Supporting Information

Anfinsen Redux: Ribonuclease Folding in the Single-Molecule Regime

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MATERIALS AND METHODS

Reagent preparation. RNase assay buffer was prepared by diluting a solution of 1.0 M 2-(N-morpholino)ethanesulfonic acid (MES) (Sigma–Aldrich M1317) and 5.0 M NaCl (Sigma–Aldrich S6546) in 500 mL of ddH_2O to a final concentration of 100 mM and 500 mM, respectively, after which the pH was adjusted to 6.0 with conc. HCl(aq). Diethyl pyrocarbonate (DEPC) (VWR AAB22753) was added to a final concentration of 0.1% (v/v). The resulting solution was vigorously stirred at room temperature for 2 h, incubated at 37 °C overnight, and autoclaved. RNase A (Sigma–Aldrich R6513) was reconstituted in nuclease-free H_2O to a final concentration of 0.3 mM, and the resulting solution was flash frozen and stored at −80 °C. Human salivary RNase was freshly isolated from saliva by centrifugation at 10,000*g* at 4 °C, followed by 1:100 dilution in RNase assay buffer. A hypersensitive fluorogenic substrate, 6-FAM–dArUdAdA–6-TAMRA (Figure S1), was dissolved in nuclease-free H_2O to a final concentration of 40 μM, and the resulting solution was stored at −20 °C. 8.0 M urea solution was prepared by dissolving nuclease-free urea (Sigma–Aldrich 51456) in RNase assay buffer. All reagents were carefully prepared using aseptic technique after spraying and wiping down equipment and surfaces with 0.3% alkyl (C12 40%; C14 50%; C16 10%) dimethyl benzyl ammonium chloride to inhibit contaminating/environmental ribonucleases.

Enzyme assays. Single-molecule enzyme assays were performed similarly to methods described previously.[11,12] Briefly, a fluorescent microscope (Olympus IX83) on the GFP channel and microwell array disks (Quanterix) were used to monitor the catalytic activity of individual RNase molecules by measuring the increase in fluorescence after the hydrolysis of 6-FAM–dArUdAdA–6-TAMRA. RNase stocks were diluted in RNase assay buffer to the picomolar range (~1–10 pM) such that each well adhered to Poisson statistics and contained 0 or 1 RNase molecules upon imaging. Of note, Poisson statistics are followed when there are approximately 1 in 10 active ("on") wells, corresponding to a Poisson lambda parameter of ~0.1 such that 90% of wells have no signal (*i.e.*, 0 enzyme molecules), ~9.5% of wells contain 1 enzyme molecule, and ~0.5% of wells contain 2 or more enzyme molecules. Nuclease-free plasticware was used in the preparation of each enzyme assay. For assays involving urea-mediated denaturation, the RNase stock was serially diluted in 8.0 M urea to produce an intermediate RNase stock solution, which was incubated for 1 h at room temperature. Afterwards, the denatured enzyme solution was brought to the desired final urea concentration (2.0 M or 4.0 M) by diluting with the appropriate volume of RNase assay buffer (without urea) or kept in 8.0 M urea as a control. 6-FAM–dArUdAdA–6-TAMRA was immediately added to each reaction mixture to a final concentration of 2.67 μM. Reactions were run for 30 min, and images were acquired every 2 min for a total of 15 frames per reaction.

Data Analysis. Image processing and data analysis were carried out similarly to methods described previously.[11,12] Briefly, a custom MATLAB script loaded each .tif image stack. The first image was used as the signal baseline to subtract from subsequent frames. A background fluorescence threshold was applied to the last image of each stack. Wells were defined by image binarization, and changes in fluorescence signal over time were plotted in histograms to visualize the distribution of enzymatic activity. Individual enzyme activities were also tracked per frame to produce enzyme trajectory plots. A custom MATLAB script implemented a Gaussian Mixture Model (GMM) to fit Gaussian distributions to the histograms.

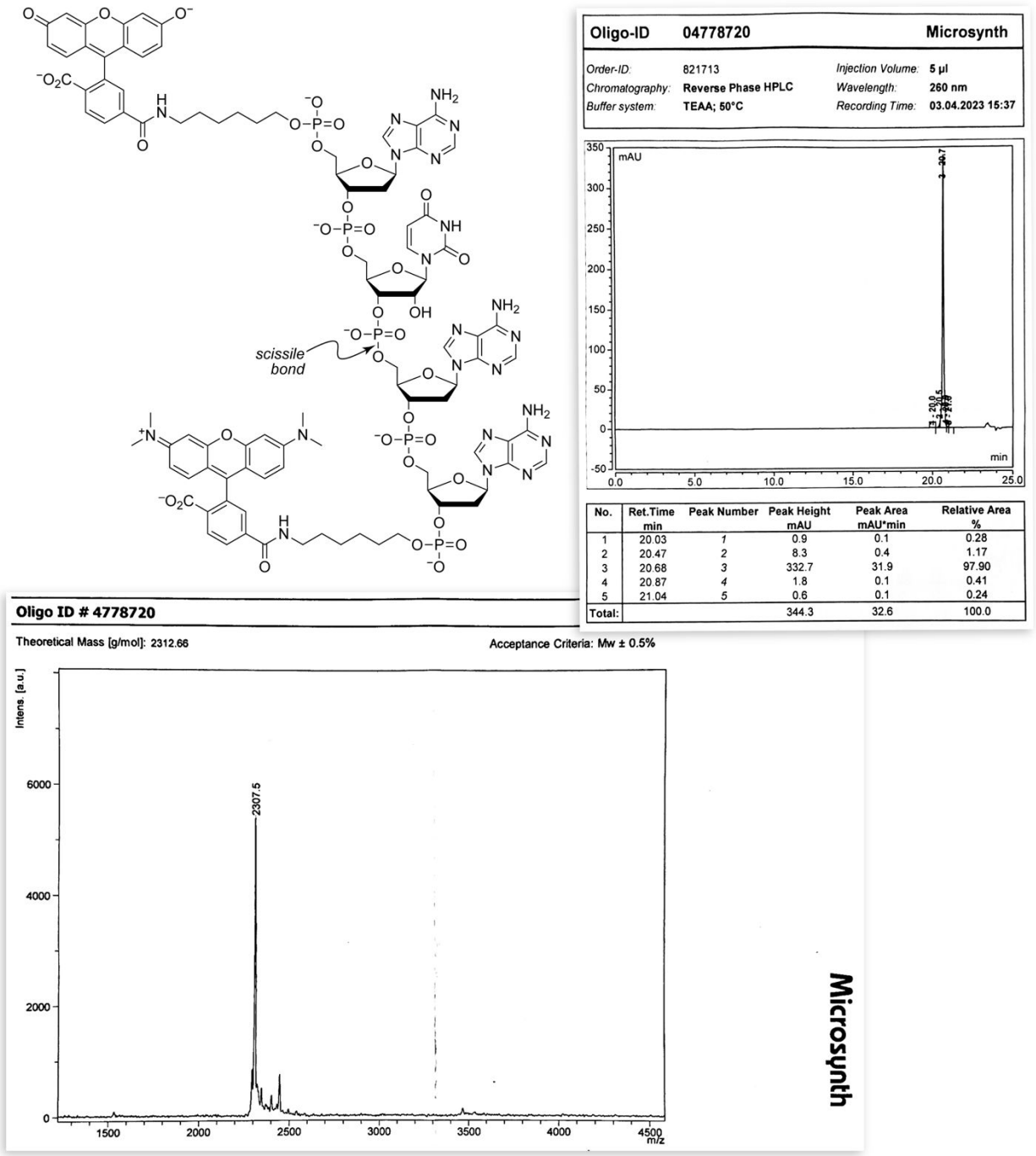
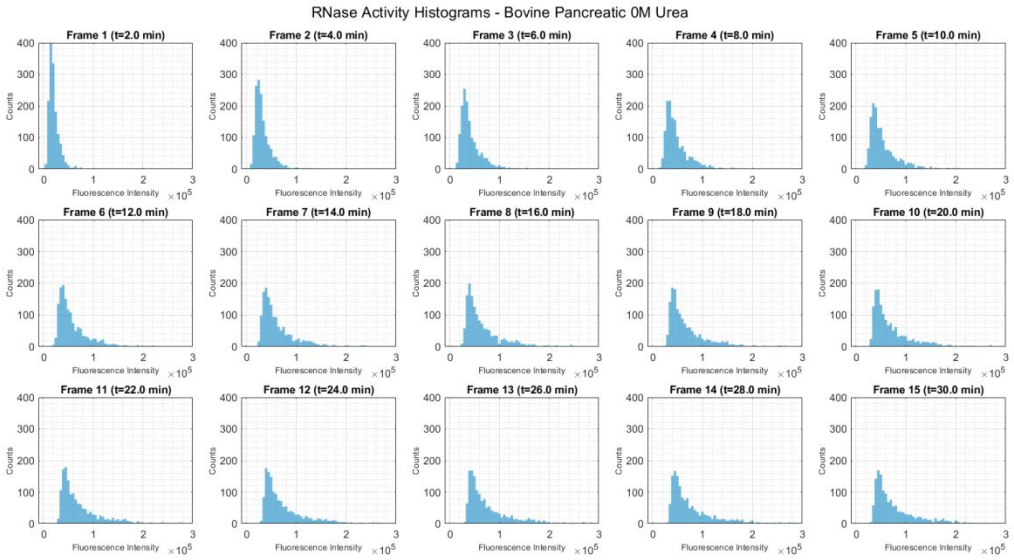
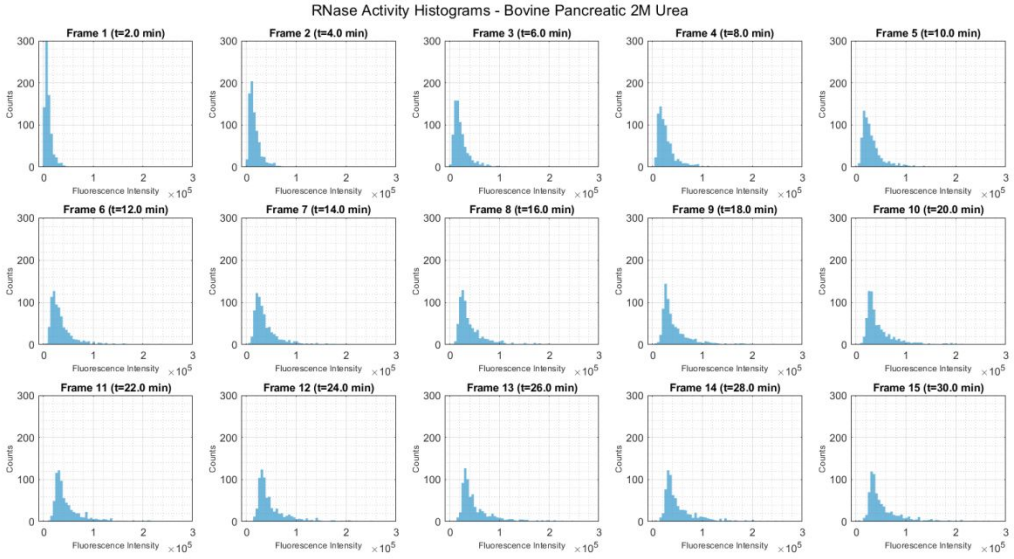


Figure S1. Structure of 6-FAM–dArU(dA)$_2$–6-TAMRA, which is the fluorogenic substrate for assays of ribonucleolytic activity and has only one scissile phosphodiester bond. The cleavage of that bond increases the fluorescence of the FAM moiety with λ_{ex} = 493 ± 5 nm and λ_{em} = 515 ± 5 nm. Insets: reversed-phase HPLC chromatogram (top) and MALDI–TOF mass spectrum (bottom) of synthetic 6-FAM–dArU(dA)$_2$–6-TAMRA, which was obtained from Microsynth (Balgach, Switzerland).


RNase Activity Histograms - Bovine Pancreatic 0M Urea
Frame 1 (t=2.0 min)
Frame 2 (t=4.0 min)
Frame 3 (t=6.0 min)
Frame 4 (t=8.0 min)
Frame 5 (t=10.0 min)
Frame 6 (t=12.0 min)
Frame 7 (t=14.0 min)
Frame 8 (t=16.0 min)
Frame 9 (t=18.0 min)
Frame 10 (t=20.0 min)
Frame 11 (t=22.0 min)
Frame 12 (t=24.0 min)
Frame 13 (t=26.0 min)
Frame 14 (t=28.0 min)
Frame 15 (t=30.0 min)
Counts
Fluorescence Intensity
×10^5


RNase Activity Histograms - Bovine Pancreatic 2M Urea
Frame 1 (t=2.0 min)
Frame 2 (t=4.0 min)
Frame 3 (t=6.0 min)
Frame 4 (t=8.0 min)
Frame 5 (t=10.0 min)
Frame 6 (t=12.0 min)
Frame 7 (t=14.0 min)
Frame 8 (t=16.0 min)
Frame 9 (t=18.0 min)
Frame 10 (t=20.0 min)
Frame 11 (t=22.0 min)
Frame 12 (t=24.0 min)
Frame 13 (t=26.0 min)
Frame 14 (t=28.0 min)
Frame 15 (t=30.0 min)
Counts
Fluorescence Intensity
×10^5

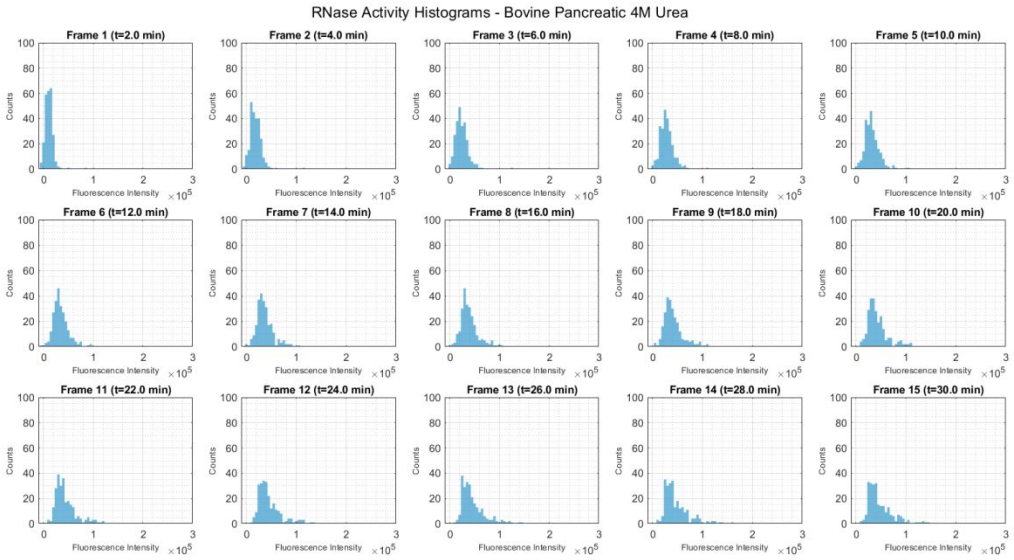
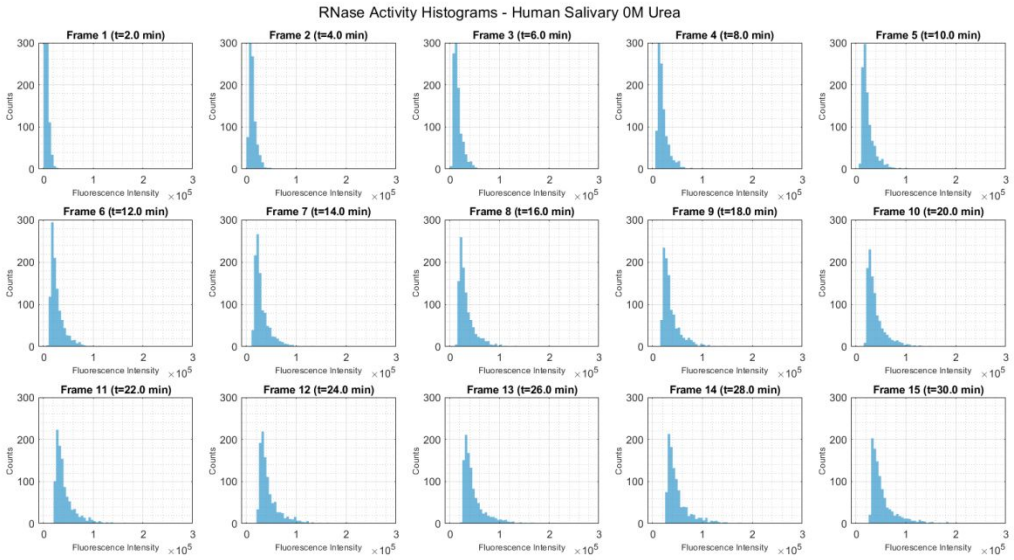
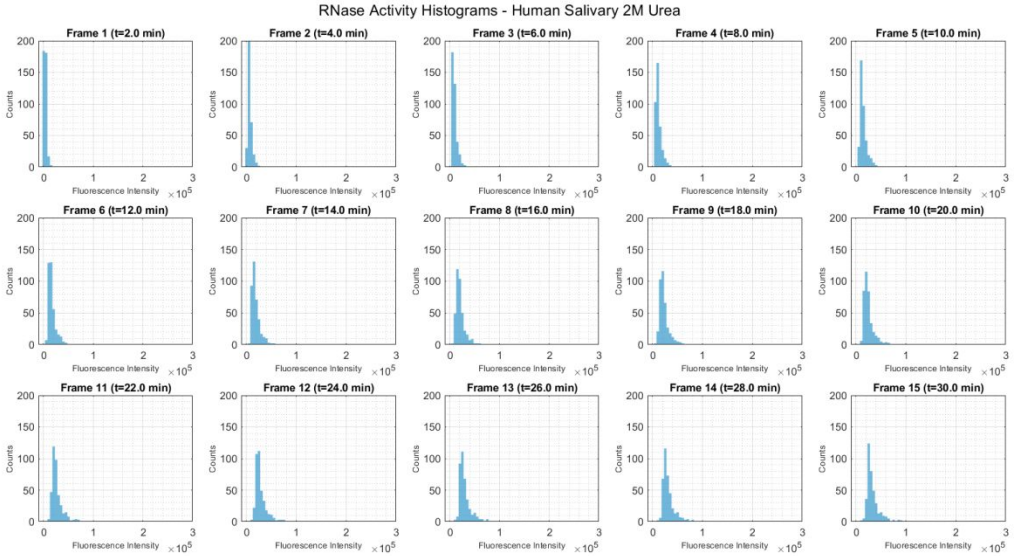


Figure S2. Full histogram series of bovine pancreatic ribonuclease (RNase A) across native ("0 M urea") and 2.0 M and 4.0 M urea renaturation conditions.


RNase Activity Histograms - Human Salivary 0M Urea
Frame 1 (t=2.0 min)
Frame 2 (t=4.0 min)
Frame 3 (t=6.0 min)
Frame 4 (t=8.0 min)
Frame 5 (t=10.0 min)
Frame 6 (t=12.0 min)
Frame 7 (t=14.0 min)
Frame 8 (t=16.0 min)
Frame 9 (t=18.0 min)
Frame 10 (t=20.0 min)
Frame 11 (t=22.0 min)
Frame 12 (t=24.0 min)
Frame 13 (t=26.0 min)
Frame 14 (t=28.0 min)
Frame 15 (t=30.0 min)
Counts
Fluorescence Intensity
×10^5


RNase Activity Histograms - Human Salivary 2M Urea
Frame 1 (t=2.0 min)
Frame 2 (t=4.0 min)
Frame 3 (t=6.0 min)
Frame 4 (t=8.0 min)
Frame 5 (t=10.0 min)
Frame 6 (t=12.0 min)
Frame 7 (t=14.0 min)
Frame 8 (t=16.0 min)
Frame 9 (t=18.0 min)
Frame 10 (t=20.0 min)
Frame 11 (t=22.0 min)
Frame 12 (t=24.0 min)
Frame 13 (t=26.0 min)
Frame 14 (t=28.0 min)
Frame 15 (t=30.0 min)
Counts
Fluorescence Intensity
×10^5

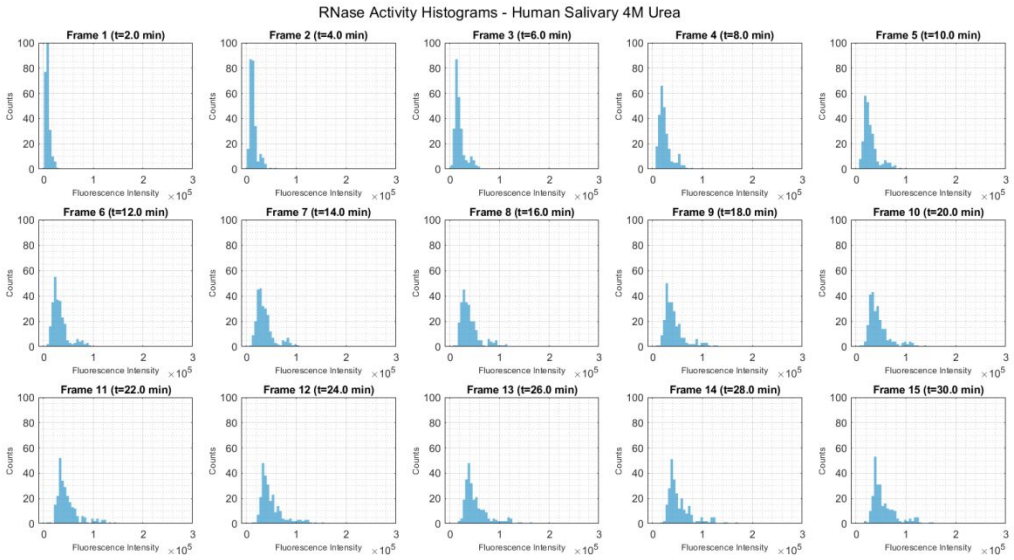
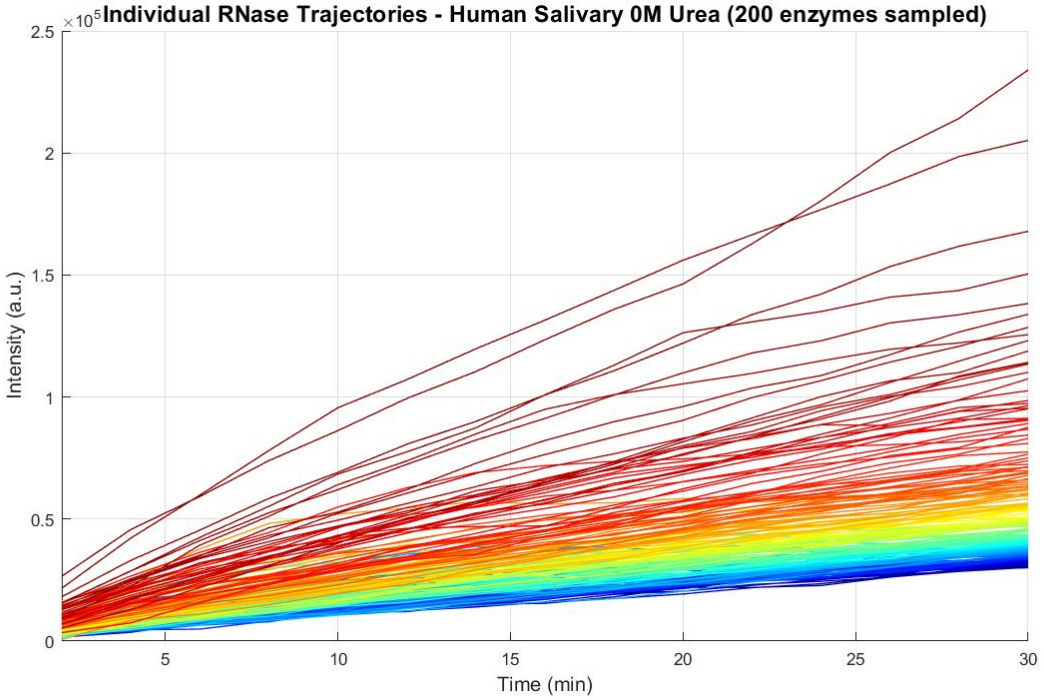
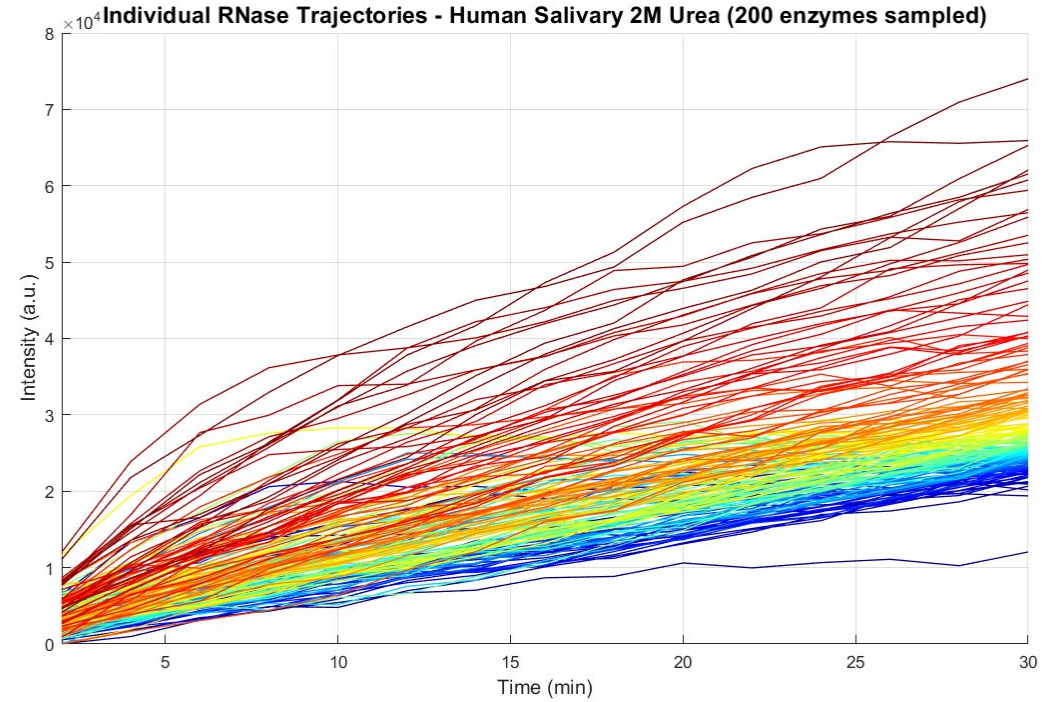


Figure S3. Full histogram series of human salivary ribonuclease across native ("0 M urea") and 2.0 M and 4.0 M urea renaturation conditions.


Individual RNase Trajectories - Human Salivary 0M Urea (200 enzymes sampled)
×10^5
2.5
2
1.5
1
0.5
0
Intensity (a.u.)
5
10
15
20
25
30
Time (min)


Individual RNase Trajectories - Human Salivary 2M Urea (200 enzymes sampled)
×10^4
8
7
6
5
4
3
2
1
0
Intensity (a.u.)
5
10
15
20
25
30
Time (min)

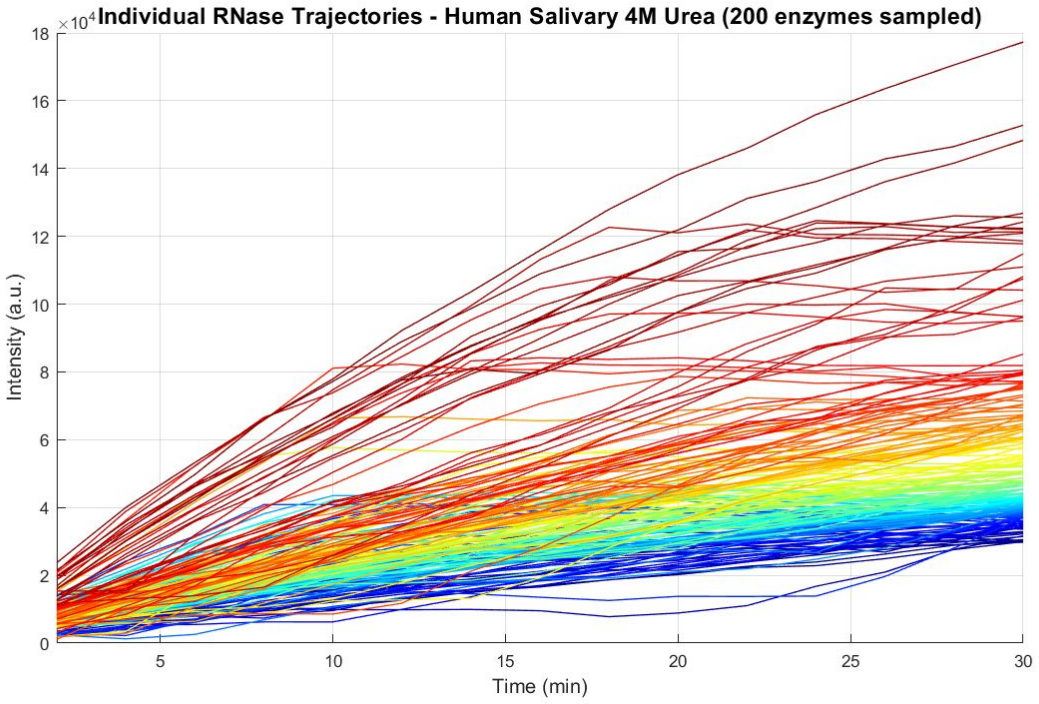
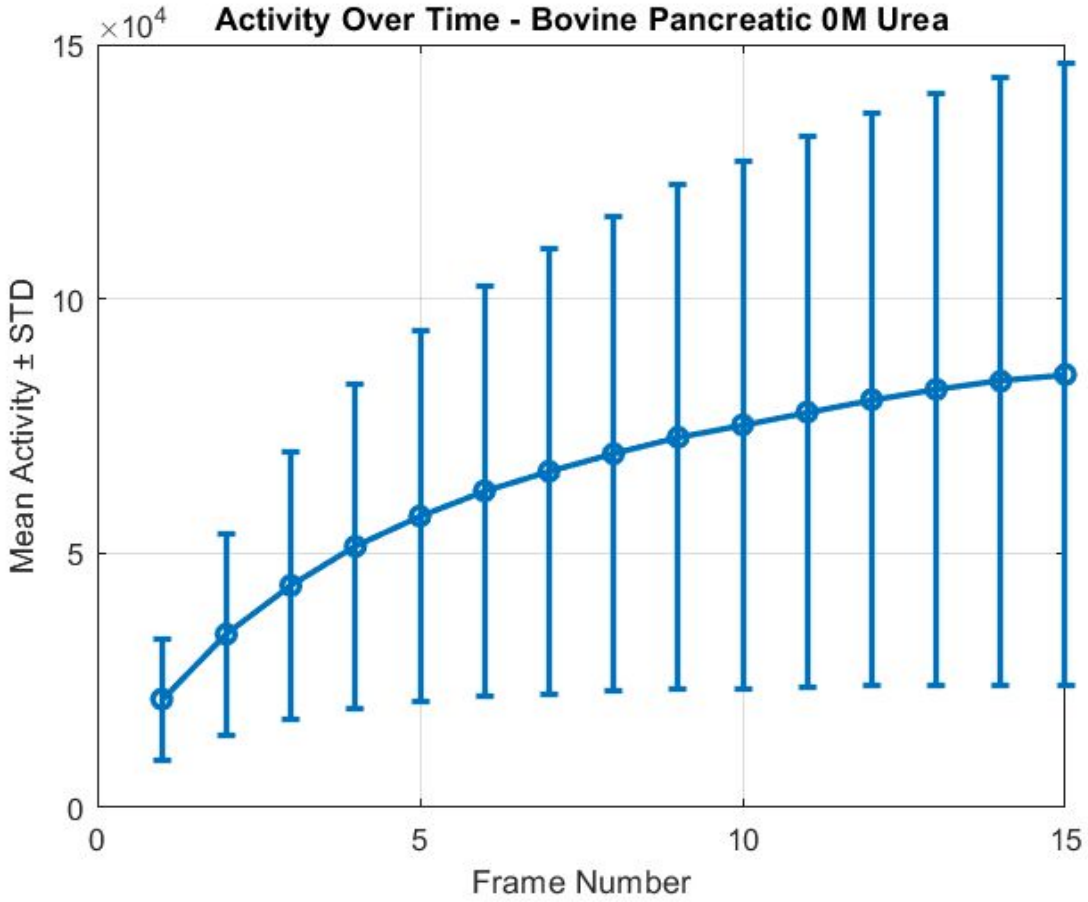
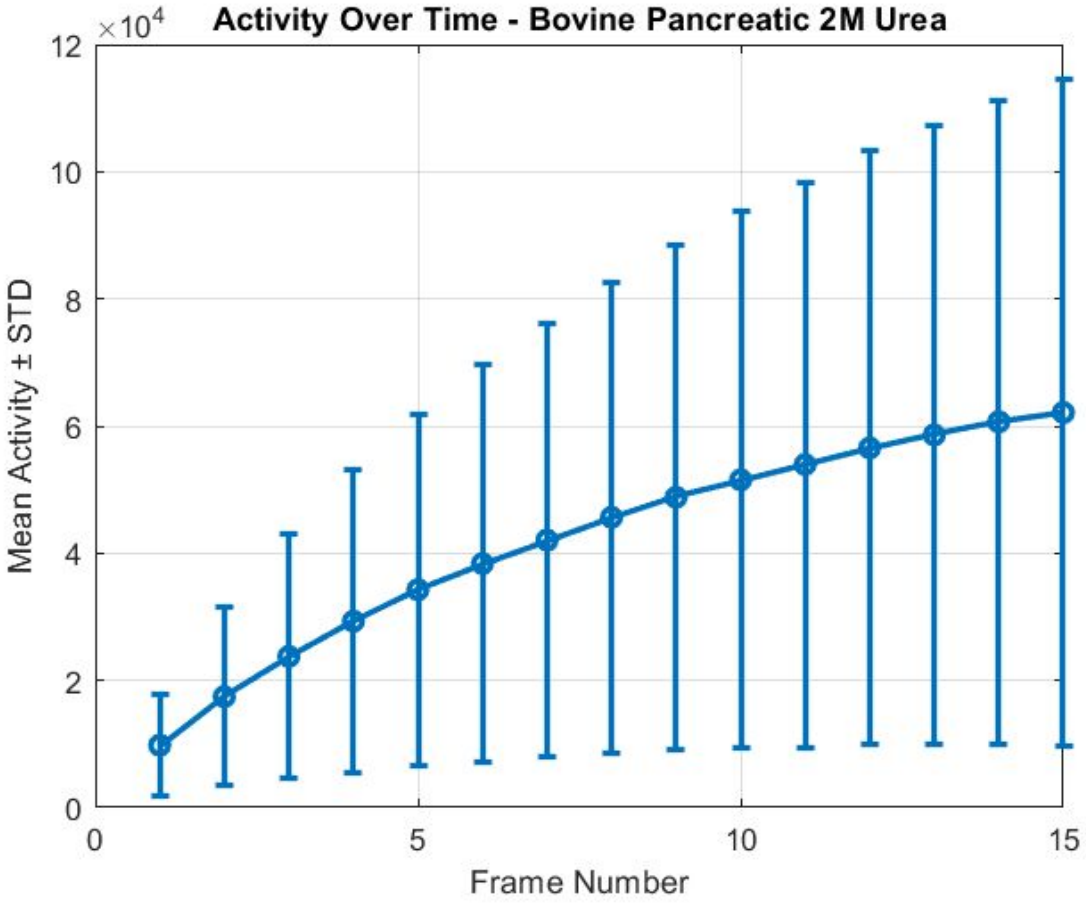


Figure S4. Individual enzyme trajectories for human salivary ribonuclease across native ("0 M urea") and 2.0 M and 4.0 M urea renaturation conditions.


Activity Over Time - Bovine Pancreatic 0M Urea
×10^4
15
10
5
0
Mean Activity ± STD
0
5
10
15
Frame Number


Activity Over Time - Bovine Pancreatic 2M Urea
×10^4
Mean Activity ± STD
Frame Number
0
2
4
6
8
10
12
0
5
10
15

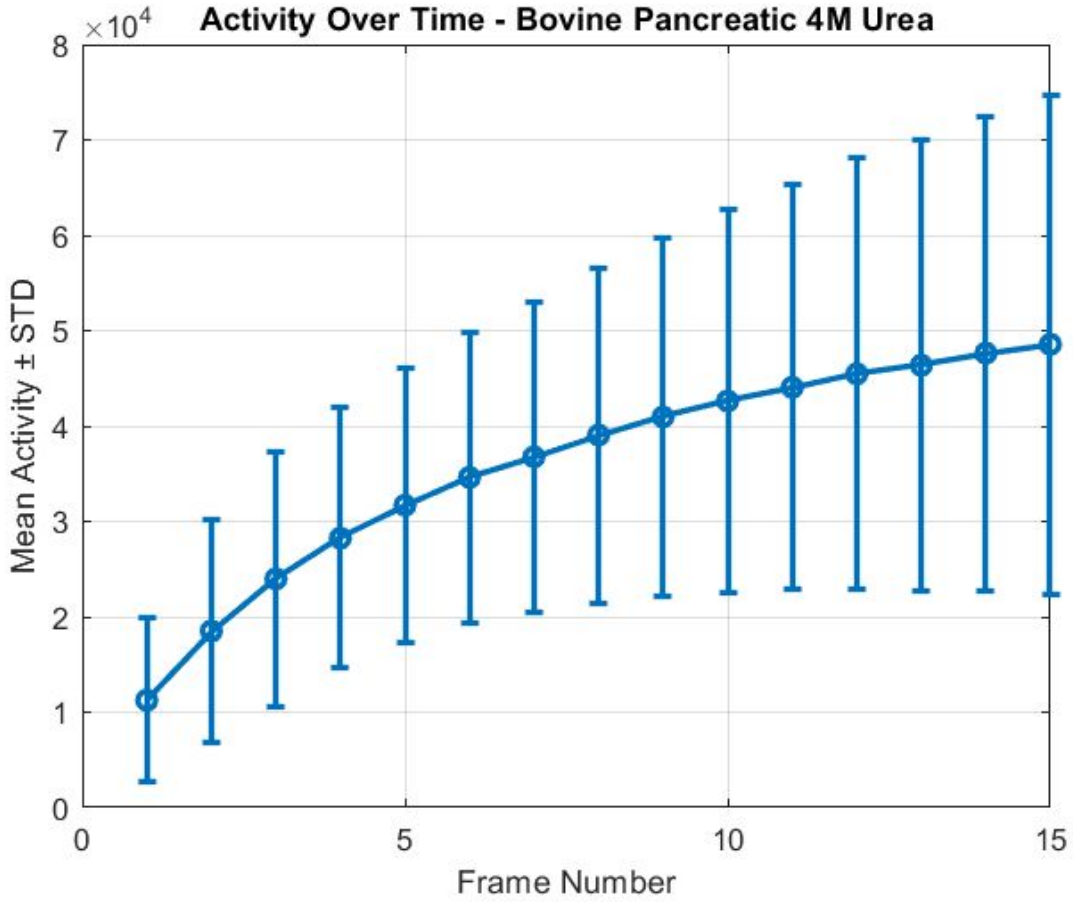
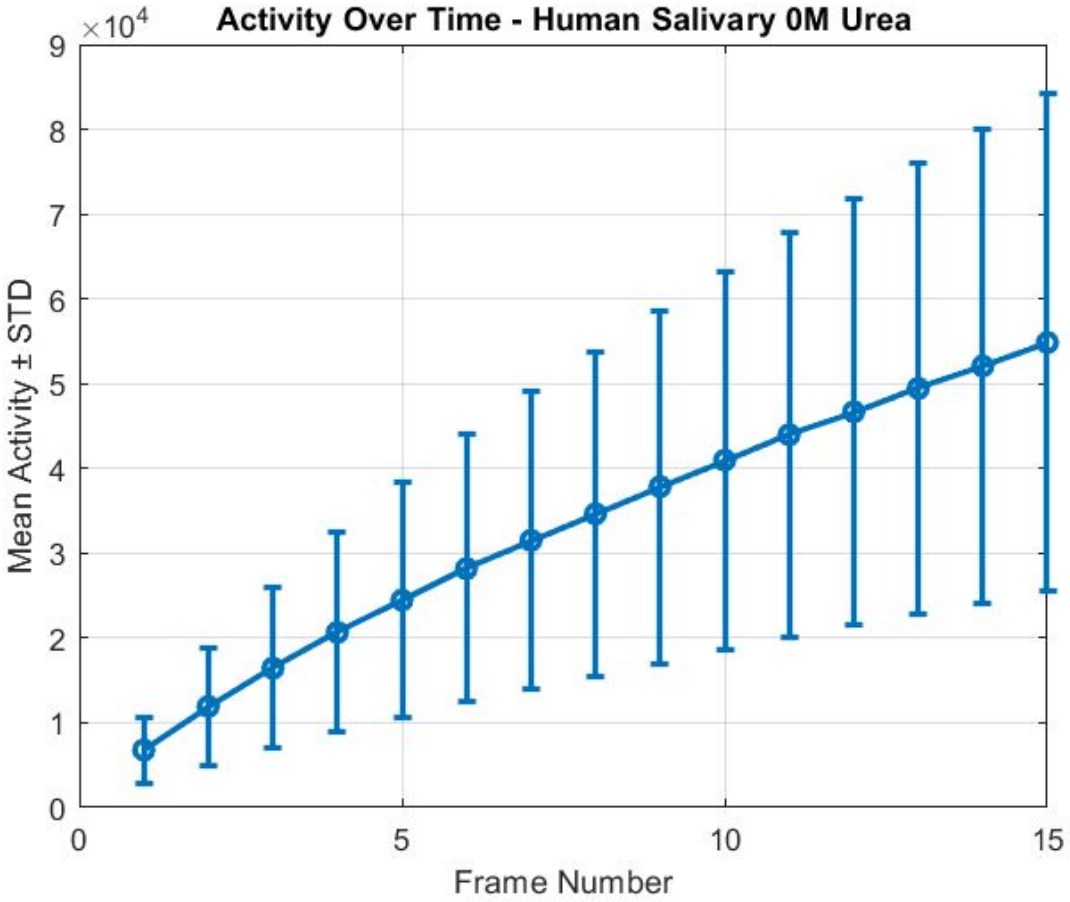
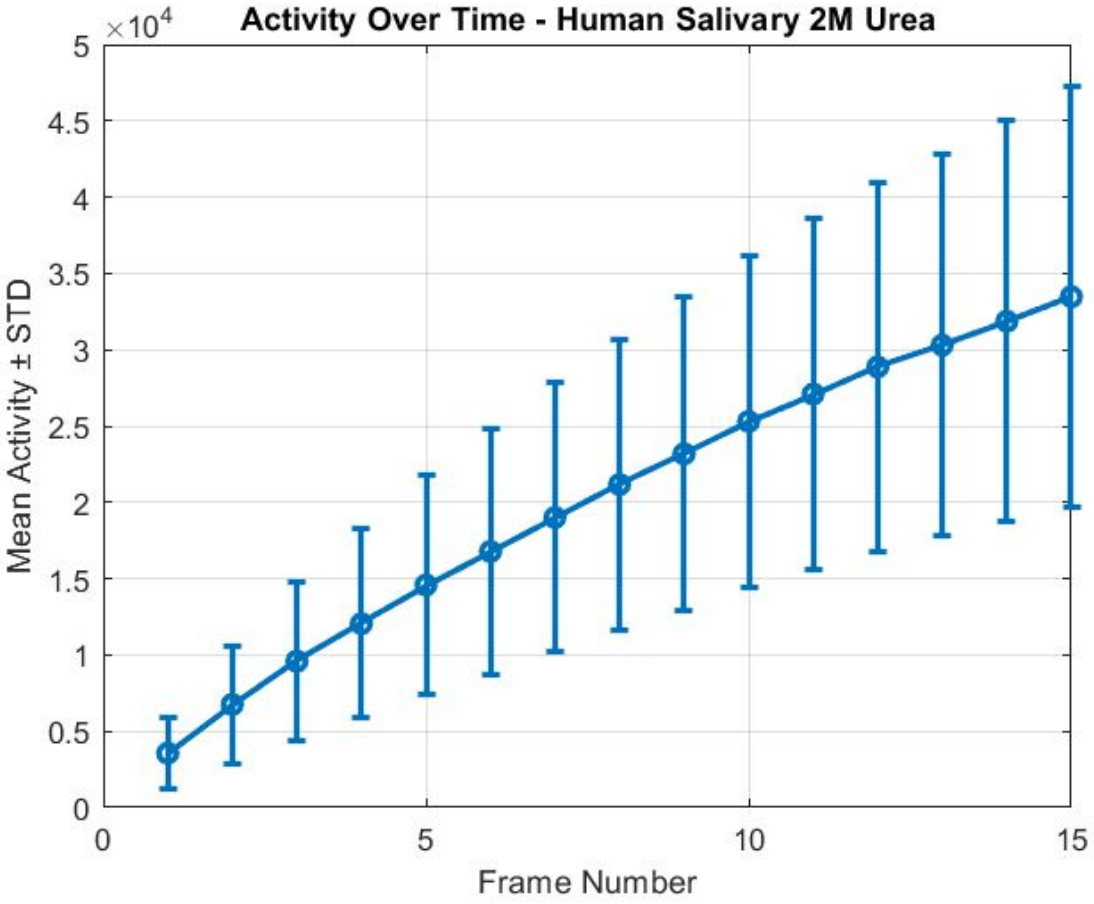


Figure S5. Activity over time of bovine pancreatic ribonuclease (RNase A) across native ("0 M urea") and 2.0 and 4.0 M urea renaturation conditions.


Activity Over Time - Human Salivary 0M Urea
×10^4
Mean Activity ± STD
Frame Number
0
1
2
3
4
5
6
7
8
9
0
5
10
15


Activity Over Time - Human Salivary 2M Urea
×10^4
Mean Activity ± STD
Frame Number
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
0
5
10
15

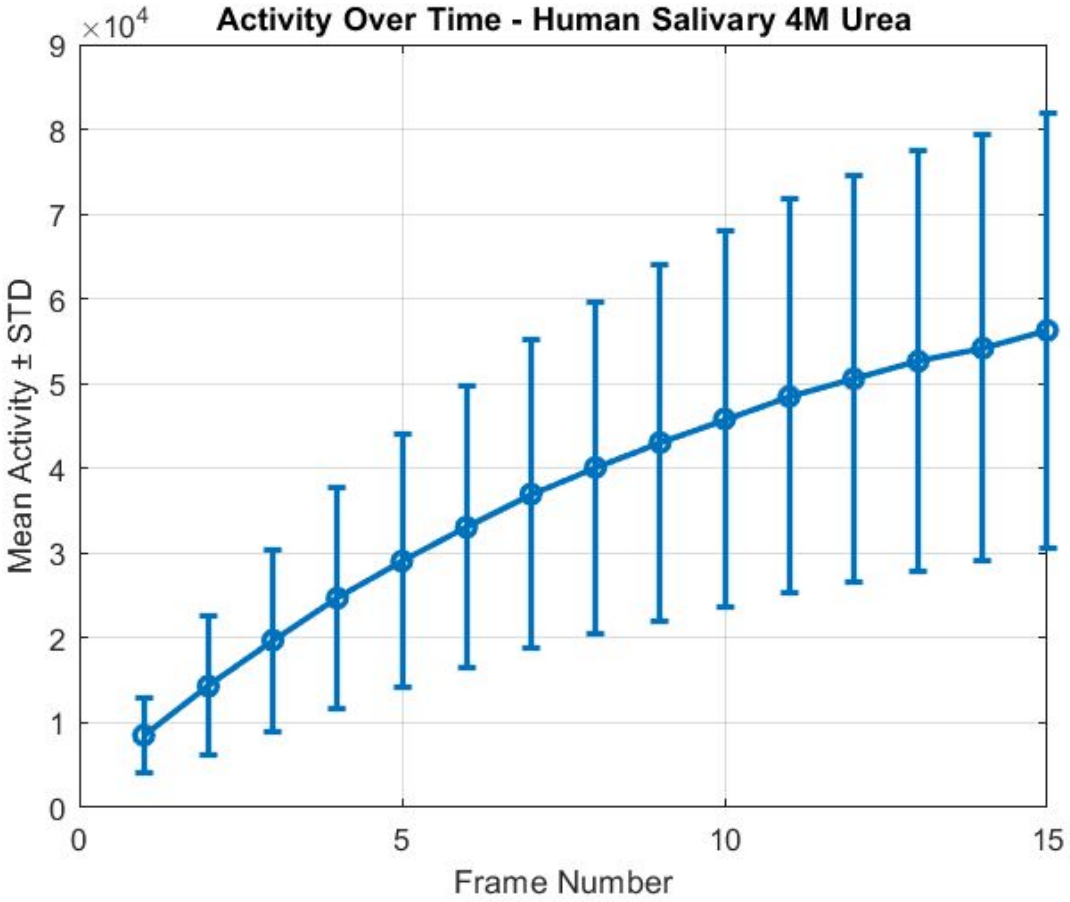


Figure S6. Activity over time of human salivary ribonuclease across native ("0 M urea") and 2.0 M and 4.0 M urea renaturation conditions.