at pH 4 and below.

### D. *Characterising the binding of ribozyme to silica*

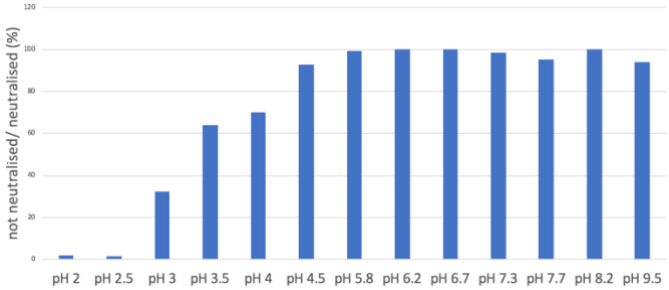


*Fig. 4: Percentage of unbound ribozyme at each pH (%)*

This shows binding of RNA to silica occurs below pH 4.5.

### E. *Effects of tween-20 on silica-catalysed strand separation*

| **0.1% tween-20** | | **No tween-20** | |
|---|---|---|---|
| Acidic | Neutralised | Acidic | Neutralised |
| 2810 | 3850 | 766 | 10494 |

*Fig. 5: Signals corresponding to number of strands separated (a.u.)*

The addition of tween-20 seems to reduce the interaction of RNA with silica. Due to silica catalysing RNA strand separation through a surface effect, not adding tween-20 may help in greater rates of strand separation.

### F. *Strand separation in silica with CHES buffer*

| **Silica** | | **No silica** | |
|---|---|---|---|
| pH 4 | pH 9 | pH 4 | pH 9 |
| 22.2 | 8.4 | 5.4 | 4.7 |

*Fig. 5: Percentage of strands separated (%)*

Silica catalyses strand separation better at low pH. This shows potential to be used to cycle the reaction mixture through multiple cycles of denaturation and extension conditions.

### G. *Finding less harmful strand separation conditions using pH and temperature*

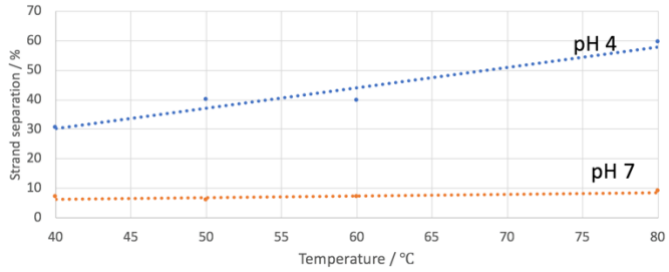


*Fig. 6: Percentage of strands separated at each temperature (%)*

At pH 7, as temperature increases, strand separation does not vary significantly. However, at pH 4, as temperature increases, strand separation increases significantly. Given that RNA is stable near pH 4, increasing the temperature at this pH could be a potential denaturation method.

*H. Effect of tween-20 on strand separation using pH and temperature*

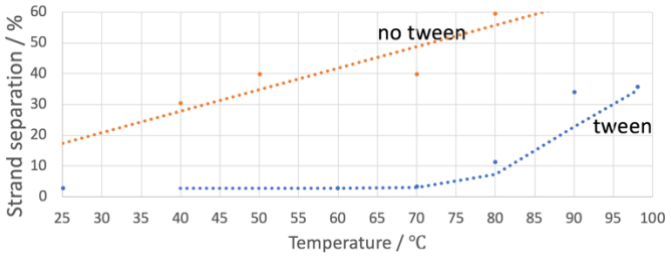


*Fig. 7: Percentage of strands separated at each temperature, with or without tween-20 (%)*

The addition of tween vastly reduces strand separation at each temperature.

*I. Understanding acid denaturation*

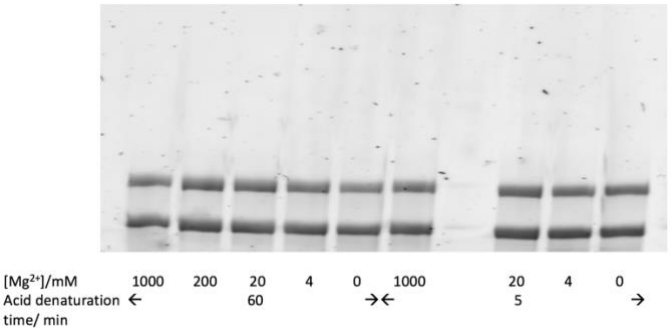


*Fig. 8: T5 and T1 after acid denaturation*

While acid denaturation aggregates ribozymes, it does not break down the ribozymes. They are still in their component parts T5 and T1.

*J. Finding conditions optimal for extension: templates*

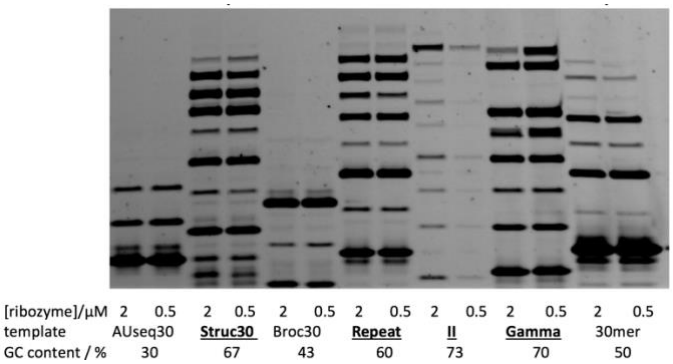


*Fig. 9: Extension on different templates*

Templates repeat, II, and gamma were shown to be replicated completely.

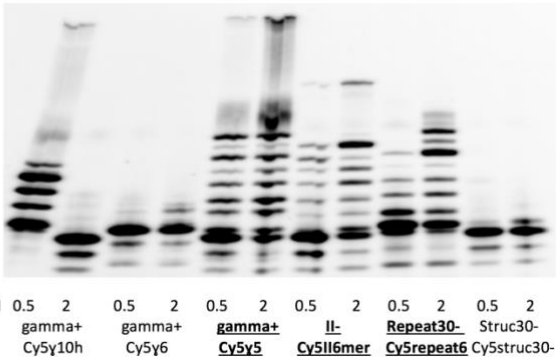


*Fig. 10: Extension on different templates (complementary strand)*

Gamma, II, and repeat30 were able to be replicated completely on both strands.

*K. Finding conditions optimal for extension: K10*

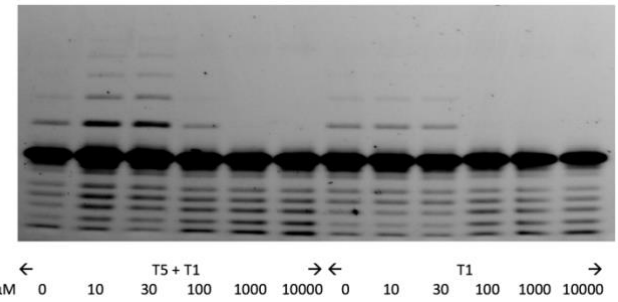


*Fig. 11: Effect of K10 on refolding*

10 μM of K10 improves extension and does not significantly affect ribozyme refolding after acid denaturation.

*L. Finding conditions optimal for extension: low temperature and high triplet concentration*

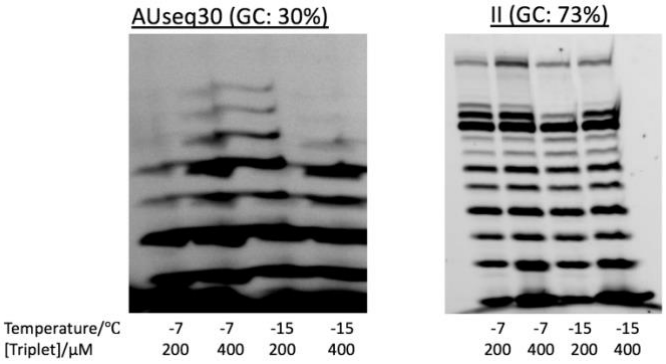


*Fig. 11: Effects of temperature and triplet concentration on extension*

For template AUseq30, at -7 °C, a higher triplet concentration aids extension. At 200 μM of triplets, a lower temperature -15 °C aids extension. For low GC content templates which are usually harder to replicate completely, lowering the temperature or using a higher triplet concentration may aid in extension.

*M. Amount of $Mg^{2+}$ which causes aggregation*

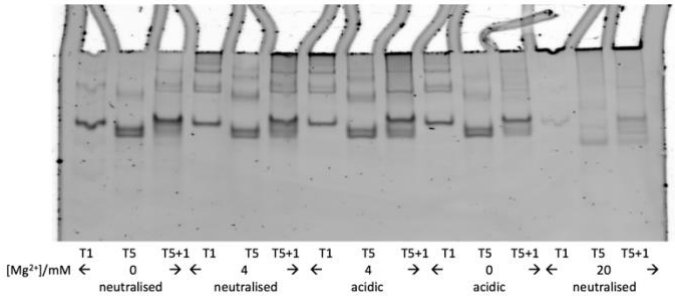


*Fig. 12: Effects of $Mg^{2+}$ concentration on ribozyme aggregation (0 to 20 mM)*

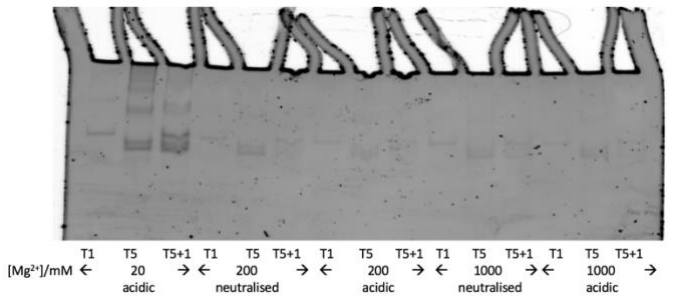


*Fig. 13: Effects of $Mg^{2+}$ concentration on ribozyme aggregation (20 to 1000 mM)*

With 0 mM $Mg^{2+}$, no ribozyme aggregation is observed. With 4 mM $Mg^{2+}$, partial ribozyme aggregation is observed. At 20 mM $Mg^{2+}$, almost complete ribozyme aggregation is observed. Hence, for extension reactions involving multiple round of acid denaturation, less than 20 mM $Mg^{2+}$ should be used.

*N. Finding out when aggregation occurs (centrifugation)*

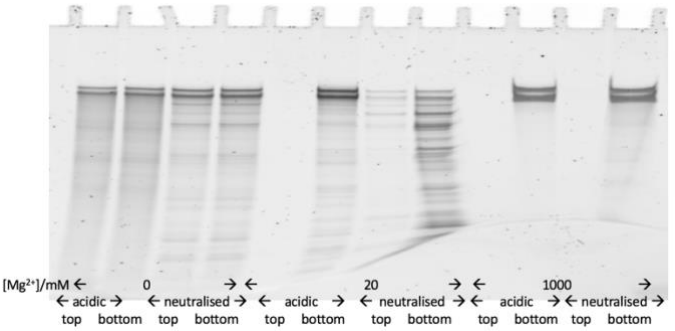


*Fig. 14: Effects of $Mg^{2+}$ concentration on ribozyme aggregation (centrifugation)*

Ribozyme aggregation occurs in acid and remains upon neutralisation.

*O. Finding out when aggregation occurs (filtration)*

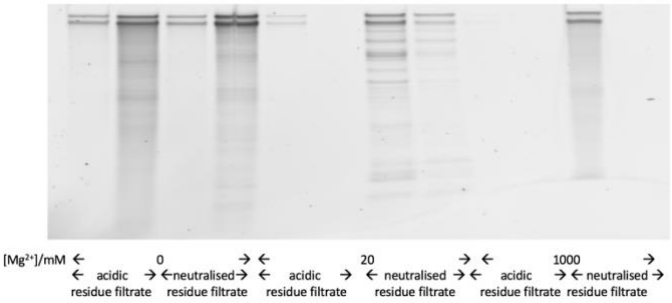


*Fig. 15: Effects of $Mg^{2+}$ concentration on ribozyme aggregation (filtration)*

This further demonstrates that aggregation happens in acid and remains upon neutralisation. Therefore, we should look into preventing aggregation from occurring in acid.

*P. The effect of sequence on aggregation*

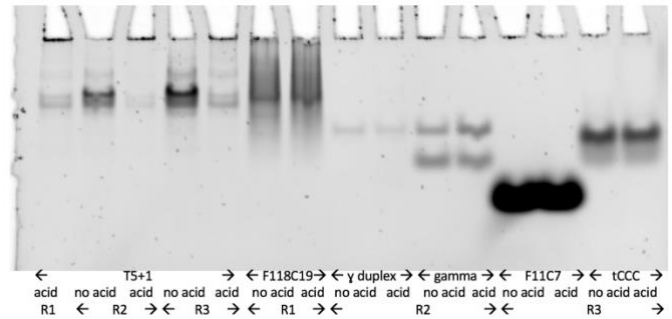


*Fig. 16: Effect of sequence on aggregation*

Ribozymes T5 and T1 at amounts with the same number of nucleotides as each sample were used as controls, and R1, R2, and R3 refer to the control that the sample should be compared to.

Shorter sequences such as the gamma duplex, single-stranded gamma, F11C7, and tCCC, seem to show no significant aggregation. F118C19, which is of a similar size as the ribozymes, also seems to be resistant to aggregation. This shows that sequence may affect aggregation.

*Q. Effect of additives and pre-folding on aggregation*

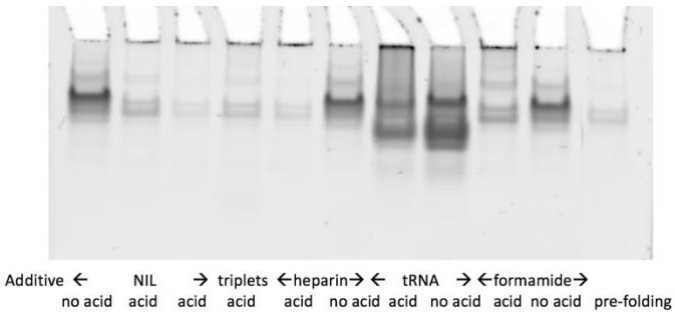


*Fig. 17: Effect of additives and pre-folding on aggregation*

Pre-folding and all additives tested do not seem to help prevent aggregation.

*R. Multiple cycles of extension (controls)*

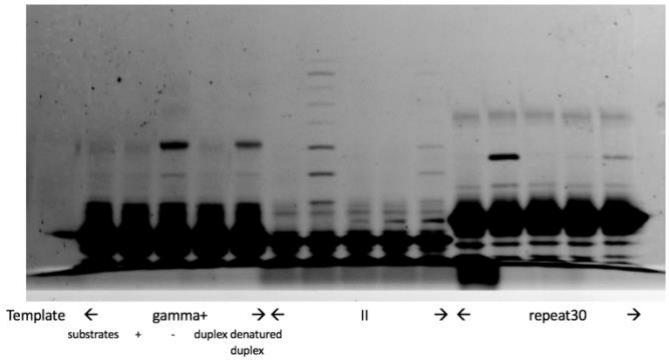


*Fig. 18: Controls for extension reactions*

Only II was able to be completely replicated overnight. Hence, II will be used in the actual multiple extension cycles. Experiments for that are still ongoing.

## IV. CONCLUSION

In summary, the problems associated with decreased ribozyme activity and aggregation caused by heat and acid denaturation were explored. To reduce the decrease in ribozyme activity, less harsh denaturation conditions were discovered. 200 mM to 500 mM of $K^+$ was shown to aid in extension and silica was shown to catalyse strand separation through a surface effect. Silica-catalysed strand separation was shown to work in CHES buffer and is optimal at around pH 4, which is also near the pH where RNA is most stable. Tween-20 was shown to affect the silica-RNA interaction and hence negatively affect silica-catalysed strand separation. Additionally, combining high temperatures of around 80-90 °C and a low pH of 4 appears to be a strategy for strand separation. It was also shown that acid denaturation does not hydrolyse and break down the ribozymes T5 and T1.

Furthermore, optimal reaction conditions were explored. The templates gamma, II, and repeat30 were found to be able to be replicated completely on both strands. 10 µM of K10 was found to improve extension and to not significantly affect ribozyme refolding. Also, lowering the temperature or increasing the triplet concentration may help in improving replication of low GC-rich sequences.

Regarding the ribozyme aggregation problem, it was found that concentrations of 20 mM $Mg^{2+}$ or more causes complete aggregation and it may be better to use around 4 mM $Mg^{2+}$. Ribozyme aggregation was shown to occur in acid, thus allowing targeting of solutions to resolve that. Sequence appears to affect the propensity to aggregate, while additives and pre-folding do not seem to help prevent aggregation.

Template II was able to be replicated overnight, and hence was used to setup experiments with multiple extension cycles.

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