E. coli RNase I exhibits a strong Ca^{2+}-dependent inherent double-stranded RNase activity

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Supplementary Data

Supplementary Experimental Procedures

Differential scanning fluorimetry

45 μM of the dsRNA substrate was incubated in buffer A as described in "Enzymatic assays". The dsRNA was then diluted on ice to 0.5 μM in 50 μL buffer A supplemented with 1 mM indicated cation and a 400-fold dilution of Quant-iT RiboGreen (Thermo Fisher Scientific). DSF assays were performed on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) from 20 °C to 95 °C at a temperature ramp of 1 °C per 30 seconds. After the fluorescent signal was normalized, the melting temperature was determined as the maxima of negative first derivative from four independent measurements (1).

DNase contamination assays

0.5 μM DNA oligonucleotides (either 3'- or 5'-end FAM labeled) were incubated with 6.7 μM RNase I in buffer A in the presence or absence of 4 mM $CaCl_2$ at 37 °C for 15 minutes. The reactions were quenched by the addition of 0.8 U Proteinase K (NEB P8107) on ice, followed by 15 min incubation at 37 °C. After diluting the samples with nuclease free water to appropriate concentrations, the oligonucleotides were separated using capillary electrophoresis and visualized using a 3730xl Genetic Analyzer (Applied Biosystems) as described (2). The fluorescent fractions were analyzed by Peak Scanner software version 1.0 (Applied Biosystems).

Enzymatic assays to determine RNase I catalytic efficiency

With the exception of the following changes, the enzymatic assays were performed essentially as described in the Materials & Methods section of the main text. RNase I was incubated with dsRNA at enzyme to substrate (E:S) ratios of 1/10, 1/20, 1/40, 1/80, and 1/100 in the absence of Ca^{2+}. When Ca^{2+} was present, these ratios were 1/250, 1/500, 1/750, and 1/1,250 to accommodate for the significantly higher activity. E:S ratios tested in ssRNA reactions in the absence and presence of Ca^{2+} were 1/1000, 1/1,500, 1/3,000, 1/4,000, 1/5,000. A master mix was assembled on ice, RNase I being the last component added, and subsequently aliquoted into separate tubes. The tube for the 0-timepoint contained 1 μL of Proteinase K to immediately stop the reaction. The remaining tubes were transferred to 37 °C and the reaction stopped by addition of 1 μl Proteinase K at the indicated timepoints. After 10 min of Proteinase K treatment at 37 °C, dsRNA samples were analyzed on 10% non-denaturing gels as described in Materials & Methods, while ssRNA samples were loaded onto 15% denaturing polyacrylamide TBE-urea gels and run in 1x TBE buffer for 45 min at 180V. The gels were stained and visualized as described in Methods & Materials. The reduction of the full-length RNA substrate band of two to three independent replicas per experiment was quantified using ImageQuant TL software and the kinetic traces were generated using KaleidaGraph software (Reading, PA). All traces were fit to a single exponential equation except for dsRNA no Ca^{2+}, which was fit to a double exponential equation. An initial rate for each condition was derived by multiplying the amplitude and the observed rate constant. This in turn was plotted against substrate concentration and fit to a Michaelis-Menten equation. An accurate derivation of the Michaelis constant (K_M) for each condition was not possible due to the fact that the data points fell on the saturating part of the

curve due to experimental limitations dictated by the detection limits of the in-gel staining of the cleavage products. Instead, we estimated K_M to be roughly 150 nM for all conditions allowing us to derive the maximum rate (v_{max}) as described in Table S4. The turnover number (k_{cat}) described as v_{max}/[Enzyme] is used to compare performance of RNase I on the various substrates.

1. Silvers, R., Keller, H., Schwalbe, H. and Hengesbach, M. (2015) Differential scanning fluorimetry for monitoring RNA stability. *Chembiochem*, **16**, 1109-1114.
2. Greenough, L., Schermerhorn, K.M., Mazzola, L., Bybee, J., Rivizzigno, D., Cantin, E., Slatko, B.E. and Gardner, A.F. (2016) Adapting capillary gel electrophoresis as a sensitive, high-throughput method to accelerate characterization of nucleic acid metabolic enzymes. *Nucleic Acids Res*, **44**, e15.

Supplementary Figure and Table legends

Figure S1. Protein gel of all RNase I variants used in this study. 5 µl of a 1:10 dilution of each protein stock were ran on a 10-20% Tris-Glycine SDS gel and stained with SimplyBlue Safe Stain. NEB Color Prestained Protein Standard P7712 was used as marker. Asterisks indicate endogenous *E. coli* MBP and degradation products.

Figure S2. Catalytic efficiency of RNase I on dsRNA substrates. (A) $6.7x10^{-3}$ µM of RNase I was incubated with various concentrations of dsRNA starting at 0.067 µM (top) to 0.67 µM (bottom) in the absence of $CaCl_2$. The digestion reaction was quenched at the timepoints indicated above the respective lanes on the gel. **(B)** Timepoints were taken as in (A) with $1.34x10^{-3}$ µM RNase I and dsRNA concentrations starting at 0.335 µM (top) to 1.675 µM (bottom). **(C)** Michaelis-Menten plots showing effect of Ca^{2+} on the activity of RNase I in the presence of dsRNA substrate.

Figure S3. Cleavage pattern for the digestion of a FAM-labeled 33mer dsRNA substrate (2 nt 3' overhangs) with RNase I. MS results showing the location of the cleavage and the relative abundance of the resulting fragment for reactions containing 0.5 µM dsRNA substrate and $1.34x10^{-3}$ µM RNase I without (**A**) or with Ca^{2+} (**B**). A representative example of cleavage products is shown in (**C**). Asterisks indicate the location of cleavage in this example. Blue bars represent uncleaved full-length (FL) oligonucleotides. Red bars represent cleaved products without a FAM label (sequence to left of the bar). Green bars represent cleaved products with a FAM label (sequence to right of the bar, including nucleotide under the bar).

Figure S4. Calcium induced RNase I activity on dsRNA with blunt and DNA ends. (**A**) A 31mer blunt-ended dsRNA digested with RNase I in the absence (lanes 1-9) or presence of $CaCl_2$ (lanes 10-18). The reactions were carried under the standard assay conditions with 0.1 µM substrate and enzyme concentrations as indicated above the lanes. Quantification of the uncleaved dsRNA substrate is shown in (**C**). (**B**) A 41mer blunt-ended dsDNA-RNA-DNA chimera digested with RNase I in the absence (lanes 1-8) or presence of $CaCl_2$ (lanes 9-16). The blunt-ended DNA-RNA-DNA chimera featuring seven deoxyribonucleotides in each of the ends was chosen to protect against potential exoribonuclease activity. Quantification of uncleaved dsRNA substrate is shown in (**D**).

Figure S5. RNase I binds but does not cleave DNA. (**A**) Electrophoretic mobility shift assays of various concentrations of RNase I (as indicated above the gel) with either dsDNA (upper panel) or ssDNA (lower panel). Quantification of unbound RNA is shown in (**B**). (**C**) DNase activity assay. Single (left) and

double (right) stranded DNA incubated either with buffer A or RNase I, with or without $CaCl_2$. The bars represent the percentage of uncleaved DNA substrate after incubation from two independent replicates.

Figure S6. Cleavage pattern for digestion of blunt-ended dsRNA substrates with RNase I. MS results showing the location of the cleavage and the relative abundance of the resulting fragment for reactions of 26mer (**A**) and 41mer (**B**) dsRNA substrates. Reactions in (**A**) were carried out with 0.67 µM RNase I without Ca^{2+} (top) or $6.7x10^{-2}$ µM RNase I with Ca^{2+} (bottom). Reactions in (**B**) were carried out with 0.134 µM RNase I without Ca^{2+} (top) or $1.34x10^{-2}$ µM RNase I with Ca^{2+} (bottom). Blue bars represent uncleaved full-length (FL) oligonucleotides. Red bars represent primary cleavage products (sequence to left of the bar). Yellow bars represent a secondary cleavage of the primary products leaving a 5'-OH (top strands), a 2',3'-cyclic phosphate (bottom strands, dark yellow), or 3'-phosphate (bottom strands, light yellow).

Figure S7. Tagless RNase I variants require Ca^{2+} to efficiently cleave dsRNA substrates. (**A**) Various dilutions of two commercially available, tagless RNase I variants incubated with 0.1 µM of 33mer dsRNA substrate with 2 nt 3' overhangs. The asterisks highlight the uncleaved full-length dsRNA substrate. Reactions in lanes 7-12 contained 4 mM $CaCl_2$. (**B**) Quantification of the gel bands shown in (**A**).

Figure S8. Catalytic efficiency of RNase I on ssRNA. $6.7x10^{-4}$ µM of RNase I was incubated with various concentrations of a 33 nt ssRNA from 0.67 µM (top) to 3.35 µM (bottom). Reactions in **(A)** were incubated in the absence and reactions in **(B)** in the presence of 4 mM $CaCl_2$. Reactions were quenched by the addition of Proteinase K at the timepoints indicated above the respective lanes. The diamond indicates a potential primary cleavage product. **(C)** Michaelis-Menten plots showing effect of Ca^{2+} on the activity of RNase I in the presence of ssRNA substrate.

Figure S9. The presence of Ca^{2+} does not make dsRNA more susceptible to digestion by single strand specific endoribonucleases. (**A**) 0.1 µM dsRNA (upper panel) or ssRNA (lower panel) substrate cleaved with 10-fold decreasing concentrations of pancreatic RNase A, from 10 µM (lanes 2, 8) to $1x10^{-3}$ µM (lanes 6, 12) either in the absence (lanes 1-6) or presence of 4 mM $CaCl_2$ (lanes 7-12). NE, no enzyme control. (**B**) Differential scanning fluorimetry was used to determine the stability of dsRNA in buffer, both in the absence and presence of divalent cations. The table shows the observed melting temperatures determined from three replicates. N.D, not determined due to no local maximum.

Figure S10. The inhibitory effect of EDTA on RNase I dsRNase activity is concentration dependent. (**A**) RNase I purified without DTT/EDTA was incubated with dsRNA in the absence (left panel) or presence (right panel) of 4 mM $CaCl_2$. Either no, 0.5 mM, or 4 mM EDTA was added to the reactions as indicated below each gel. Quantification of uncleaved dsRNA substrate in the reactions containing $1.34x10^{-2}$ µM RNase I in the absence of Ca^{2+} is shown in (**B**). The asterisk marks the no enzyme control. EDTA barely affects cleavage of ssRNA by RNase I, regardless if Ca^{2+} is present (**C** and **D**).

Figure S11. Structural analysis of the RNase I Ca^{2+} binding site and RNase I mutant function. (**A**) The zoomed-in section of the Ca^{2+} binding site in the crystal structure of RNase I (2PQX; Rodriguez et al., 2008) highlights the highly conserved amino acid residues that coordinate the Ca^{2+}. RNase I_{D241L} concentrations from 6.7 µM to $6.7x10^{-4}$ µM incubated with 0.1 µM of a 31mer blunt-ended dsRNA substrate in the absence (**B**) or presence of 4 mM $CaCl_2$ (**C**). The relative amount of uncleaved full-length dsRNA substrate is shown in (**D**). (**E**) The distance between the Ca^{2+} binding site and the catalytic center of RNase I is approximately 18 Å. Two β-strands (red) of the RNase I central β-sheet are either directly contacting residues that bind to Ca^{2+} (β6 and the residues D264 and L265) or are in close proximity to the

Ca^{2+} binding site (β1 positioned directly upstream of the Q49 containing α1 helix (green)). Other β-strands are shown in orange.

Figure S12. Deconvoluted ESI-MS spectra of 33mer dsRNA substrate (2 nt 3' overhangs) digested with RNase I. Reactions were carried out with $6.7x10^{-2}$ μM RNase I without Ca^{2+} (**A**) or $6.7x10^{-3}$ μM RNase I with Ca^{2+} (**B**). The composition of each peak was determined by comparison with calculated monoisotopic mass. Output mass range of 7,000 to 11,000 Da. Monoisotopic masses corresponding to uncleaved full-length (FL) top and bottom strands are shown.

Figure S13. Deconvoluted ESI-MS spectra of FAM-labeled 33mer dsRNA substrate (2 nt 3' overhangs) digested with RNase I. Reactions were carried out with $1.34x10^{-3}$ μM RNase I without (**A**) or with Ca^{2+} (**B**). The composition of each peak was determined by comparison with calculated monoisotopic mass. Output mass range of 2,000 to 12,000 Da. Monoisotopic masses corresponding to uncleaved full-length (FL) top and bottom strands are shown.

Figure S14. Deconvoluted ESI-MS spectra of 26mer dsRNA substrate (blunt ends) digested with RNase I. Reactions were carried out with 0.67 μM RNase I without Ca^{2+} (**A**) or $6.7x10^{-2}$ μM RNase I with Ca^{2+} (**B**). The composition of each peak was determined by comparison with calculated monoisotopic mass. Output mass range of 5,000 to 9,000 Da. Monoisotopic masses corresponding to uncleaved full-length (FL) top and bottom strands are shown.

Figure S15. Deconvoluted ESI-MS spectra of 41mer dsRNA substrate (blunt ends) digested with RNase I. Reactions were carried out with 0.134 μM RNase I without Ca^{2+} (**A**) or $1.34x10^{-2}$ μM RNase I with Ca^{2+} (**B**). The composition of each peak was determined by comparison with calculated monoisotopic mass. Output mass range of 8,500 to 13,500 Da. Monoisotopic masses corresponding to uncleaved full-length (FL) top and bottom strands are shown.

Figure S16. Deconvoluted ESI-MS spectra of 33mer dsRNA substrate (2 nt 3' overhangs) digested with RNase I in absence of DTT and EDTA. Reactions were carried out with $6.7x10^{-3}$ μM RNase I (–DTT/–EDTA) without (**A**) or with Ca^{2+} (**B**). The composition of each peak was determined by comparison with calculated monoisotopic mass. Output mass range of 7,000 to 11,000 Da. Monoisotopic masses corresponding to uncleaved full-length (FL) top and bottom strands are shown.

Table S1: A list of substrates used in this study. Size, type, and chemical structure of the oligo ends are indicated in the table.

Table S2. Assigned fragments based on ESI-MS analysis of 0.5 μM 33mer dsRNA substrate (2 nt 3' overhangs) digested with RNase I. (**A**) Without Ca^{2+}. (**B**) With Ca^{2+}. Calculated and found monoisotopic masses are shown. Mass errors are expressed in ppm. n.f., not found. >p, 2',3'-cyclic phosphate. "% Rel" is the relative signal strength compared to the highest peak, which is set to be 100%.

Table S3. Assigned fragments based on ESI-MS analysis of 0.5 μM 33mer dsRNA substrate (2 nt 3' overhangs) digested with RNase I purified in the absence of DTT and EDTA. (**A**) Without Ca^{2+}. (**B**) With Ca^{2+}. Calculated and found monoisotopic masses are shown. Mass errors are expressed in ppm. n.f.,

not found. >p, 2',3'-cyclic phosphate. "% Rel" is the relative signal strength compared to the highest peak, which is set to be 100%.

Table S4. Kinetic parameters for RNase I digestion of ds- and ssRNA substrates in the presence or absence of Ca^{2+}.

Figure S1

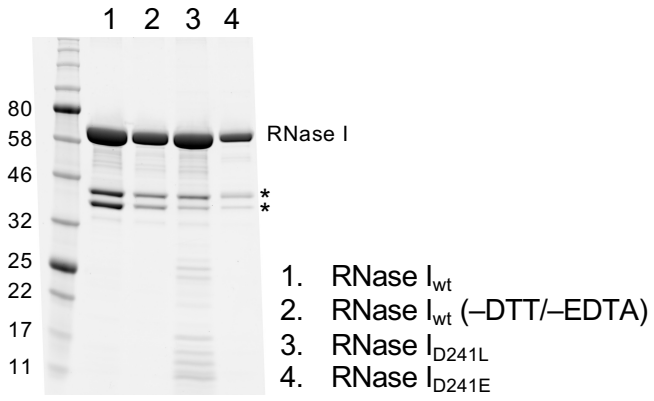
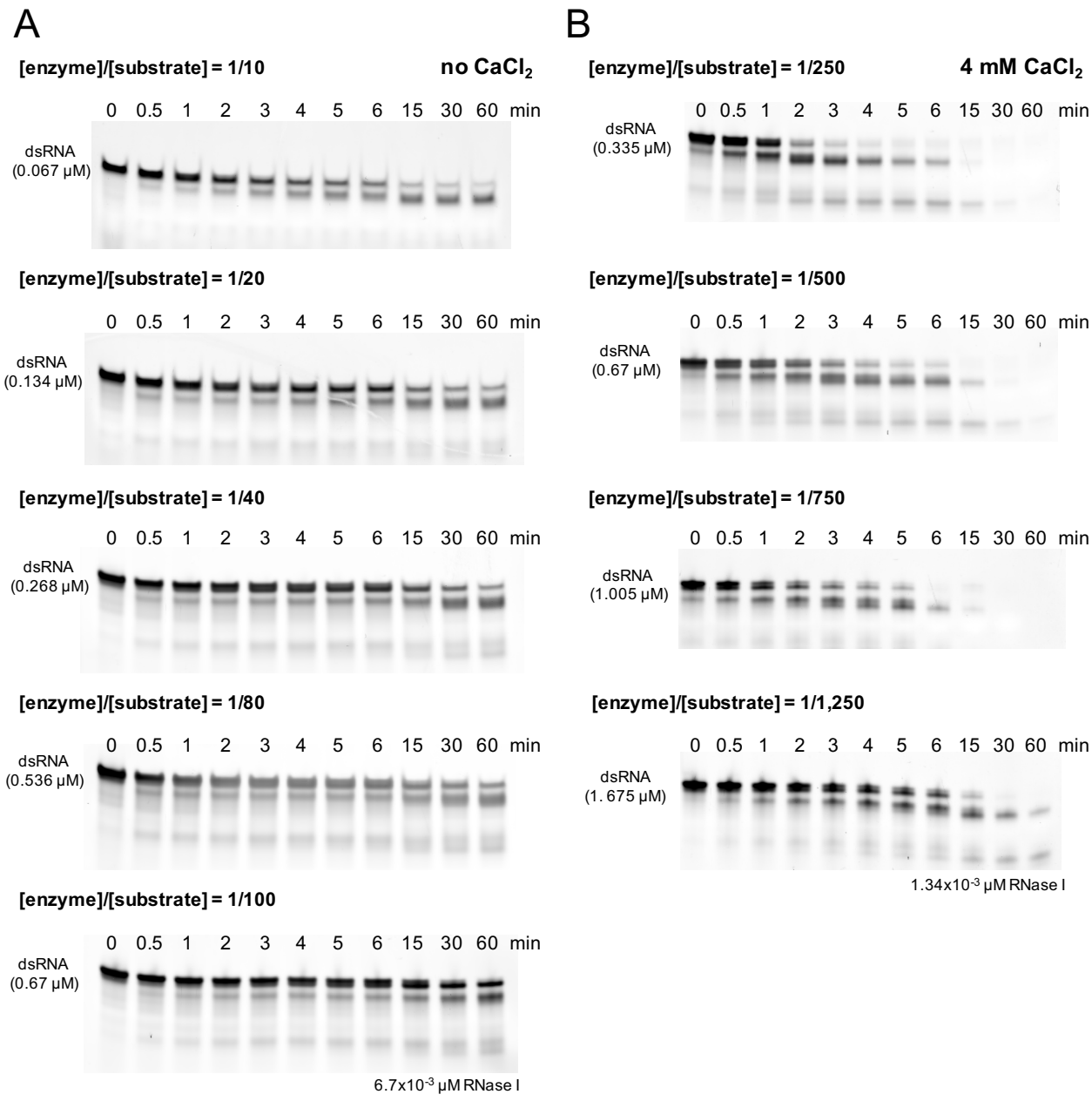

1 2 3 4
80
58
46
32
25
22
17
11
RNase I
*
*
1. RNase I_{wt}
2. RNase I_{wt} (–DTT/–EDTA)
3. RNase I_{D241L}
4. RNase I_{D241E}

Figure S2


A
[enzyme]/[substrate] = 1/10
no CaCl2
0 0.5 1 2 3 4 5 6 15 30 60 min
dsRNA (0.067 µM)
[enzyme]/[substrate] = 1/20
dsRNA (0.134 µM)
[enzyme]/[substrate] = 1/40
dsRNA (0.268 µM)
[enzyme]/[substrate] = 1/80
dsRNA (0.536 µM)
[enzyme]/[substrate] = 1/100
dsRNA (0.67 µM)
6.7x10-3 µM RNase I
B
[enzyme]/[substrate] = 1/250
4 mM CaCl2
dsRNA (0.335 µM)
[enzyme]/[substrate] = 1/500
dsRNA (0.67 µM)
[enzyme]/[substrate] = 1/750
dsRNA (1.005 µM)
[enzyme]/[substrate] = 1/1,250
dsRNA (1. 675 µM)
1.34x10-3 µM RNase I

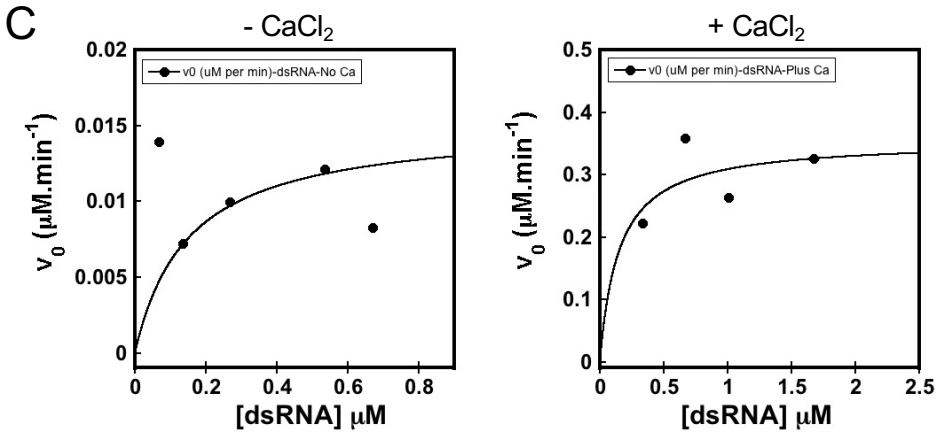

C
- CaCl2
v0 (uM per min)-dsRNA-No Ca
v0 (µM.min-1)
[dsRNA] µM
+ CaCl2
v0 (uM per min)-dsRNA-Plus Ca
v0 (µM.min-1)
[dsRNA] µM

Figure S3

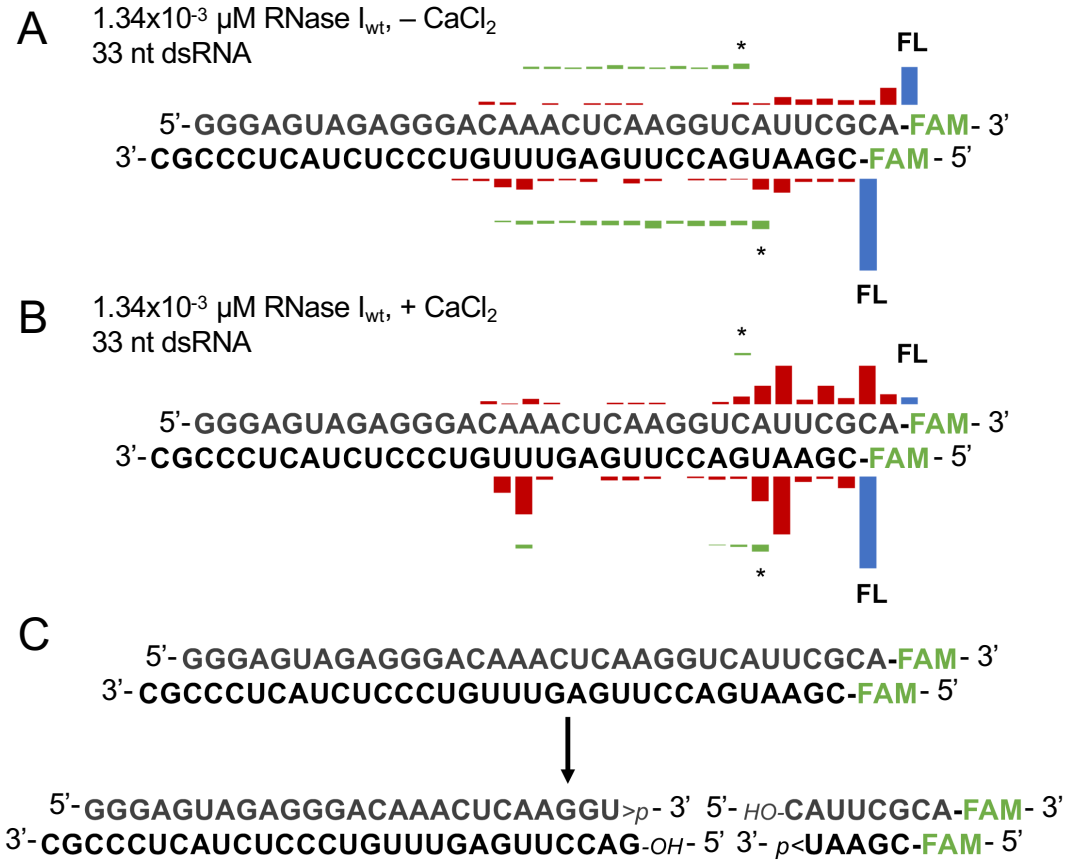
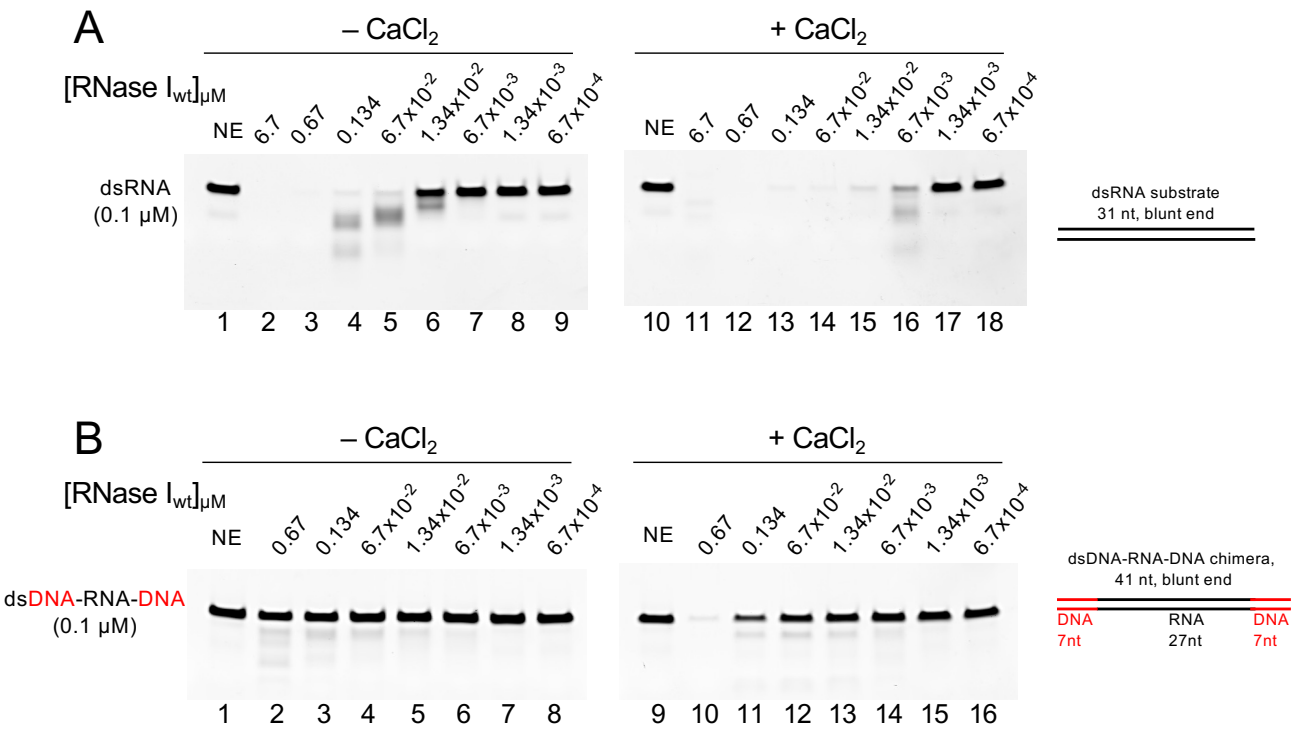
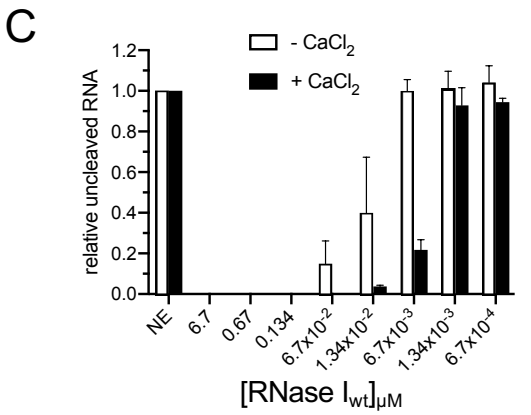

A
1.34x10-3 µM RNase Iwt, – CaCl2
33 nt dsRNA
FL
5’-GGGAGUAGAGGGACAAACUCAAGGUCAUUCGCA-FAM- 3’
3’-CGCCCUCAUCUCCCUGUUUGAGUUCCAGUAAGC-FAM- 5’
FL
B
1.34x10-3 µM RNase Iwt, + CaCl2
33 nt dsRNA
FL
5’-GGGAGUAGAGGGACAAACUCAAGGUCAUUCGCA-FAM- 3’
3’-CGCCCUCAUCUCCCUGUUUGAGUUCCAGUAAGC-FAM- 5’
FL
C
5’-GGGAGUAGAGGGACAAACUCAAGGUCAUUCGCA-FAM- 3’
3’-CGCCCUCAUCUCCCUGUUUGAGUUCCAGUAAGC-FAM- 5’
5’-GGGAGUAGAGGGACAAACUCAAGGU>p- 3’ 5’- HO-CAUUCGCA-FAM- 3’
3’-CGCCCUCAUCUCCCUGUUUGAGUUCCAG-OH- 5’ 3’- p<UAAGC-FAM- 5’

Figure S4


A
– CaCl2
+ CaCl2
[RNase Iwt]µM
NE 6.7 0.67 0.134 6.7x10-2 1.34x10-2 6.7x10-3 1.34x10-3 6.7x10-4
dsRNA
(0.1 µM)
1 2 3 4 5 6 7 8 9
10 11 12 13 14 15 16 17 18
dsRNA substrate
31 nt, blunt end
B
– CaCl2
+ CaCl2
[RNase Iwt]µM
NE 0.67 0.134 6.7x10-2 1.34x10-2 6.7x10-3 1.34x10-3 6.7x10-4
dsDNA-RNA-DNA
(0.1 µM)
1 2 3 4 5 6 7 8
9 10 11 12 13 14 15 16
dsDNA-RNA-DNA chimera,
41 nt, blunt end
DNA 7nt
RNA 27nt
DNA 7nt


C
- CaCl2
+ CaCl2
relative uncleaved RNA
0.0 0.2 0.4 0.6 0.8 1.0 1.2
NE 6.7 0.67 0.134 6.7x10-2 1.34x10-2 6.7x10-3 1.34x10-3 6.7x10-4
[RNase Iwt]µM

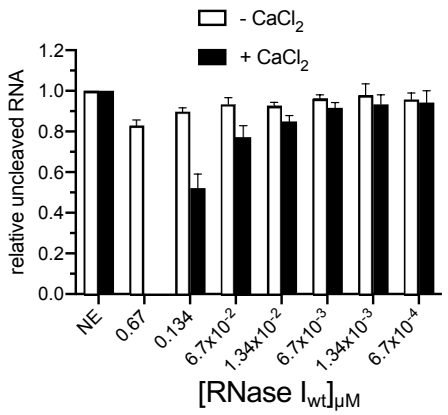

D
- CaCl2
+ CaCl2
relative uncleaved RNA
0.0 0.2 0.4 0.6 0.8 1.0 1.2
NE 0.67 0.134 6.7x10-2 1.34x10-2 6.7x10-3 1.34x10-3 6.7x10-4
[RNase Iwt]µM

Figure S5

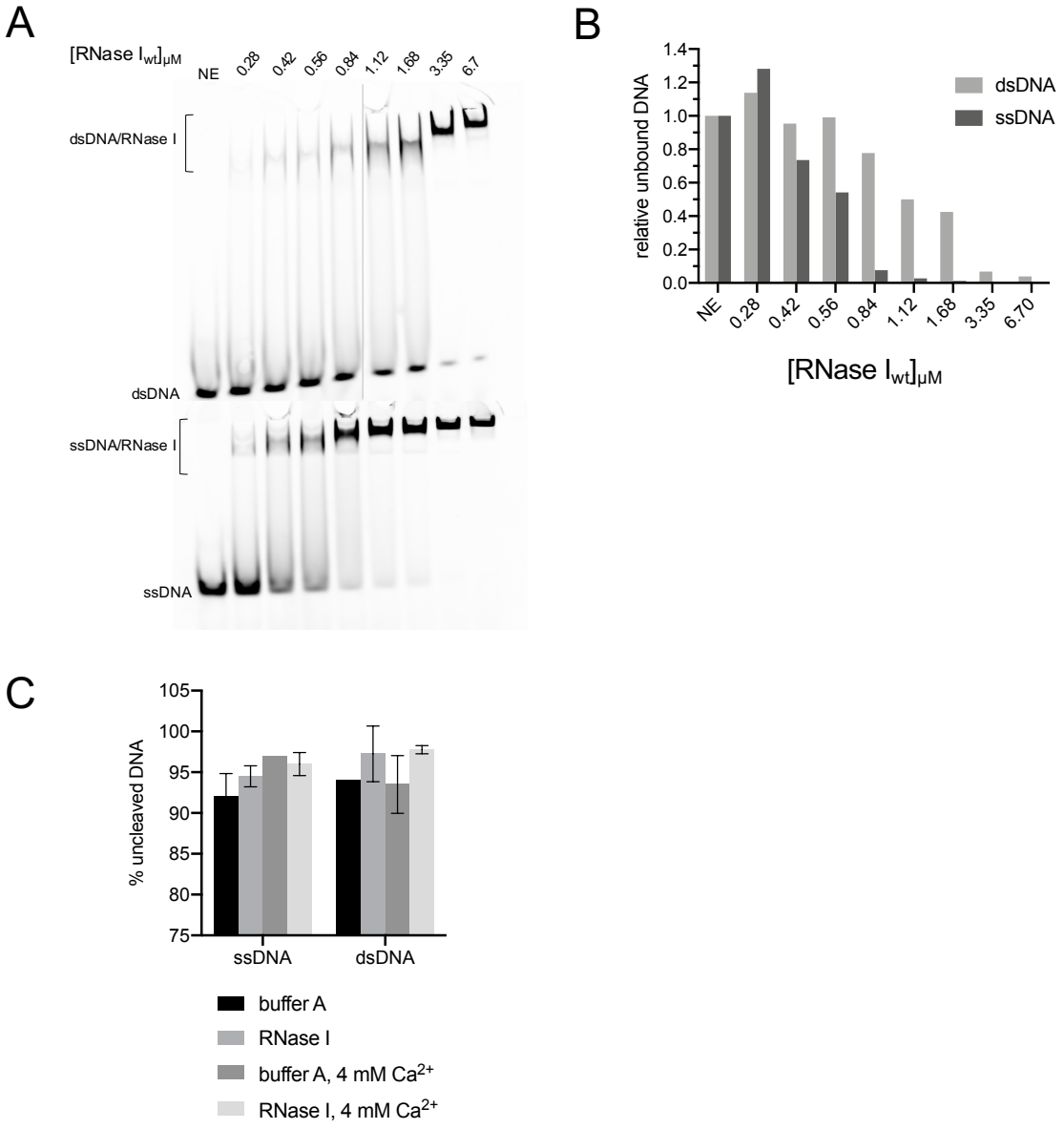

A
[RNase I$_{wt}$]$_{\mu M}$
NE
0.28
0.42
0.56
0.84
1.12
1.68
3.35
6.7
dsDNA/RNase I
dsDNA
ssDNA/RNase I
ssDNA
B
relative unbound DNA
dsDNA
ssDNA
[RNase I$_{wt}$]$_{\mu M}$
C
% uncleaved DNA
ssDNA
dsDNA
buffer A
RNase I
buffer A, 4 mM Ca^{2+}
RNase I, 4 mM Ca^{2+}

Figure S6

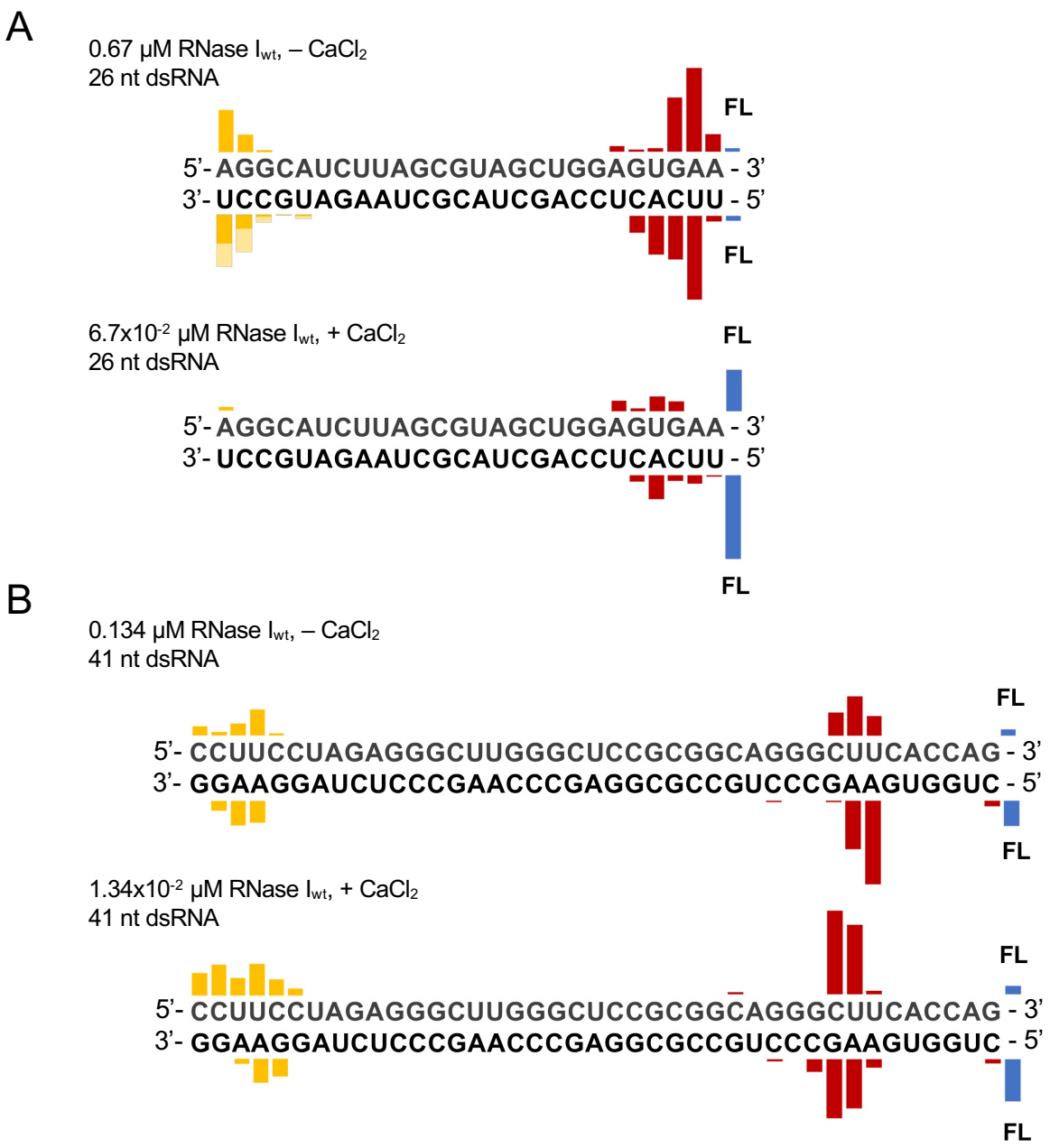

A
0.67 μM RNase I$_{wt}$, – CaCl$_2$
26 nt dsRNA
FL
5'- AGGCAUCUUAGCGUAGCUGGAGUGAA - 3'
3'- UCCGUAGAAUCGCAUCGACCUCACUU - 5'
FL
6.7x10^{-2} μM RNase I$_{wt}$, + CaCl$_2$
26 nt dsRNA
FL
5'- AGGCAUCUUAGCGUAGCUGGAGUGAA - 3'
3'- UCCGUAGAAUCGCAUCGACCUCACUU - 5'
FL
B
0.134 μM RNase I$_{wt}$, – CaCl$_2$
41 nt dsRNA
FL
5'- CCUUCCUAGAGGGCUUGGGCUCCGCGGCAGGGCUUCACCAG - 3'
3'- GGAAGGAUCUCCCGAACCCGAGGCGCCGUCCCGAAGUGGUC - 5'
FL
1.34x10^{-2} μM RNase I$_{wt}$, + CaCl$_2$
41 nt dsRNA
FL
5'- CCUUCCUAGAGGGCUUGGGCUCCGCGGCAGGGCUUCACCAG - 3'
3'- GGAAGGAUCUCCCGAACCCGAGGCGCCGUCCCGAAGUGGUC - 5'
FL

Figure S7

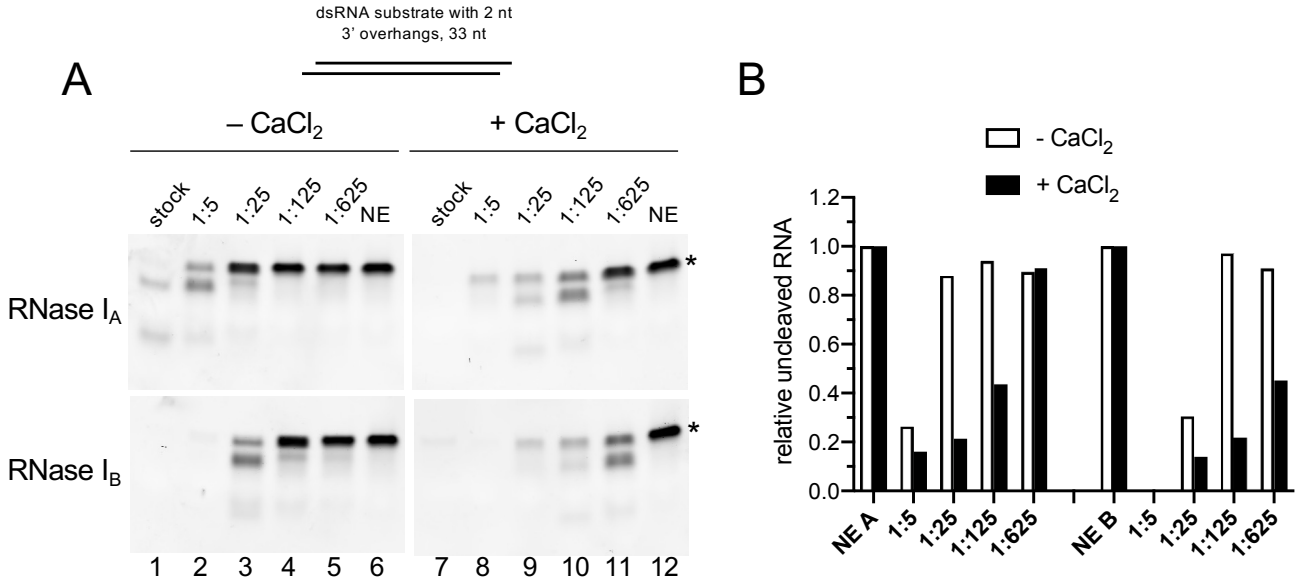
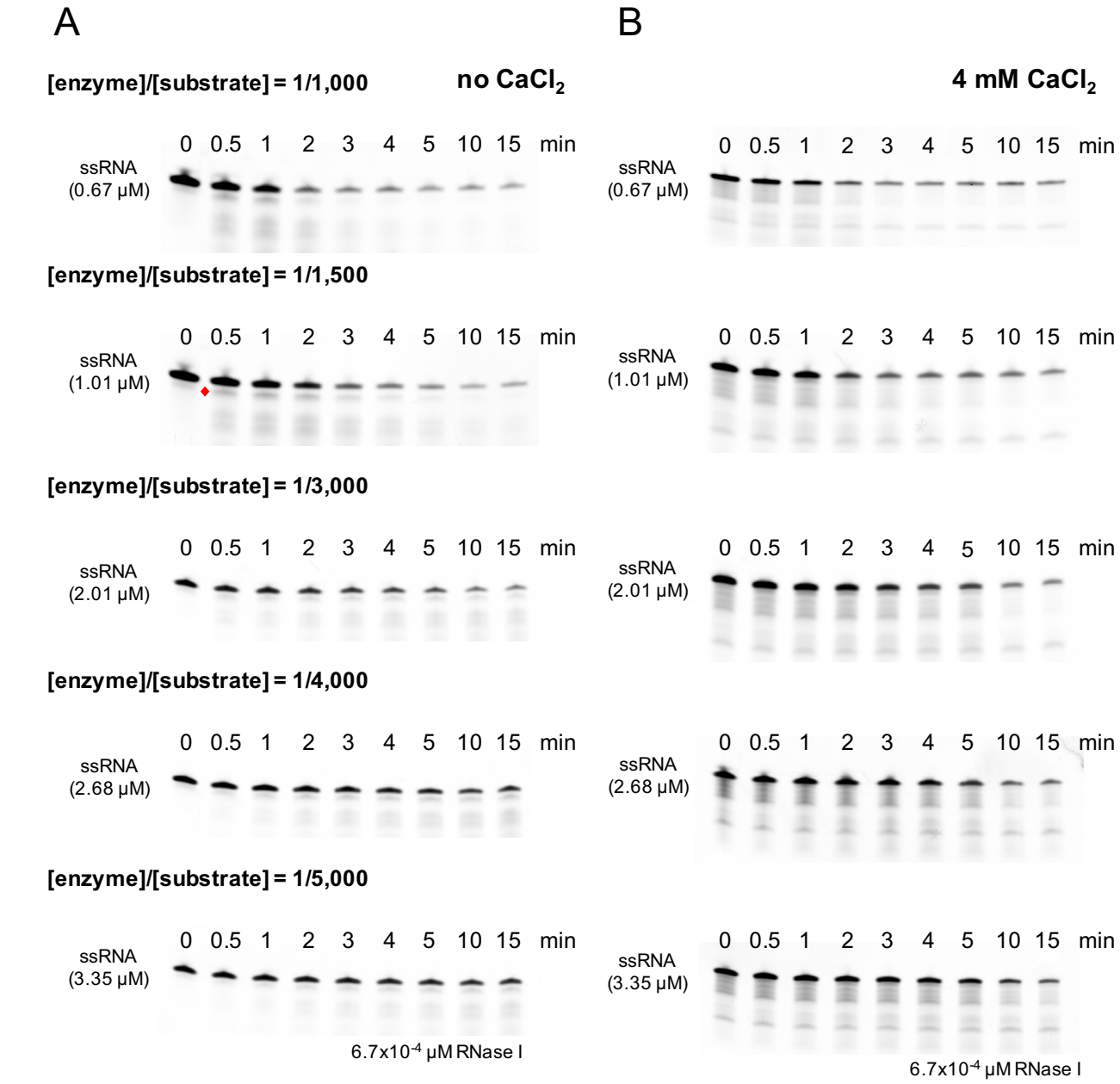

dsRNA substrate with 2 nt
3' overhangs, 33 nt
A
– CaCl2
+ CaCl2
stock
1:5
1:25
1:125
1:625
NE
RNase IA
RNase IB
*
1 2 3 4 5 6 7 8 9 10 11 12
B
- CaCl2
+ CaCl2
relative uncleaved RNA
1.2
1.0
0.8
0.6
0.4
0.2
0.0
NE A
1:5
1:25
1:125
1:625
NE B
1:5
1:25
1:125
1:625

Figure S8


A
B
[enzyme]/[substrate] = 1/1,000
no CaCl2
4 mM CaCl2
0 0.5 1 2 3 4 5 10 15 min
ssRNA (0.67 µM)
[enzyme]/[substrate] = 1/1,500
ssRNA (1.01 µM)
[enzyme]/[substrate] = 1/3,000
ssRNA (2.01 µM)
[enzyme]/[substrate] = 1/4,000
ssRNA (2.68 µM)
[enzyme]/[substrate] = 1/5,000
ssRNA (3.35 µM)
6.7x10-4 µM RNase I
6.7x10-4 µM RNase I

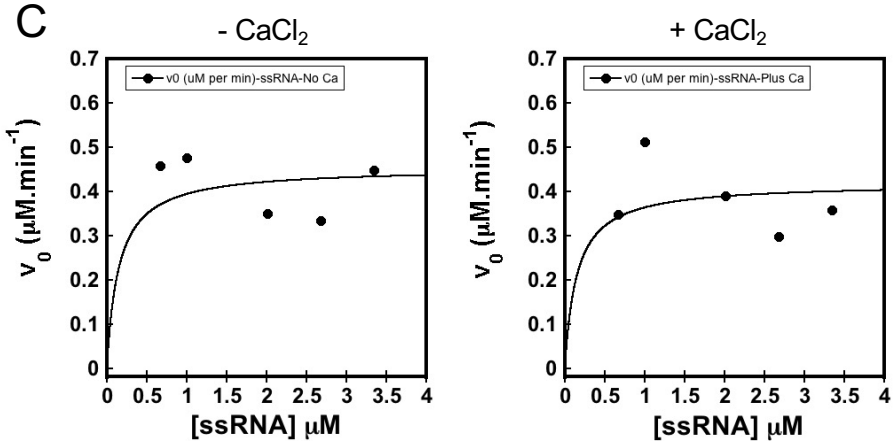
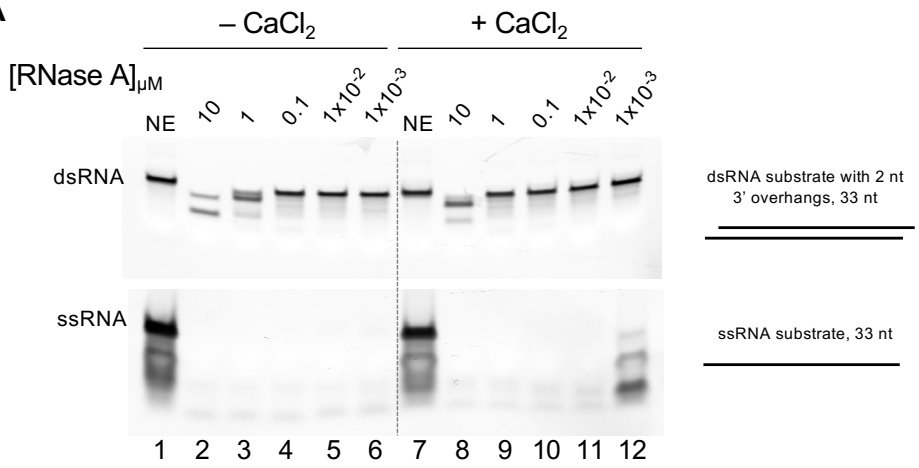
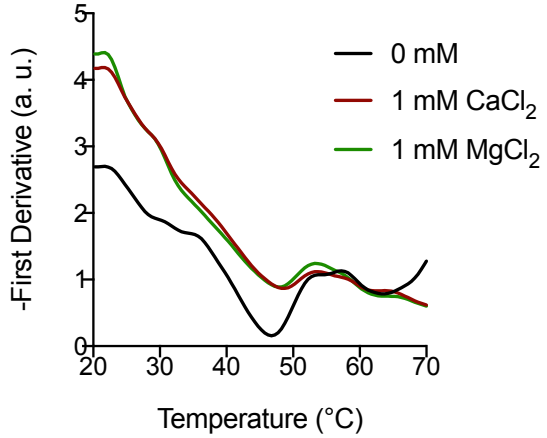

C
- CaCl2
+ CaCl2
v0 (uM per min)-ssRNA-No Ca
v0 (uM per min)-ssRNA-Plus Ca
v0 (µM.min-1)
[ssRNA] µM

Figure S9

A



B



Cation	T_m (℃)
0 mM	55.4 ± 2.2
1 mM $CaCl_2$	53.8 ± 0.4
1 mM $MgCl_2$	53.4 ± 0.9

Figure S10

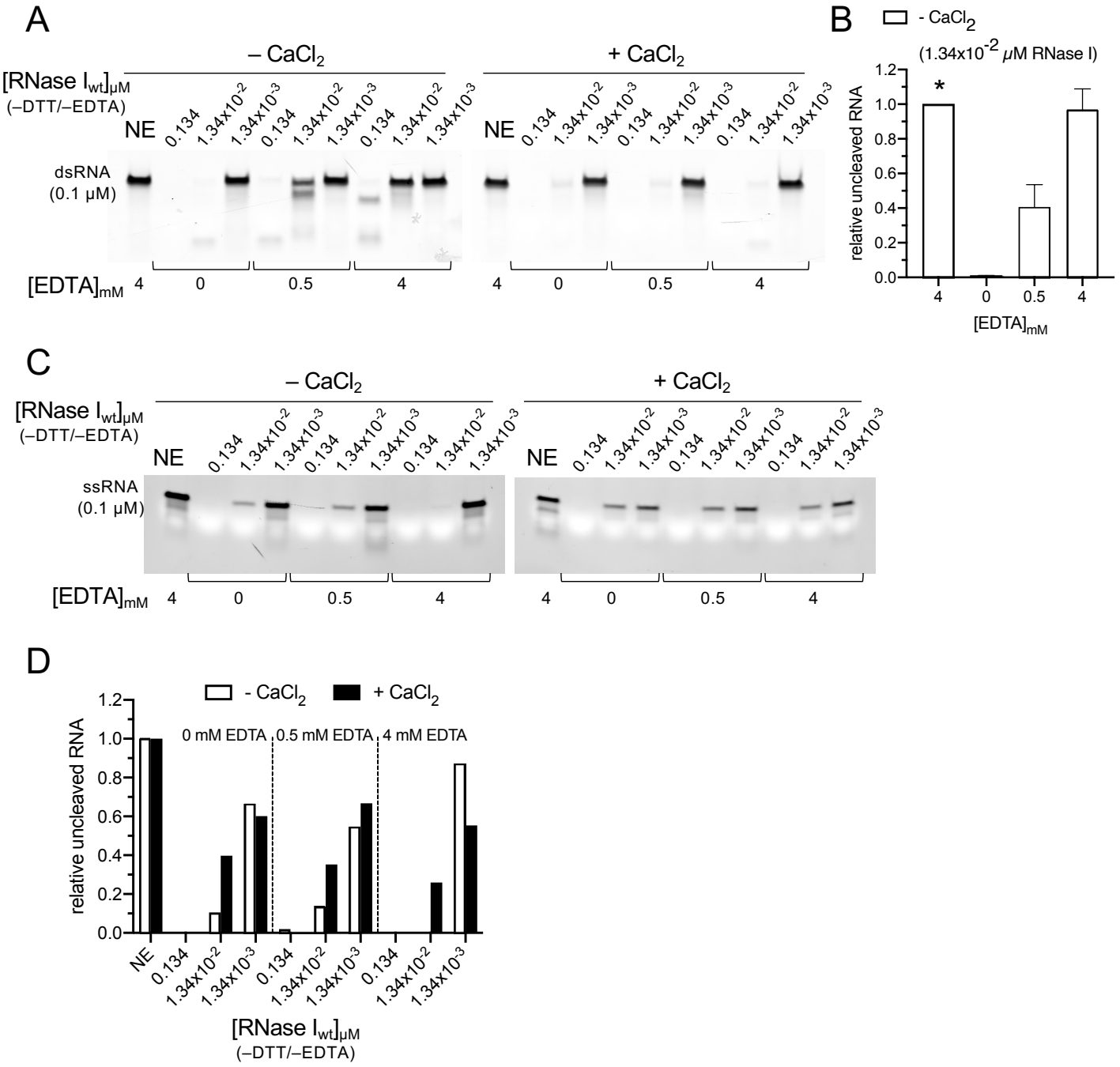

A
– CaCl2
+ CaCl2
[RNase Iwt]µM
(–DTT/–EDTA)
NE
0.134
1.34x10-2
1.34x10-3
dsRNA
(0.1 µM)
[EDTA]mM
4
0
0.5
4
B
- CaCl2
(1.34x10-2 µM RNase I)
relative uncleaved RNA
*
[EDTA]mM
C
– CaCl2
+ CaCl2
[RNase Iwt]µM
(–DTT/–EDTA)
ssRNA
(0.1 µM)
[EDTA]mM
D
- CaCl2
+ CaCl2
0 mM EDTA
0.5 mM EDTA
4 mM EDTA
relative uncleaved RNA
[RNase Iwt]µM
(–DTT/–EDTA)

Figure S11

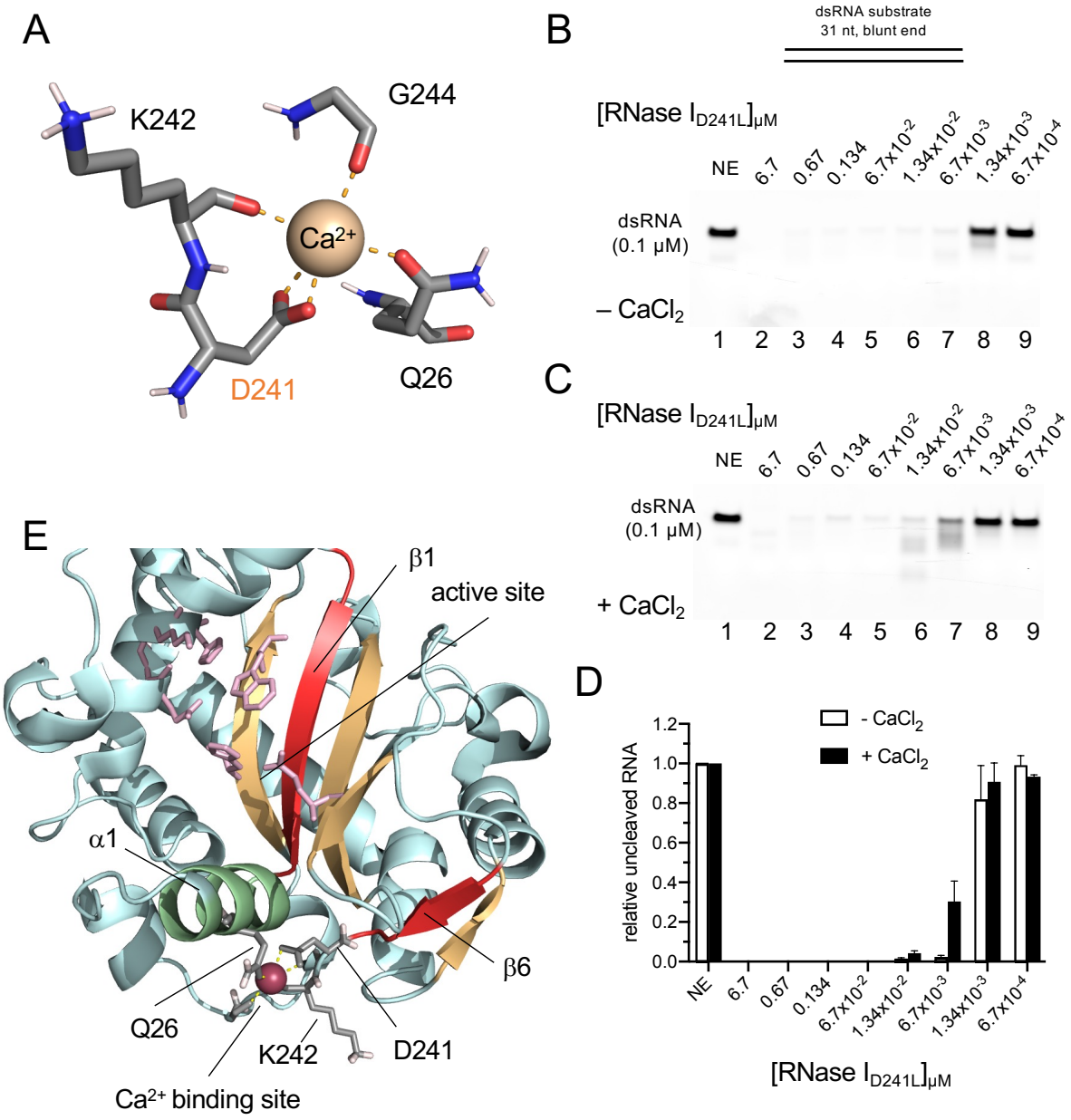
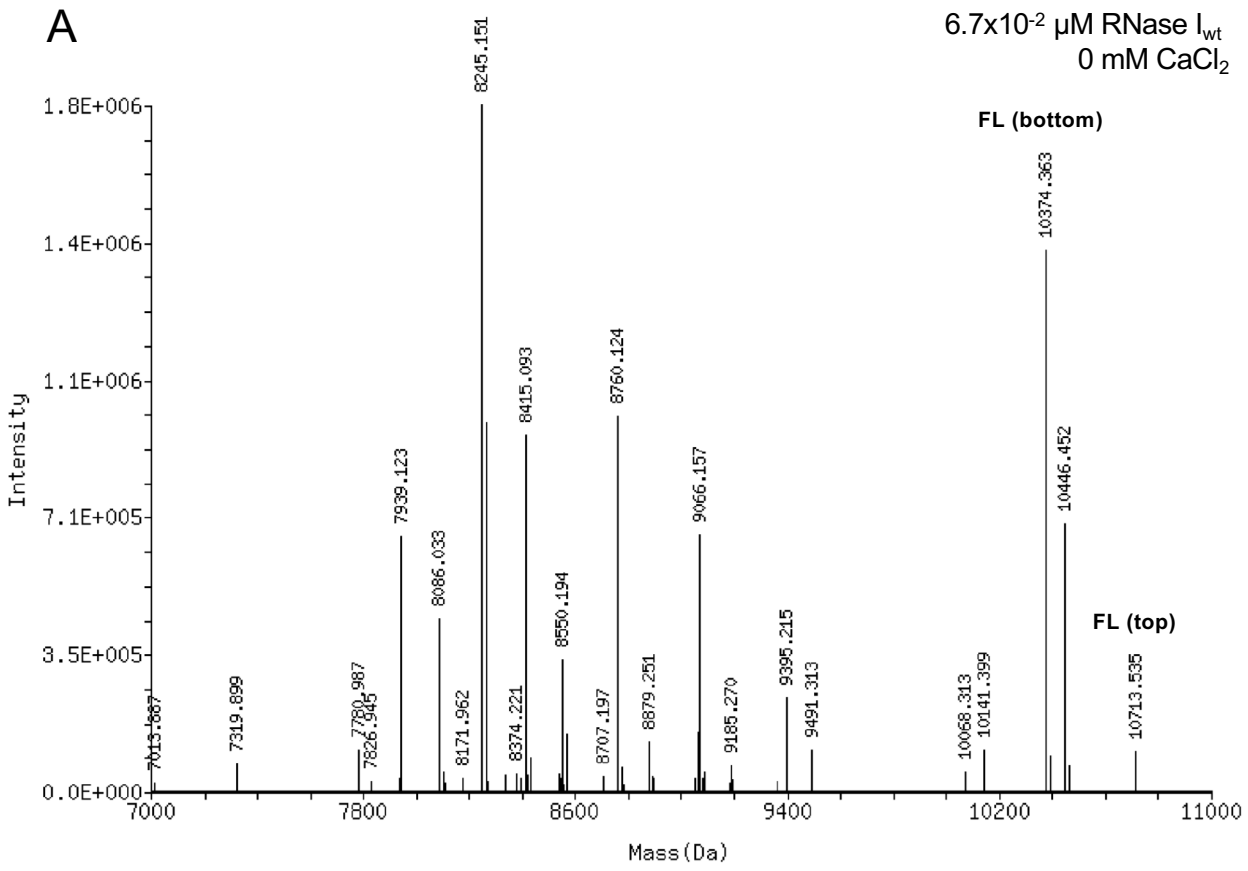

A
K242
G244
Ca2+
D241
Q26
B
dsRNA substrate
31 nt, blunt end
[RNase ID241L]μM
NE
6.7
0.67
0.134
6.7x10-2
1.34x10-2
6.7x10-3
1.34x10-3
6.7x10-4
dsRNA
(0.1 μM)
– CaCl2
1 2 3 4 5 6 7 8 9
C
[RNase ID241L]μM
NE
6.7
0.67
0.134
6.7x10-2
1.34x10-2
6.7x10-3
1.34x10-3
6.7x10-4
dsRNA
(0.1 μM)
+ CaCl2
1 2 3 4 5 6 7 8 9
D
- CaCl2
+ CaCl2
relative uncleaved RNA
1.2
1.0
0.8
0.6
0.4
0.2
0.0
NE
6.7
0.67
0.134
6.7x10-2
1.34x10-2
6.7x10-3
1.34x10-3
6.7x10-4
[RNase ID241L]μM
E
β1
active site
α1
β6
Q26
K242
D241
Ca2+ binding site

Figure S12


A
6.7x10-2 μM RNase Iwt
0 mM CaCl2
FL (bottom)
FL (top)
Intensity
Mass(Da)
1.8E+006
1.4E+006
1.1E+006
7.1E+005
3.5E+005
0.0E+000
7000
7800
8600
9400
10200
11000
8245.151
10374.363
8415.093
8760.124
7939.123
10446.452
9066.157
8086.033
8550.194
9395.215
8879.251
9491.313
10068.313
10141.399
10713.535
9185.270
8707.197
8374.221
8171.962
7780.987
7826.945
7319.899
7013.887

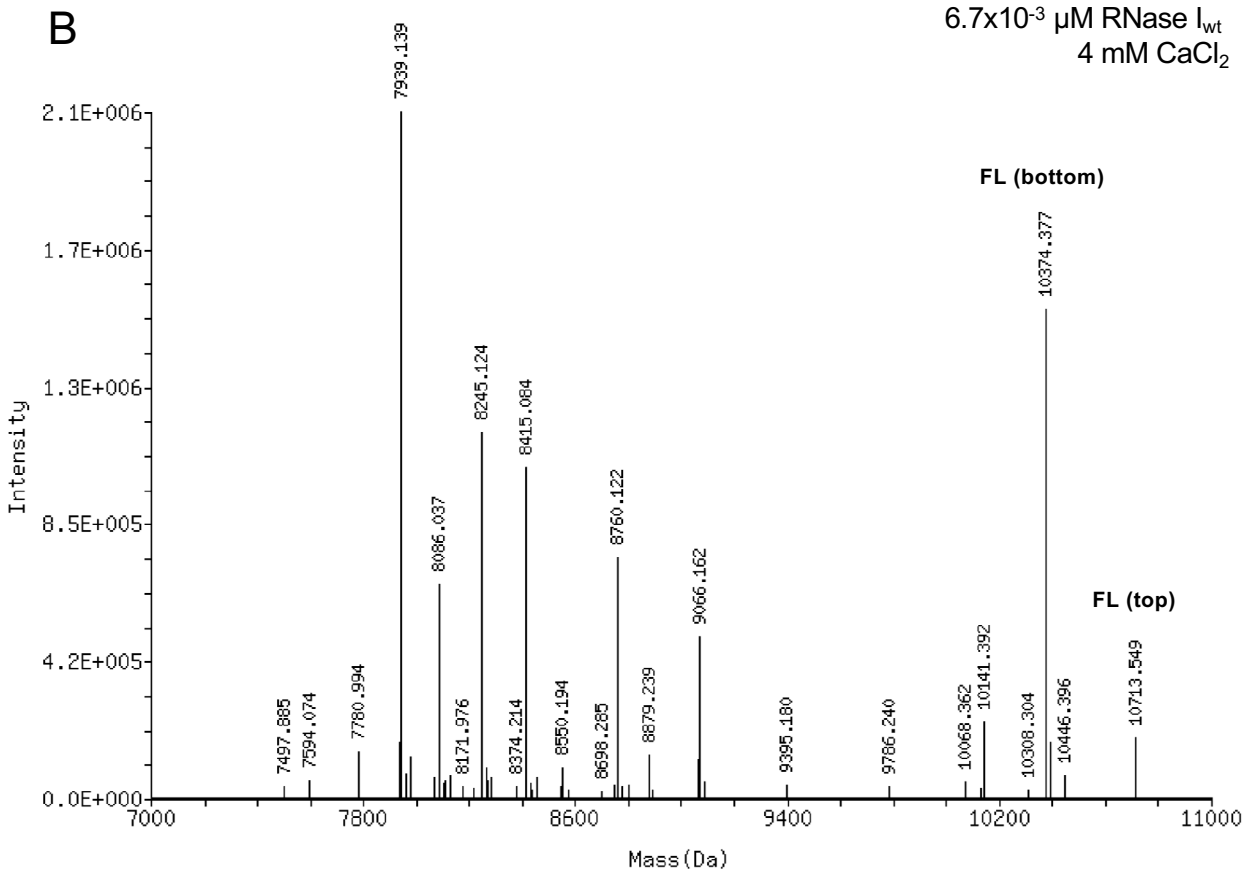

B
6.7x10-3 μM RNase Iwt
4 mM CaCl2
FL (bottom)
FL (top)
Intensity
Mass(Da)
2.1E+006
1.7E+006
1.3E+006
8.5E+005
4.2E+005
0.0E+000
7000
7800
8600
9400
10200
11000
7939.139
10374.377
8245.124
8415.084
8086.037
8760.122
9066.162
10141.392
10713.549
8550.194
8879.239
10446.396
9395.180
9786.240
10068.362
10308.304
8698.285
8374.214
8171.976
7780.994
7594.074
7497.885

Figure S13

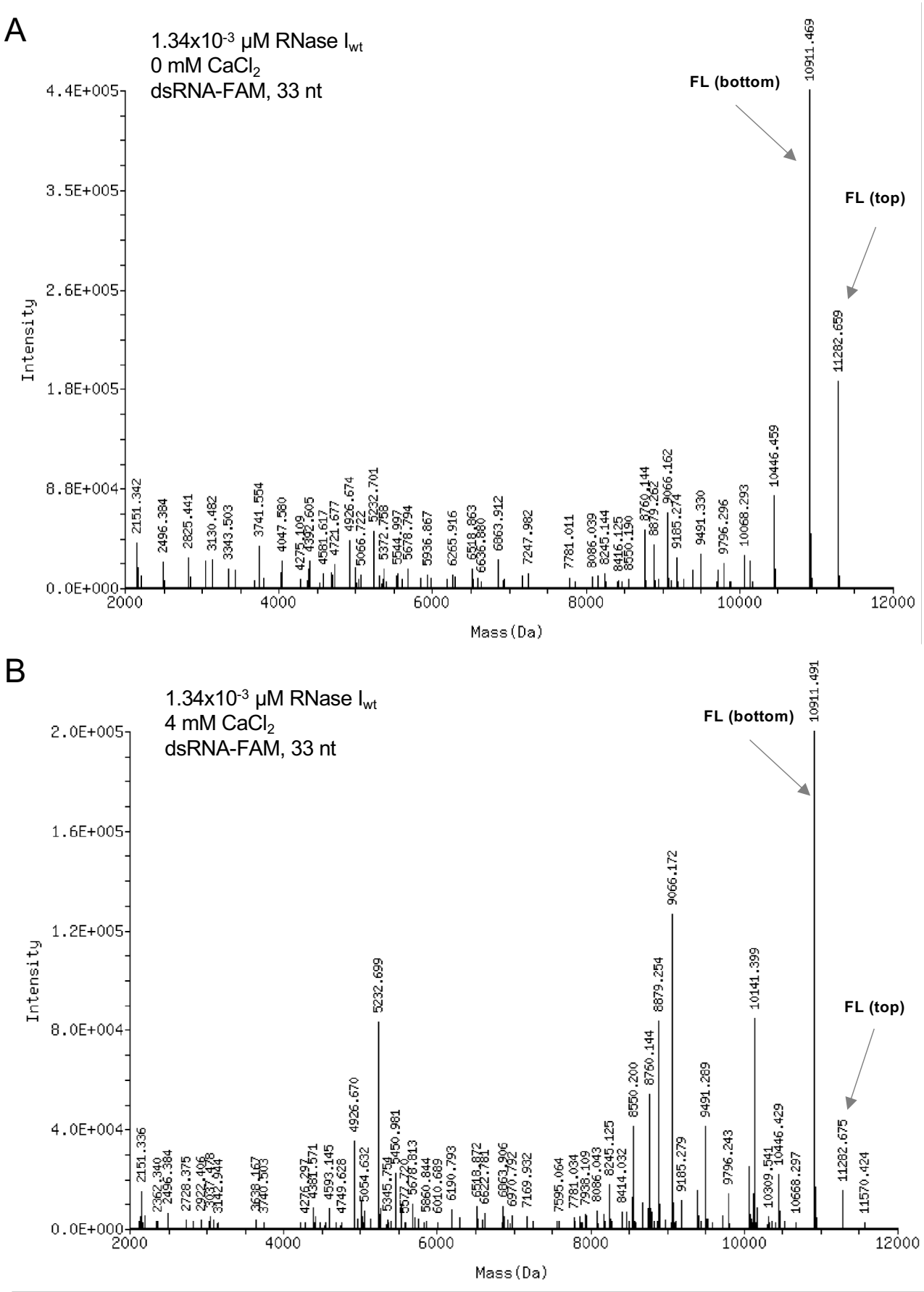
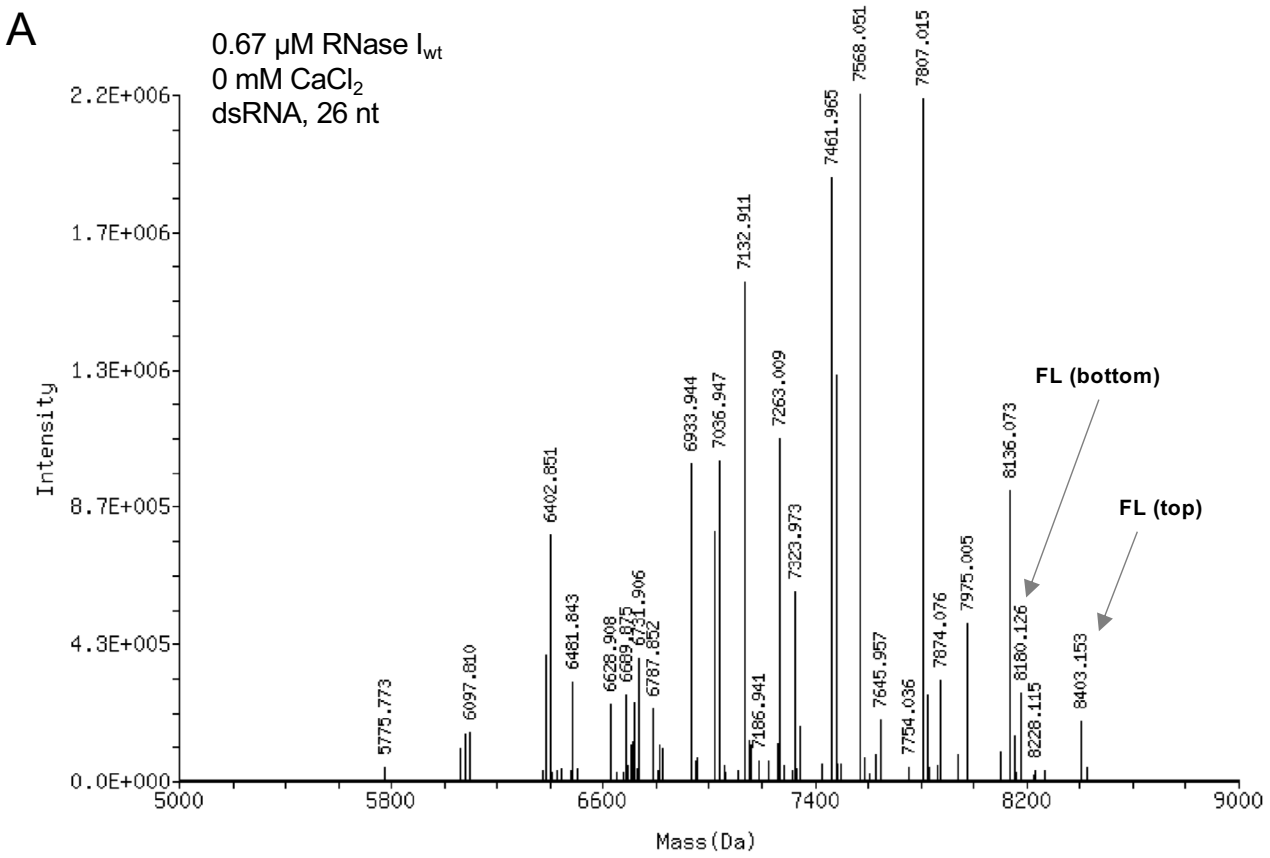

A
1.34x10-3 µM RNase Iwt
0 mM CaCl2
dsRNA-FAM, 33 nt
FL (bottom)
FL (top)
10911.469
11282.659
10446.459
Intensity
Mass(Da)
B
1.34x10-3 µM RNase Iwt
4 mM CaCl2
dsRNA-FAM, 33 nt
FL (bottom)
FL (top)
10911.491
9066.172
8879.254
10141.399
5232.699
11282.675
Intensity
Mass(Da)

Figure S14


A
0.67 μM RNase I$_{wt}$
0 mM CaCl$_2$
dsRNA, 26 nt
Intensity
Mass(Da)
FL (bottom)
FL (top)
5775.773
6097.810
6402.851
6481.843
6628.908
6689.875
6731.906
6787.852
6933.944
7036.947
7132.911
7186.941
7263.009
7323.973
7461.965
7568.051
7645.957
7754.036
7807.015
7874.076
7975.005
8136.073
8180.126
8228.115
8403.153

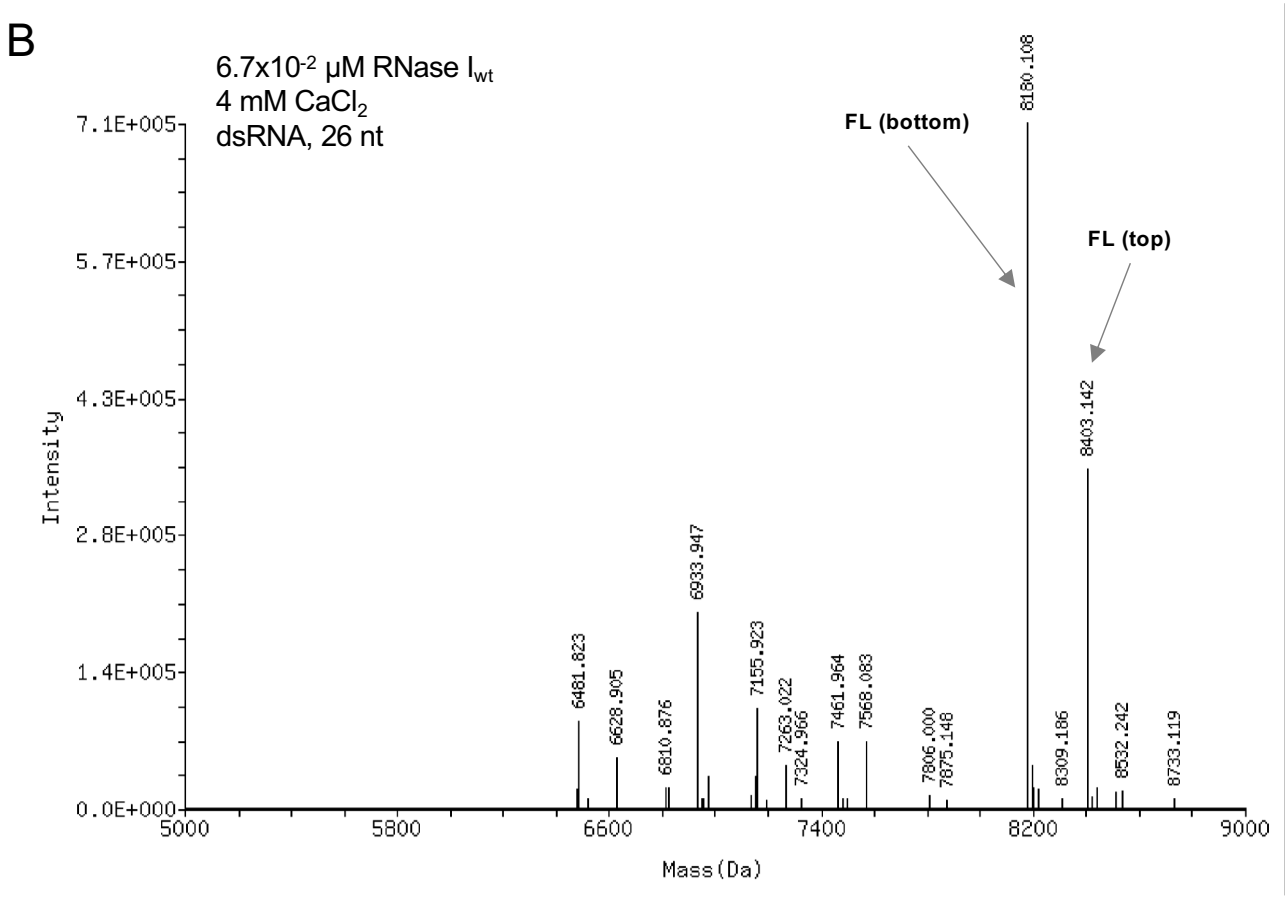

B
6.7x10^{-2} μM RNase I$_{wt}$
4 mM CaCl$_2$
dsRNA, 26 nt
Intensity
Mass(Da)
FL (bottom)
FL (top)
6481.823
6628.905
6810.876
6933.947
7155.923
7263.022
7324.966
7461.964
7568.083
7806.000
7875.148
8180.108
8309.186
8403.142
8532.242
8733.119

Figure S15

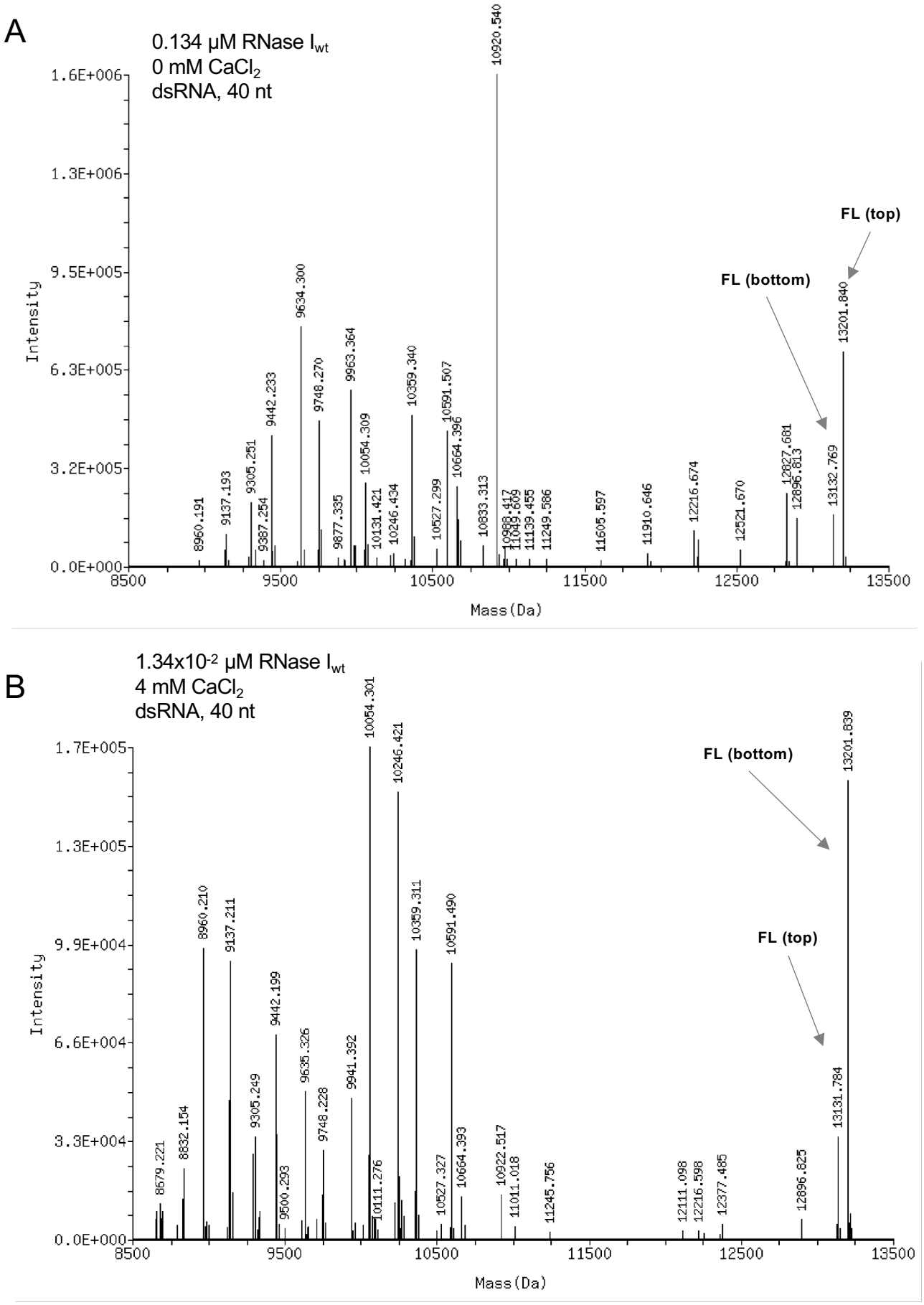
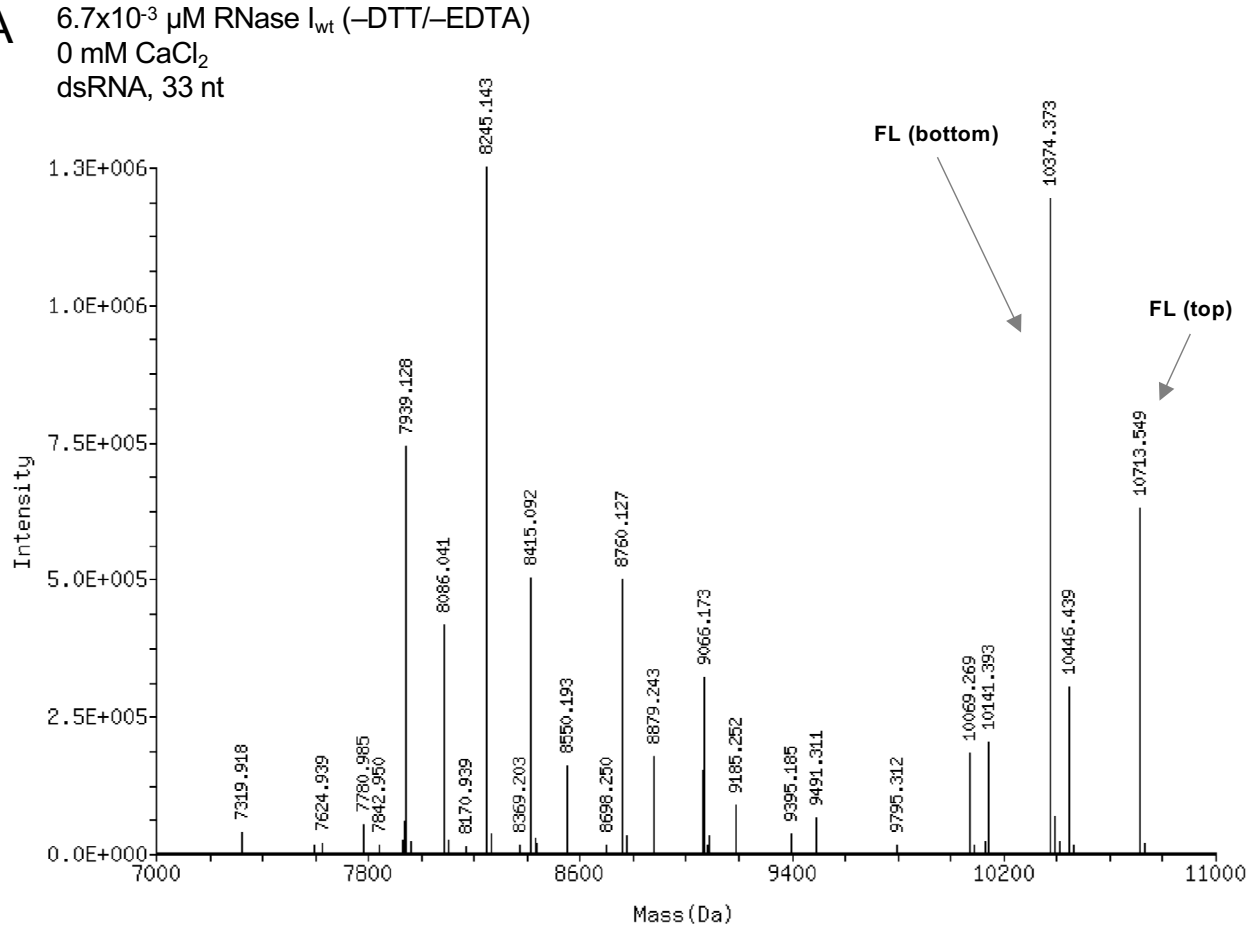

A
0.134 μM RNase I$_{wt}$
0 mM CaCl$_2$
dsRNA, 40 nt
FL (top)
FL (bottom)
Intensity
Mass(Da)
B
1.34x10^{-2} μM RNase I$_{wt}$
4 mM CaCl$_2$
dsRNA, 40 nt
FL (bottom)
FL (top)
Intensity
Mass(Da)

Figure S16

A



B

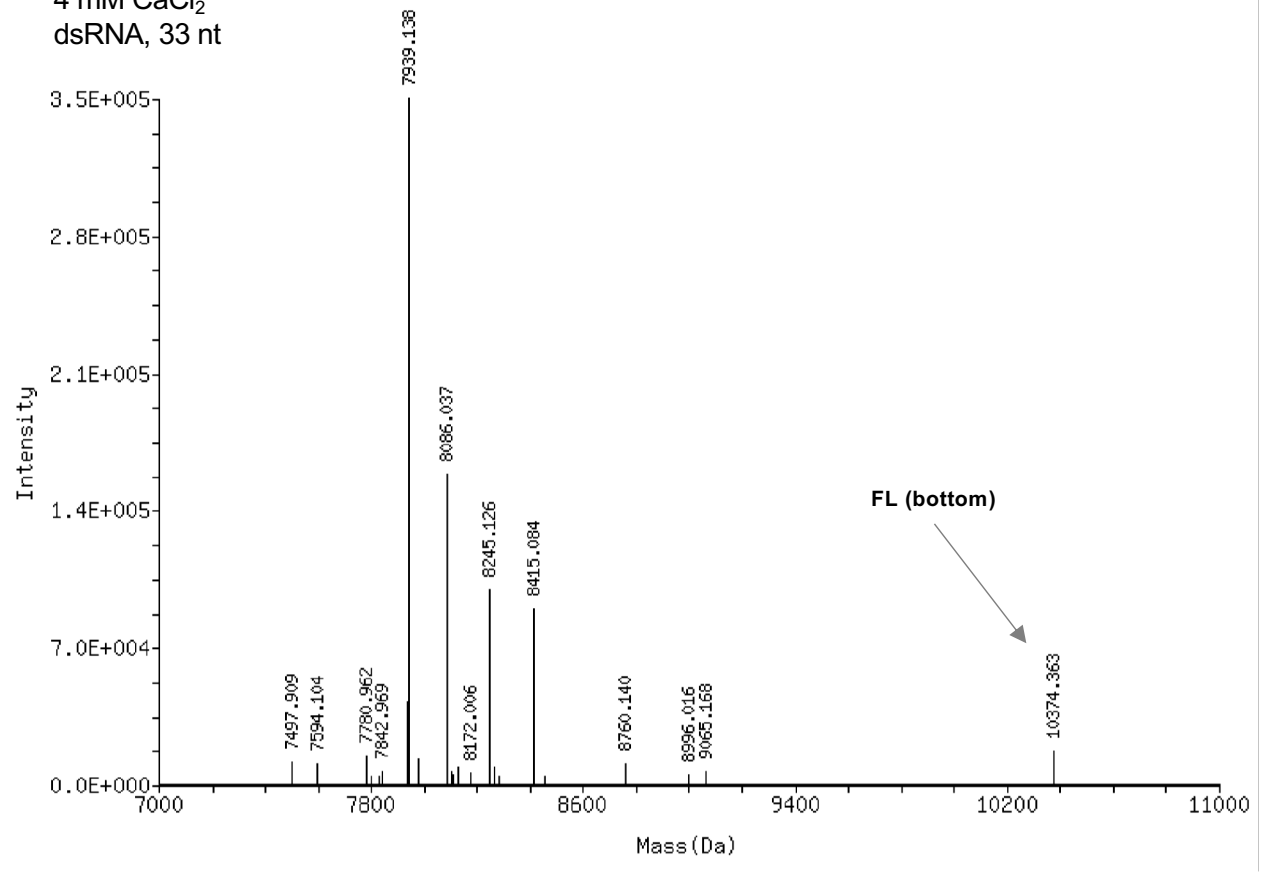


Table S1

RNA/DNA substrates	Oligo Sequence	Size (nt)	Type	5' end	3' end
R270(33t)	GGGAGUAGAGGGACAAACUCAAGGUCAUUCGCA	33	RNA	hydroxyl	hydroxyl
R271(33b)	CGAAUGACCUUGAGUUUGUCCCUCUACUCCCGC	33	RNA	hydroxyl	hydroxyl
R272(31t)	AGUGGGAGAGGGACAAACUCAAGGUCAUUCG	31	RNA	hydroxyl	hydroxyl
R273(31b)	CGAAUGACCUUGAGUUUGUCCCUCUCCCACU	31	RNA	hydroxyl	hydroxyl
R274(26t)	AGGCAUCUUAGCGUAGCUGGAGUGAA	26	RNA	hydroxyl	hydroxyl
R275(26b)	UUCACUCCAGCUACGCUAAGAUGCCU	26	RNA	hydroxyl	hydroxyl
R276(41t)	CCUUCCUAGAGGGCUUGGGCUCCGCGGCAGGGCUUCACCAG	41	RNA	hydroxyl	hydroxyl
R277(41b)	CUGGUGAAGCCCUGCCGCGGAGCCCAAGCCCUCUAGGAAGG	41	RNA	hydroxyl	hydroxyl
R279(41t)	CCTTCCTAGAGGGCUUGGGCUCCGCGGCAGGGCUTCACCAG	41	DNA-RNA-DNA	hydroxyl	hydroxyl
R280(41b)	CTGGTGAAGCCCUGCCGCGGAGCCCAAGCCCUCUAGGAAGG	41	DNA-RNA-DNA	hydroxyl	hydroxyl
R337(33t)	GGGAGUAGAGGGACAAACUCAAGGUCAUUCGCA-FAM	33	RNA	hydroxyl	FAM
R338(33b)	FAM-CGAAUGACCUUGAGUUUGUCCCUCUACUCCCGC	33	RNA	FAM	hydroxyl
D434	AGTGGGAGAGGGACAAACTCAAGGTCATTCG	31	DNA	hydroxyl	hydroxyl

R270/271: 33 nt dsRNA with 2 nt 3' overhangs
R274/275: 26 nt dsRNA with blunt ends (for MS)
R276/277: 41 nt dsRNA with blunt ends (for MS)
R272/273: 31 nt dsRNA with blunt ends
D434/R273: 31 nt double stranded DNA/RNA hybrid with blunt ends
R279/280: 41 nt double stranded DNA-RNA-DNA chimera with blunt ends
R337/338: 33 nt dsRNA with 2 nt 3' overhangs and FAM labeled 3' (top) and 5' (bottom) ends (for MS)
t: top strand
b: bottom strand

Table S2

A

$6.7x10^{-2}$ µM RNase I_{wt}, - $CaCl_2$		Calc.	Found	Error/ppm	% Rel.
RNA top strand	5'-GGGAGUAGAGGGACAAACUCAAGGUCAUUCGCA-3'	10713.518	10713.535	-1.59	4.4
Top 5'>p frags	GGGAGUAGAGGGACAAACUCAAGGUCAUUCGC>p	10446.421	10446.455	-3.24	31.1
	GGGAGUAGAGGGACAAACUCAAGGUCAUUCG>p	10141.380	10141.399	-1.88	5.4
	GGGAGUAGAGGGACAAACUCAAGGUCAUUC>p	9796.333	n.f.	n.f.	n.f.
	GGGAGUAGAGGGACAAACUCAAGGUCAUU>p	9491.291	9491.311	-2.09	5.6
	GGGAGUAGAGGGACAAACUCAAGGUCAU>p	9185.266	9185.238	3.04	3.9
	GGGAGUAGAGGGACAAACUCAAGGUCA>p	8879.241	8879.253	-1.40	7.2
	GGGAGUAGAGGGACAAACUCAAGGUC>p	8550.188	8550.194	-0.69	19.3
	GGGAGUAGAGGGACAAACUCAAGGU>p	8245.147	8245.153	-0.75	100
	GGGAGUAGAGGGACAAACUCAAGG>p	7939.122	7939.124	-0.31	36.4
RNA bottom strand	3'-CGCCCUCAUCUCCCUGUUUGAGUUCCAGUAAGC-5'	10374.340	10374.362	-2.12	77.4
Bottom 3' frags	CGCCCUCAUCUCCCUGUUUGAGUUCCAGUAAG	10069.299	10069.319	-2.02	2.0
	CGCCCUCAUCUCCCUGUUUGAGUUCCAGUAA	9724.251	n.f.	n.f.	n.f.
	CGCCCUCAUCUCCCUGUUUGAGUUCCAGUA	9395.199	9395.209	-1.09	13.2
	CGCCCUCAUCUCCCUGUUUGAGUUCCAGU	9066.146	9066.155	-0.96	36.5
	CGCCCUCAUCUCCCUGUUUGAGUUCCAG	8760.121	8760.124	-0.34	54.9
	CGCCCUCAUCUCCCUGUUUGAGUUCCA	8415.074	8415.092	-2.19	51.5
	CGCCCUCAUCUCCCUGUUUGAGUUCC	8086.021	8086.034	-1.60	25.1

B

$6.7x10^{-3}$ µM RNase I_{wt}, + $CaCl_2$		Calc.	Found	Error/ppm	% Rel.
RNA top strand	5'-GGGAGUAGAGGGACAAACUCAAGGUCAUUCGCA-3'	10713.518	10713.549	-2.89	8.8
Top 5'>p frags	GGGAGUAGAGGGACAAACUCAAGGUCAUUCGC>p	10446.421	10446.396	2.41	3.2
	GGGAGUAGAGGGACAAACUCAAGGUCAUUCG>p	10141.380	10141.392	-1.19	11.1
	GGGAGUAGAGGGACAAACUCAAGGUCAUUC>p	9796.333	n.f.	n.f.	n.f.
	GGGAGUAGAGGGACAAACUCAAGGUCAUU>p	9491.291	n.f.	n.f.	n.f.
	GGGAGUAGAGGGACAAACUCAAGGUCAU>p	9185.266	n.f.	n.f.	n.f.
	GGGAGUAGAGGGACAAACUCAAGGUCA>p	8879.241	8879.239	0.18	6.2
	GGGAGUAGAGGGACAAACUCAAGGUC>p	8550.188	8550.194	-0.69	4.4
	GGGAGUAGAGGGACAAACUCAAGGU>p	8245.147	8245.124	2.77	53.3
	GGGAGUAGAGGGACAAACUCAAGG>p	7939.122	7939.139	-2.20	100
RNA bottom strand	3'-CGCCCUCAUCUCCCUGUUUGAGUUCCAGUAAGC-5'	10374.340	10374.377	-3.57	71.3
Bottom 3' frags	CGCCCUCAUCUCCCUGUUUGAGUUCCAGUAAG	10069.299	n.f.	n.f.	n.f.
	CGCCCUCAUCUCCCUGUUUGAGUUCCAGUAA	9724.251	n.f.	n.f.	n.f.
	CGCCCUCAUCUCCCUGUUUGAGUUCCAGUA	9395.199	9395.180	2.00	1.8
	CGCCCUCAUCUCCCUGUUUGAGUUCCAGU	9066.146	9066.162	-1.73	23.5
	CGCCCUCAUCUCCCUGUUUGAGUUCCAG	8760.121	8760.122	-0.11	35.0
	CGCCCUCAUCUCCCUGUUUGAGUUCCA	8415.074	8415.084	-1.24	48.2
	CGCCCUCAUCUCCCUGUUUGAGUUCC	8086.021	8086.037	-1.97	31.1
	CGCCCUCAUCUCCCUGUUUGAGUUC	7780.980	7780.994	-1.82	6.8

Table S3

A

$6.7x10^{-3}$ µM RNase $I_{(-DTT/-EDTA)}$, - $CaCl_2$		Calc.	Found	Error/ppm	% Rel.
RNA top strand	5'-GGGAGUAGAGGGACAAACUCAAGGUCAUUCGCA-3'	10713.518	10713.549	-2.89	50.2
Top 5'>p frags	GGGAGUAGAGGGACAAACUCAAGGUCAUUCGC>p	10446.421	10446.439	-1.70	24.2
	GGGAGUAGAGGGACAAACUCAAGGUCAUUCG>p	10141.380	10141.393	-1.29	16.1
	GGGAGUAGAGGGACAAACUCAAGGUCAUUC>p	9796.333	n.f.	n.f.	n.f.
	GGGAGUAGAGGGACAAACUCAAGGUCAUU>p	9491.291	9491.311	-2.09	5.0
	GGGAGUAGAGGGACAAACUCAAGGUCAU>p	9185.266	9185.252	1.51	7.0
	GGGAGUAGAGGGACAAACUCAAGGUCA>p	8879.241	8879.243	-0.27	14.1
	GGGAGUAGAGGGACAAACUCAAGGUC>p	8550.188	8550.193	-0.57	12.6
	GGGAGUAGAGGGACAAACUCAAGGU>p	8245.147	8245.143	0.46	100
	GGGAGUAGAGGGACAAACUCAAGG>p	7939.122	7939.128	-0.82	59.3
RNA bottom strand	3'-CGCCCUCAUCUCCCUGUUUGAGUUCCAGUAAGC-5'	10374.340	10374.373	-3.18	95.5
Bottom 3' frags	CGCCCUCAUCUCCCUGUUUGAGUUCCAGUAAG	10069.299	10069.269	2.95	14.5
	CGCCCUCAUCUCCCUGUUUGAGUUCCAGUAA	9724.251	n.f.	n.f.	n.f.
	CGCCCUCAUCUCCCUGUUUGAGUUCCAGUA	9395.199	9395.185	1.47	2.8
	CGCCCUCAUCUCCCUGUUUGAGUUCCAGU	9066.146	9066.162	-1.73	25.5
	CGCCCUCAUCUCCCUGUUUGAGUUCCAG	8760.121	8760.127	-0.68	39.8
	CGCCCUCAUCUCCCUGUUUGAGUUCCA	8415.074	8415.092	-2.19	40.0
	CGCCCUCAUCUCCCUGUUUGAGUUCC	8086.021	8086.037	-1.97	33.1
	CGCCCUCAUCUCCCUGUUUGAGUUC	7780.980	7780.985	-0.67	4.1

B

$6.7x10^{-3}$ µM RNase $I_{(-DTT/-EDTA)}$, + $CaCl_2$		Calc.	Found	Error/ppm	% Rel.
RNA top strand	5'-GGGAGUAGAGGGACAAACUCAAGGUCAUUCGCA-3'	10713.518	n.f.	n.f.	n.f.
Top 5'>p frags	GGGAGUAGAGGGACAAACUCAAGGUCAUUCGC>p	10446.421	n.f.	n.f.	n.f.
	GGGAGUAGAGGGACAAACUCAAGGUCAUUCG>p	10141.380	n.f.	n.f.	n.f.
	GGGAGUAGAGGGACAAACUCAAGGUCAUUC>p	9796.333	n.f.	n.f.	n.f.
	GGGAGUAGAGGGACAAACUCAAGGUCAUU>p	9491.291	n.f.	n.f.	n.f.
	GGGAGUAGAGGGACAAACUCAAGGUCAU>p	9185.266	n.f.	n.f.	n.f.
	GGGAGUAGAGGGACAAACUCAAGGUCA>p	8879.241	n.f.	n.f.	n.f.
	GGGAGUAGAGGGACAAACUCAAGGUC>p	8550.188	n.f.	n.f.	n.f.
	GGGAGUAGAGGGACAAACUCAAGGU>p	8245.147	8245.126	2.52	28.3
	GGGAGUAGAGGGACAAACUCAAGG>p	7939.122	7939.138	-2.08	100
RNA bottom strand	3'-CGCCCUCAUCUCCCUGUUUGAGUUCCAGUAAGC-5'	10374.340	10374.373	-3.18	4.8
Bottom 3' frags	CGCCCUCAUCUCCCUGUUUGAGUUCCAGUAAG	10069.299	n.f.	n.f.	n.f.
	CGCCCUCAUCUCCCUGUUUGAGUUCCAGUAA	9724.251	n.f.	n.f.	n.f.
	CGCCCUCAUCUCCCUGUUUGAGUUCCAGUA	9395.199	n.f.	n.f.	n.f.
	CGCCCUCAUCUCCCUGUUUGAGUUCCAGU	9066.146	n.f.	n.f.	n.f.
	CGCCCUCAUCUCCCUGUUUGAGUUCCAG	8760.121	8760.140	-2.17	2.9
	CGCCCUCAUCUCCCUGUUUGAGUUCCA	8415.074	8415.084	-1.24	25.6
	CGCCCUCAUCUCCCUGUUUGAGUUCC	8086.021	8086.037	-1.97	45.1
	CGCCCUCAUCUCCCUGUUUGAGUUC	7780.980	7780.962	2.29	4.2

Table S4

Substrate	Ca^{2+}	v_{max} (mM • min^{-1})	[RNase I] mM	k_{cat} (min^{-1})
dsRNA	+	0.36	$1.34x10^{-3}$	$2.68\ x10^{2}$
dsRNA	-	0.02	$6.70x10^{-3}$	3.0
ssRNA	+	0.42	$6.70x10^{-4}$	$6.27\ x10^{2}$
ssRNA	-	0.45	$6.70x10^{-4}$	$6.71\ x10^{2}$