Supporting Information

An Expanded Substrate Scope for Cross-Chiral Ligation Enables Efficient Synthesis of Long L-RNAs

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S1. Supplementary Text.

MATERIALS AND METHODS

General

All DNA oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). All RNA oligonucleotides were prepared by solid-phase oligonucleotide synthesis using an Expedite 8909 DNA Synthesizer, except for ppp-L-RNA_8-CG, which was purchased from ChemGenes (Wilmington, MA). D-RNA phosphoramidites, solid supports, and other oligonucleotide synthesis reagents were purchased from Glen Research (Sterling, VA). L-Nucleoside phosphoramidites and solid supports were purchased from ChemGenes (Wilmington, MA). Superscript II reverse transcriptase and RNAse I was purchased from ThermoFisher (Waltham, MA). Recombinant T7-RNA polymerase was expressed in house as previously described.[1] RNase A was purchased from New England Biolabs (Ipswich, MA). Sulfo-Cy3 N-Hydroxysuccinimide (NHS) ester and Sulfo-Cy5 NHS esters were purchased from Lumiprobe (Cockeysville, MD). All other reagents were purchased from Sigma Aldrich (St. Louis, MO).

Oligonucleotide synthesis and purification

All oligonucleotides used in this study are shown in Table S1. All L-RNAs were synthesized in house on 8909 DNA synthesizer using manufacturer recommended protocols. 5′-Fluorescein labeling of acceptor strands was accomplished using the 6-FAM phosphoramidite (Glen Research, Sterling, VA). The 3′-biotin modification was installed using the 3′-biotinTEG CPG (Glen Research, Sterling, VA). Sulfo-Cy3/5 NHS esters were conjugated to the 3′ end of the indicated L-RNAs via a 3′ amino modification installed at the time of synthesis. The conjugation reaction has been described in our previous work.[2] Prior to use, all synthetic oligonucleotides were purified by 20% denaturing polyacrylamide gel electrophoresis (PAGE; 19:1, acrylamide/bisacrylamide). Pure oligonucleotides were excised from the gel and eluted overnight at room temperature in a buffer consisting of 10 mM Tris (pH 7.6), 200 mM NaCl, and 10 mM EDTA. The filtered supernatants were concentrated using either a 3 kDa pore size Amicon Ultra Centrifugal Filter device (MilliporeSigma, Burlington, MA) or a SepPak C18 cartridge (Waters, Milford, MA) following the manufacturer's recommended procedures. All purified oligonucleotides were desalted by ethanol precipitation prior to use. Oligonucleotide concentrations were determined by absorbance at 260 nm on a NanoDrop 2000c (ThermoFisher, Waltham, MA) and the identity of all oligonucleotides was confirmed using a Thermo Scientific Q Exactive Focus ESI mass spectrometer. Deconvoluted

mass spectrometry data was generated using UniDec 7.0.2.[3] Oligonucleotide purity was determined by ESI-MS using relative peak intensities.

In vitro transcription of RNA

DNA templates used to prepare cross-chiral ribozymes D-16.12t, D-27.3t, and D-27.6t were generated by a cross-extension of two overlapping synthetic oligonucleotides (Table S2). The pair of overlapping oligonucleotides (200 pmol each) was added to 60 µL water and the solution was heated at 70 °C for 2 minutes followed by slow cooling to room temperature. The cooled solution was supplemented with reagents to achieve a final volume of 100 µL containing 3 mM $MgCl_2$, 75 mM KCl, 10 mM dithiothreitol (DTT), 50 mM Tris (pH 8.3), and 0.5 mM each of the four dNTPs. 10 U/µL Superscript II reverse transcriptase was added to initiate the extension reaction, which was allowed to proceed at 42 °C for 45 minutes. The DNA was then desalted via ethanol precipitation and the resulting pellet was directly resuspended in a transcription solution (1 mL) containing 25 mM $MgCl_2$, 2 mM spermidine, 10 mM DTT, 40 mM Tris (pH 7.9), and 5 mM of each of the four NTPs. The transcription reaction was initiated by the addition of 10 U/µL T7 RNA polymerase and 0.001 U/µL Inorganic Pyrophosphatase (IPP) and the mixture was incubated at 37 °C for 2 hours. At that point, 10 U TURBO DNase was added and the reaction mixture was incubated for an additional 30 minutes at 37 °C. The reaction mixture was then ethanol precipitated and the RNA was purified by denaturing PAGE (10%, 19:1 acrylamide/bisacrylamide) as described above.

Synthesis of phosphoimidazolides

Adenosine 5′-phosphoimidazolide (ImpA), guanosine 5′-phosphoimidazolide (ImpG), uridine 5′-phosphoimidazolide (ImpU), cytidine 5′-phosphoimidazolide (ImpC), and adenosine 5′-diphosphate (ADP)-imidazolide (ImppA) were prepared in the same manner using a procedure adapted from Hafner et al.[4] A solution containing 2,2’-dipyridyldisulfide (220 mg, 1 mmol) in 3 mL of dry DMF was added dropwise into a stirring solution (12 mL) of triphenylphosphine (262 mg, 1 mmol), imidazole (170 mg, 2.5 mmol), and triethylamine (0.9 mL, 2.5 mmol) in dry DMF under argon. A suspension of 0.5 mmol nucleotide 5′-mono- or 5′-diphosphate in 15 mL dry DMF was then added dropwise to the same stirring solution above. The reaction was stirred for an additional 5 hours under argon. The completed reaction was precipitated via the dropwise addition to a stirring solution of sodium perchlorate (1.1g, 9 mmol) in 110 mL of acetone and 55 mL of anhydrous diethyl ether. The precipitant was collected via centrifugation at 5000 rpm for 5 minutes then washed with acetone followed by diethyl ether. The crude phosphoimidazolide products were

dried under vacuum and characterized by mass spectrometry. These reactions typically resulted in the conversion of >80% of the nucleotide into the corresponding phosphoimidazolide (Figure S16), and the crude product was used without further purification.

General procedure of the synthesis of 5′-N-ylated RNAs

5′-monophosphorylated RNAs (5 nmol) were dissolved in 50 µL of an aqueous solution containing 50 mM $MgCl_2$ and 100 mM crude phosphoimidazolide product (based on theoretical yield). The reaction was incubated at 52 °C for 5 hours with an additional 25 µL of 100 mM phosphoimidazolide added at the 3 hour mark. To monitor the progress of the reaction, an aliquot was taken and resolved by denaturing PAGE (20%, 19:1 acrylamide:bis-acrylamide) and visualized by staining with 1 x SYBR Gold DNA stain (ThermoFisher, Waltham, MA). Completed reactions were then desalted using Amicon Ultra Centrifugal Filter MWCO 3k (Millipore-Sigma, Burlington, MA) and the oligonucleotide products were purified by denaturing PAGE as described above.

Analysis of cross-chiral ligation activity

The components for cross-chiral ligation were assembled in a reaction mixture containing 2 µM of the L-RNA acceptor (L-$Acceptor_{hp}$ for the ligation complex depicted in Figure 1a), 4 µM of L-RNA donor, 20 µM of the D-RNA ribozyme (16.12t, 27.3t, or 27.6t), 250 mM NaCl, and 50 mM Tris (pH 8.5). The mixture was heated at 70 °C for 2 minutes and cooled down to room temperature slowly. The reaction was initiated by the addition of an equal volume of a solution containing 500 mM $MgCl_2$, 250 mM NaCl, and 50 mM Tris (pH 8.5), and the reaction was incubated at 23 °C. Aliquots (2 µL) were taken at the indicated times and quenched with a solution (18 µL) containing 90% v/v formamide and 10 mM EDTA. The reaction products were then analyzed by denaturing PAGE (20%, 19:1 acrylamide/bisacrylamide). The gel was imaged by fluorescence emission (Cy5 excitation/emission: 635 nm/≥665 nm; Cy3 excitation/emission: 532 nm/≥575 nm; FAM excitation/emission: 473 nm/≥510 nm) using Typhoon FLA-9500 Multimode Molecular Imager and quantified using ImageQuant TL software (version 8.2) (General Electric Co., Boston, MA).

Michaelis–Menten parameters were determined using a similar reaction conditions as above, except that the concentration of the L-RNA ligation complex (L-$Acceptor_{hp}$ and indicated donor) was lowered to 0.05 µM and the concentrations of the D-RNA ribozyme was varied between 0 and 50 µM. Values for k_{obs} were obtained for each enzyme concentration based on the initial rates, then fit to the Michaelis-Menten equation: $k_{obs} = k_{cat}[E] / (K_m + [E])$.[5]

Cross-Chiral synthesis of L-27.6t ribozyme

A 500 µL reaction mixture containing 5 µM L-27.6t$_A$, 5 µM Appp-L-27.6t$_D$, 5 µM L-27.6$_S$, 10 µM of D-RNA ligase ribozyme, 250 mM NaCl, and 50 mM Tris (pH 8.5) was heated to 70 °C for 2 minutes and cooled slowly to room temperature. The reaction was initiated by the addition of an equal volume (500 µL) of a solution containing 500 mM $MgCl_2$, 250 mM NaCl, and 50 mM Tris (pH 8.5) and was incubated at 23 °C for 5 hours. The reaction mixture was concentrated using a 3 kDa pore size Amicon Ultra Centrifugal Filter device and desalted by ethanol precipitation. The obtained pellet was resuspended in 50 µL of phosphate buffered saline PBS (137 mM NaCl, 10 mM phosphate, 2.7 mM KCl; pH 7.4) to which 50 U of RNase I was added to degrade the remaining D-RNA ribozyme. The mixture was incubated at 37 °C for 30 minutes, concentrated by ethanol precipitation and purified by denaturing PAGE (20%, 19:1 acrylamide/bisacrylamide) as described above.

Determination of cross-chiral ligation regiospecificity

The L-27.6t ribozyme was used to ligate a D-RNA substrate complex consisting of 20 µM D-RNA$_A$, 10 µM Appp-D-RNA$_D$, 20 µM D-RNA$_S$ (Table S1). The ligation reaction was performed as described above. The ligated product was purified by PAGE, desalted by ethanol precipitation, and dissolved in water. RNase A digestion was carried out in a reaction mixture containing 100 nM of the purified ligation product, 50 mM Tris (pH 7.6), and the indicated amounts of RNase A, which was incubated for 5 minutes at 23 °C. The reaction was quenched via the addition of 10 µg/µL tRNA and was analyzed immediately by denaturing PAGE (20%, 19:1 acrylamide/bisacrylamide) as described above. An authentic all-3′,5′-linked RNA (D-RNA$_{16}$) having an identical sequence to the ligated RNA product was treated with RNase A in a similar manner and used for comparison (Figure S10).[5]

S2. Supplementary Figures.

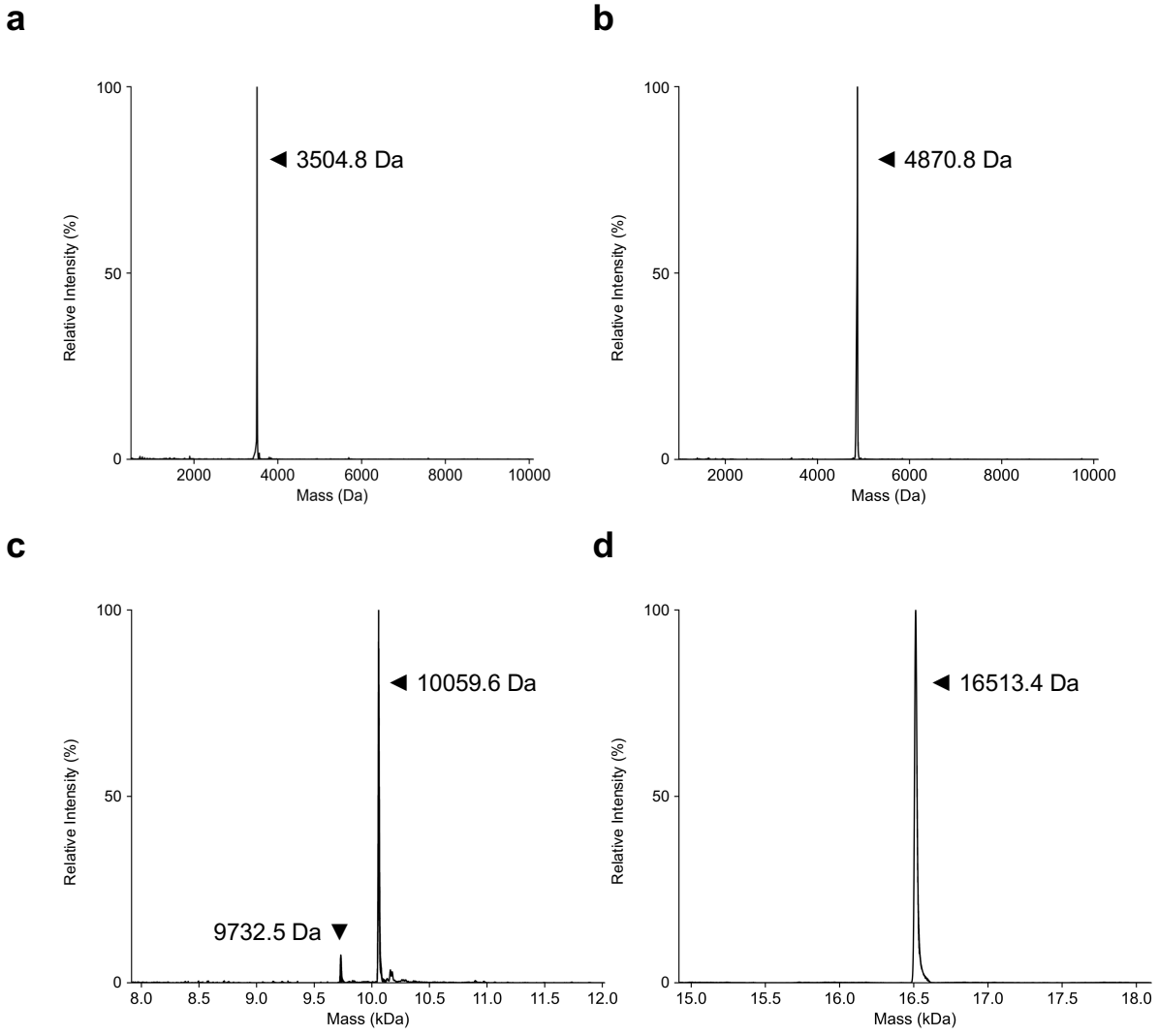

Figure S1. ESI-MS characterization of 5′-adenylated L-RNA donors. (a) ESI-MS data for App-L-RNA_8. Calculated: 3504.3 Da, observed: 3504.8 Da. (b) ESI-MS data for App-L-RNA_{14}. Calculated: 4870.8 Da, observed: 4870.8 Da. (c) ESI-MS data for App-L-RNA_{30}. Calculated: 10060.1 Da, observed: 10059.6 Da. (d) ESI-MS data for App-L-RNA_{50}. Calculated: 16513.8 Da, observed: 16513.4 Da.

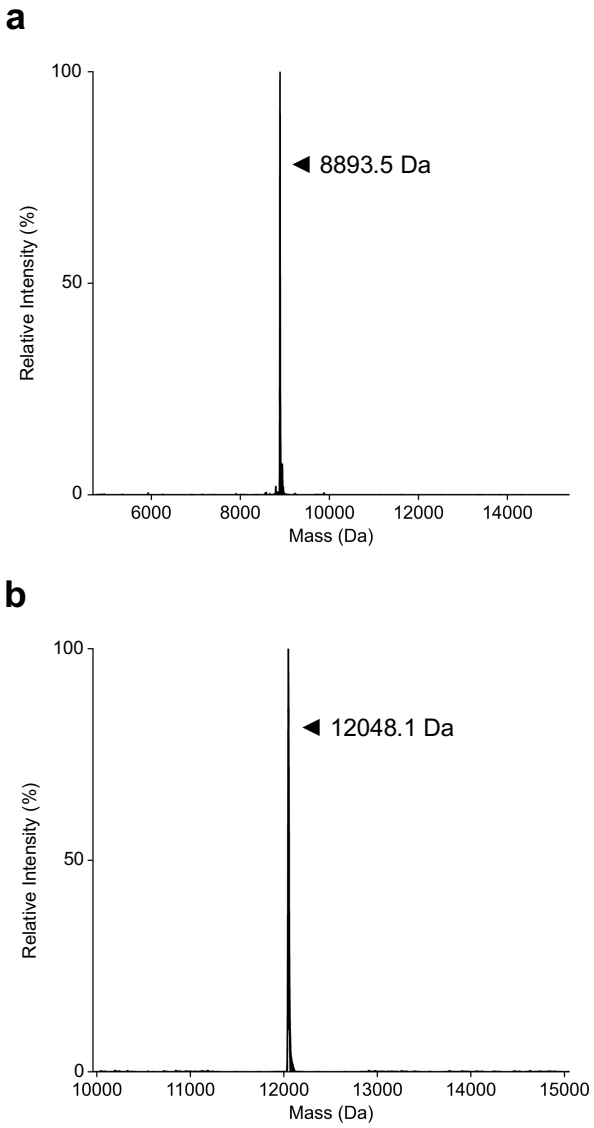

Figure S2. Cross-chiral ligation of L-Acceptor$_{hp}$ and App-L-RNA$_8$. (a) ESI-MS data for L-Acceptor$_{hp}$. Calculated: 8891.6 Da, Observed: 8893.5 Da. (b) ESI-MS data for ligated product of L-Acceptor$_{hp}$ and App-L-RNA$_8$. Calculated: 12049.4 Da, Observed: 12048.1.

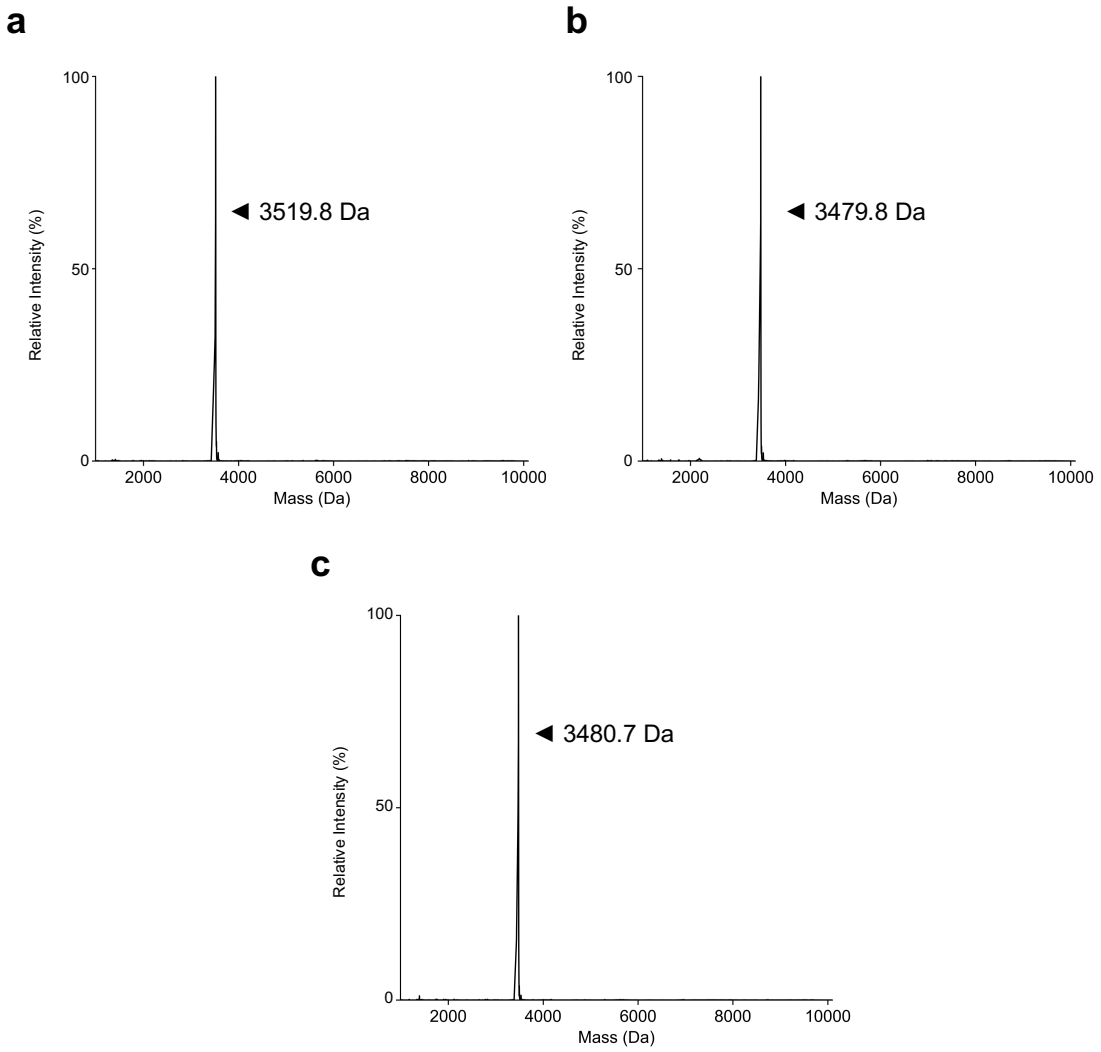

Figure S3. ESI-MS characterization of 5′-N-ylated L-RNA donors. (a) ESI-MS data for Gpp-L-RNA_8. Calculated: 3520.3 Da, observed: 3519.8 Da. (b) ESI-MS data for Cpp-L-RNA_8. Calculated: 3480.3 Da, observed: 3479.8 Da. (c) ESI-MS data for Upp-L-RNA_8. Calculated: 3481.3 Da, observed: 3480.7 Da.

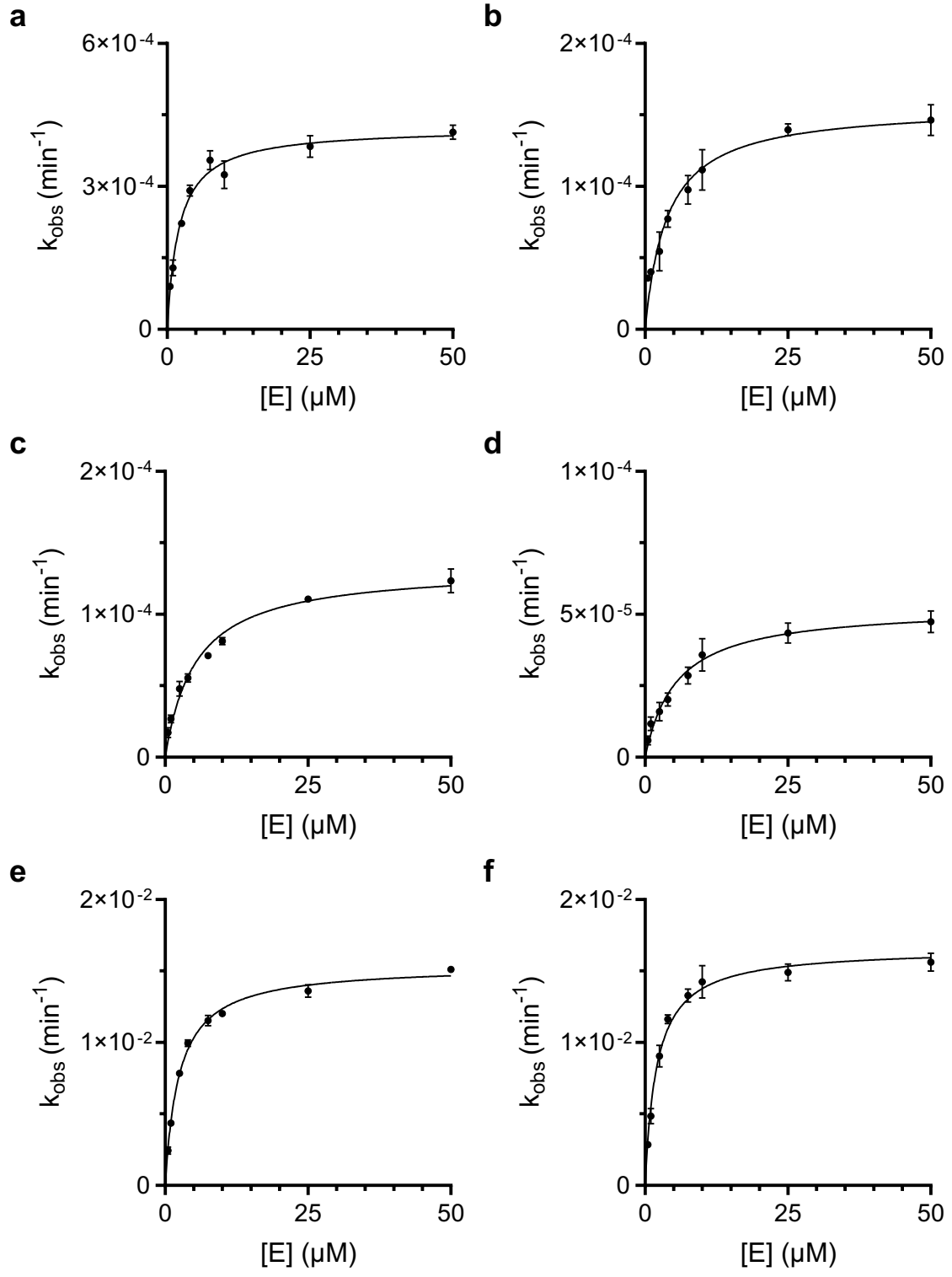

Figure S4. Fitted Michaelis-Menten kinetics analysis for (a) App-L-RNA_8, (b) Gpp-L-RNA_8, (c) Cpp-L-RNA_8, (d) Upp-L-RNA_8, (e) ppp-L-RNA_8, and (f) Appp-L-RNA_8.

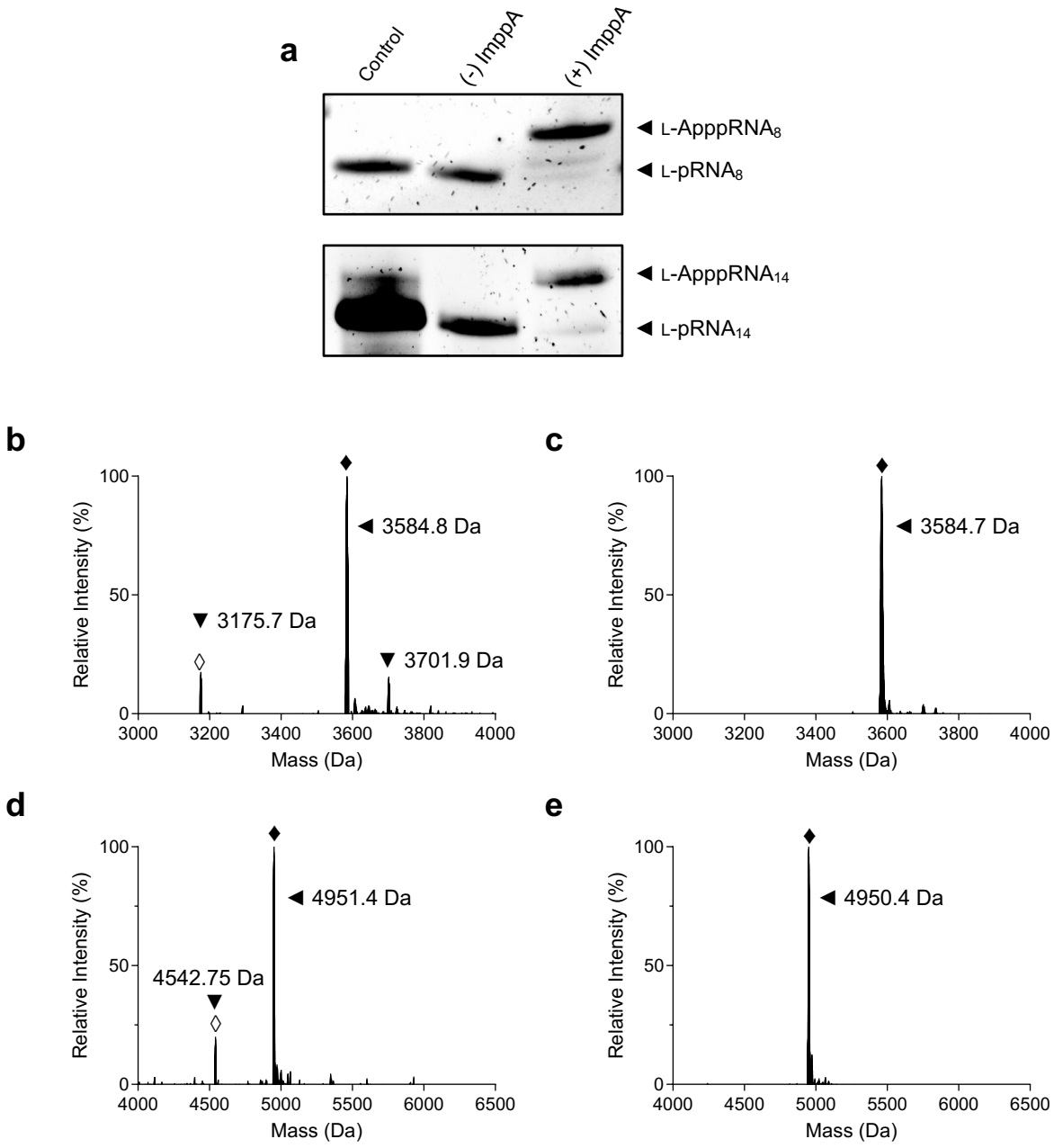

Figure S5. (a) Representative denaturing PAGE analysis of the reaction of ImppA with p-L-RNA_8 (top) and p-L-RNA_{14} (bottom). (b) ESI-MS of crude Appp-L-RNA_8. Calculated: 3584.7 Da, observed: 3584.8 Da. (c) ESI-MS of PAGE purified Appp-L-RNA_8. Calculated: 3584.7 Da, observed: 3584.7 Da. (d) ESI-MS of crude Appp-L-RNA_{14}. Calculated: 4950.7 Da, observed: 4951.4 Da. (e) ESI-MS of PAGE purified Appp-L-RNA_{14}. Calculated: 4950.7 Da, observed: 4950.4 Da. Open diamond = unreacted 5′-phosphorylated starting material; filled diamond = 5′-adenosyltriphosphate product.

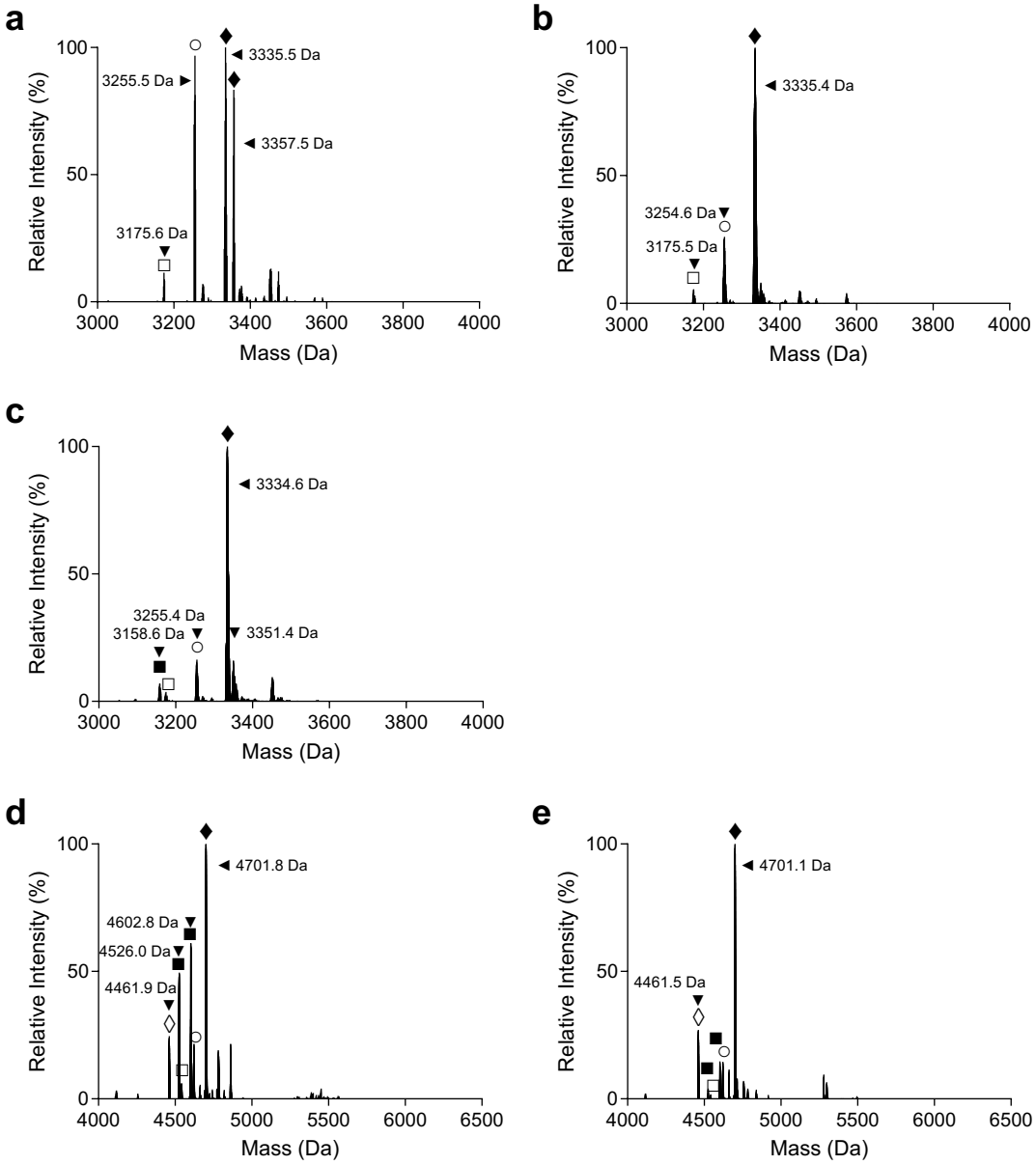

Figure S6. ESI-MS characterization of 5′-triphosphorylated L-RNA donors prepared by Ludwig-Eckstein chemistry. (a) ESI-MS of crude ppp-L-RNA_8. Calculated: 3335.2 Da, observed: 3335.5 Da (plus sodium adducts). (b) ESI-MS of PAGE purified ppp-L-RNA_8. Calculated: 3335.2 Da, observed: 3335.4 Da. (c) ESI-MS of HPLC purified ppp-L-RNA_8-CG. Calculated: 3335.2 Da, observed: 3334.6 Da. (d) ESI-MS of crude ppp-L-RNA_{14}. Calculated: 4701.1 Da, observed: 4701.8 Da. (e) ESI-MS of PAGE purified ppp-L-RNA_{14}. Calculated: 4701.1 Da, observed: 4701.1 Da. Open diamond = unreacted starting material; filled diamond = 5′-triphosphorylated product; open square = 5′-monophosphate; filled square = 5′-H-phosphonate; open circle = 5′-diphosphate.

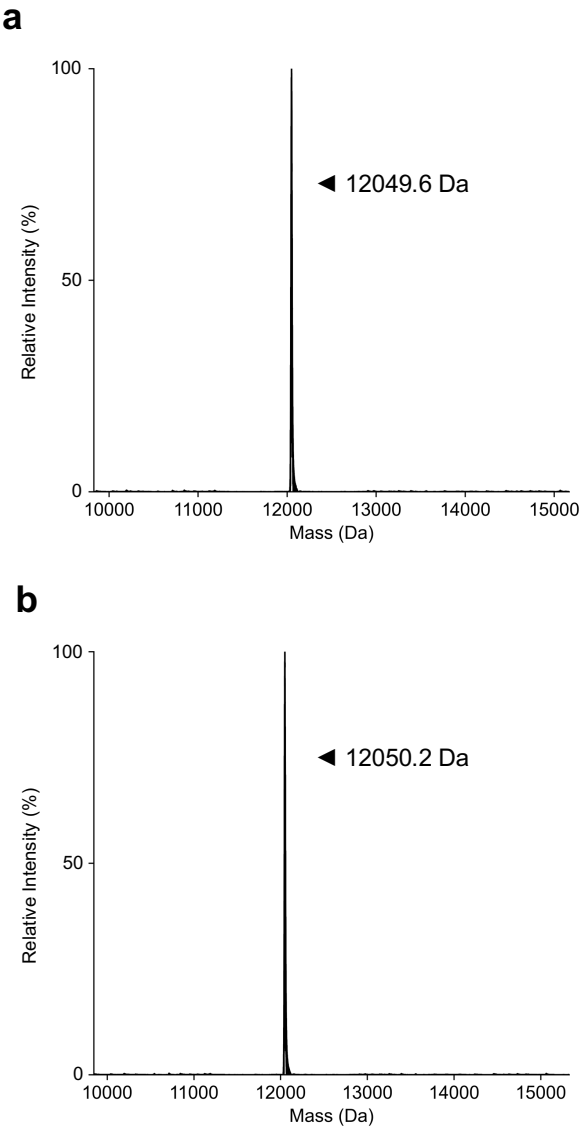

Figure S7. (a) ESI-MS data for the ligated product of L-Acceptor$_{hp}$ and ppp-L-RNA$_8$. Calculated: 12,048.7 Da, observed: 12048.6 Da. (b) ESI-MS data for the ligated product of L-Acceptor$_{hp}$ and Appp-L-RNA$_8$. Calculated: 12048.7 Da, observed: 12050.2 Da.

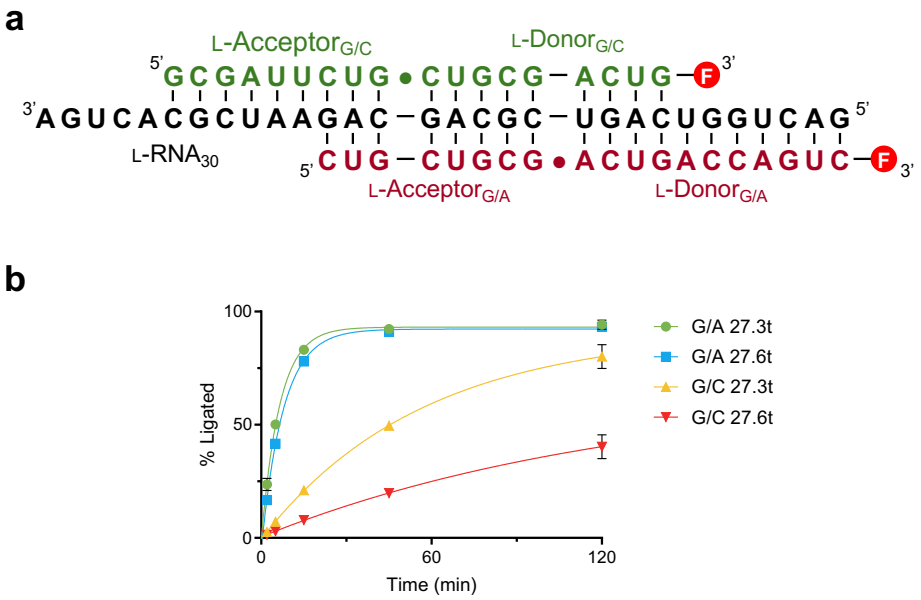

Figure S8. (a) Sequence and structure of the ligation complexes used to assess the generality of 27.3t and 27.6t towards ligation of 5′-adenosyltriphosphates. The ligation junction is indicated by a dot and the circled "F" indicates the dye label (Cy3). L-RNA$_{30}$ was used as the template for both sets of donor and acceptor. (b) Kinetic time course of the indicated D-RNA ribozyme ligating Appp-L-Donor$_{G/C}$ to L-Acceptor$_{G/C}$ (G/C) or Appp-L-Donor$_{G/A}$ to L-Acceptor$_{G/A}$ (G/A). Reaction conditions are the same as in Figure 3a. Error bars show standard deviation (n = 3).

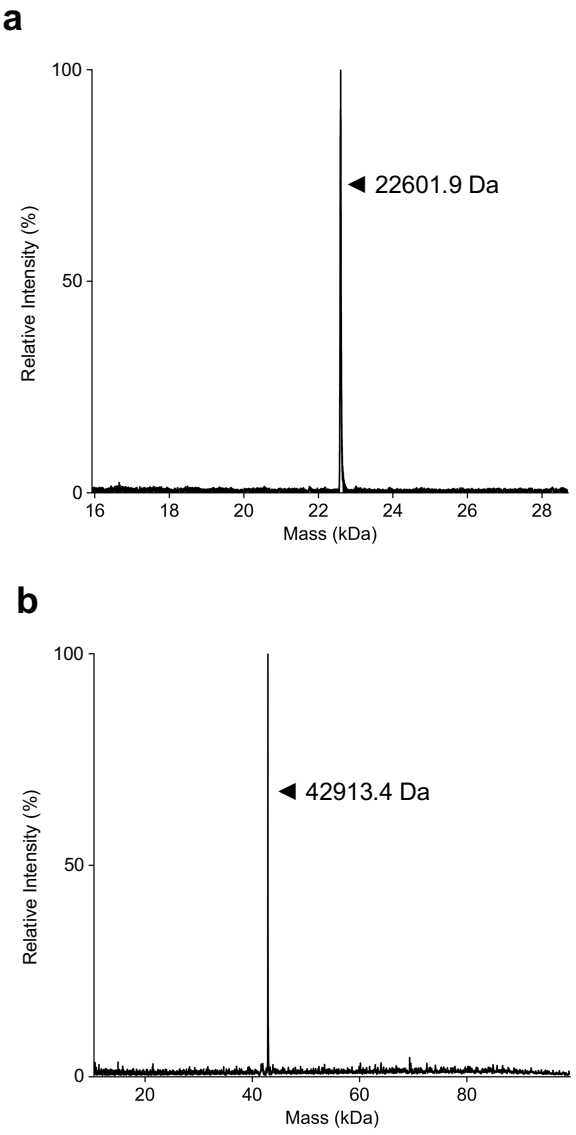

Figure S9. (a) ESI-MS characterization of donor Appp-L-27.6t$_D$. Calculated: 22598.4 Da, observed: 22601.9 Da. (b) ESI-MS characterization of L-27.6t prepared by cross-chiral ligation. Calculated: 42912.8 Da, observed: 42913.4 Da.

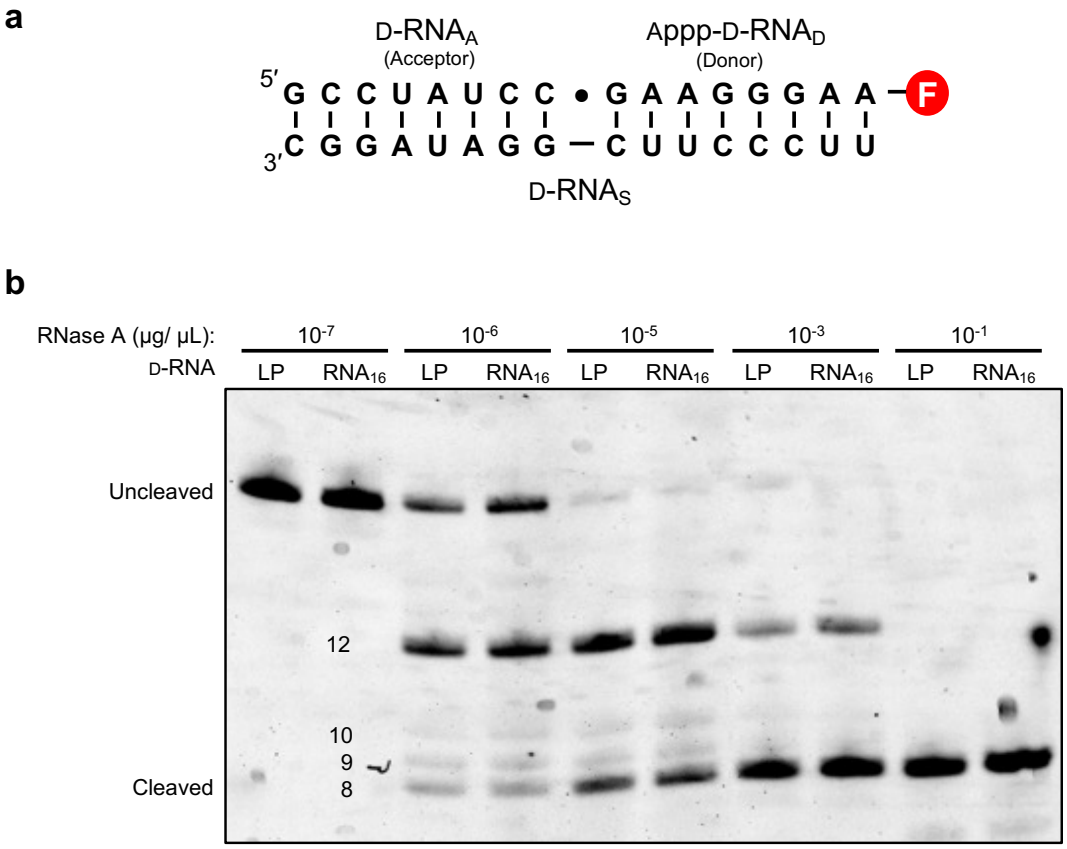

Figure S10. Analysis of the regiospecificity of cross-chiral ligation. (a) Sequence and structure of the ligation complex used to assess the regiospecificity of L-27.6t. The ligation junction is indicated by a dot, which is also the RNaseA cleavage site closest to the 3′-end of the product. Circled "F" indicates the dye label (Cy3). (b) Representative denaturing PAGE analysis of the RNase A digestion of the ligated product (LP; D-RNA$_{LP}$) depicted in panel (a) and the authentic product of the same sequence (D-RNA$_{16}$) demonstrating similar digestion patterns.

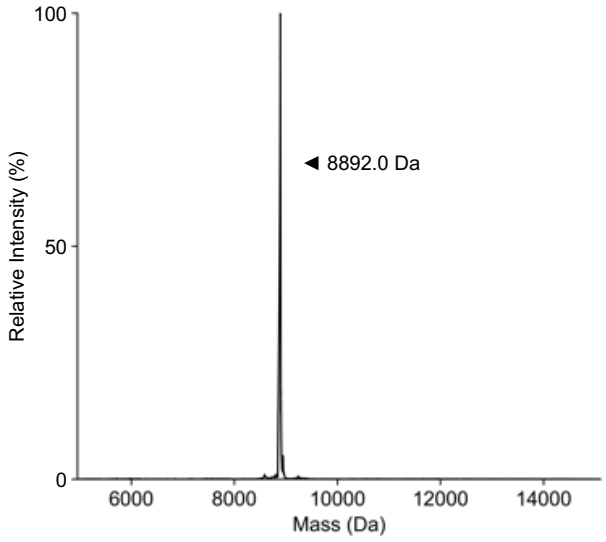

Figure S11. ESI-MS characterization of L-Acceptor$_{hp}$ used to generate the ligation complex depicted in Figure 1a. Calculated: 8891.6 Da, observed: 8892.0 Da.

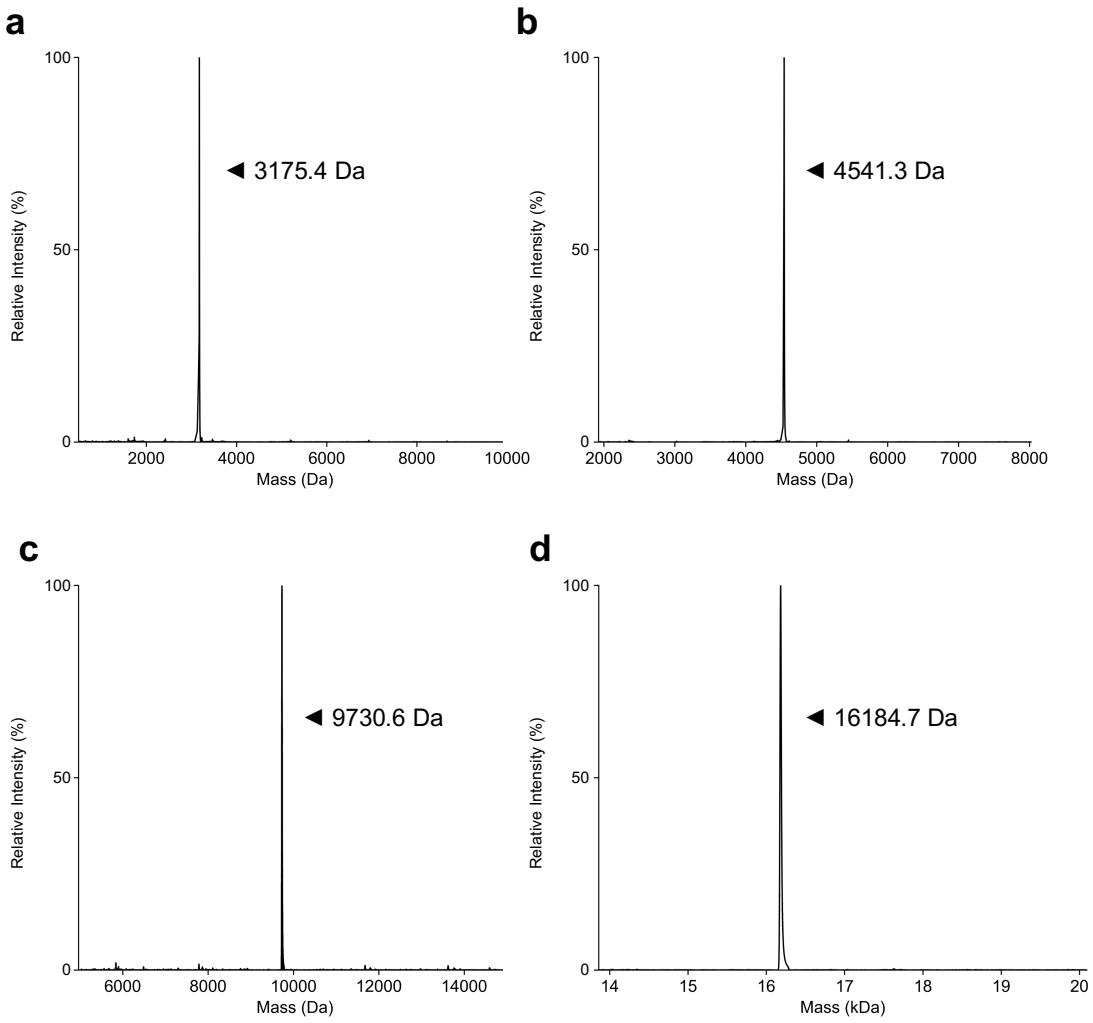

Figure S12. ESI-MS of 5′-phosphorylated L-RNAs used to generate donor substrates. (a) ESI-MS of p-L-RNA_8. Calculated: 3175.1 Da, observed: 3175.4 Da. (b) ESI-MS of p-L-RNA_{14}. Calculated: 4541.6 Da, observed: 4541.3 Da. (c) ESI-MS of p-L-RNA_{30}. Calculated: 9730.8 Da, observed: 9730.6 Da. (d) ESI-MS of p-L-RNA_{50}. Calculated: 16184.6 Da, observed: 16184.7 Da.

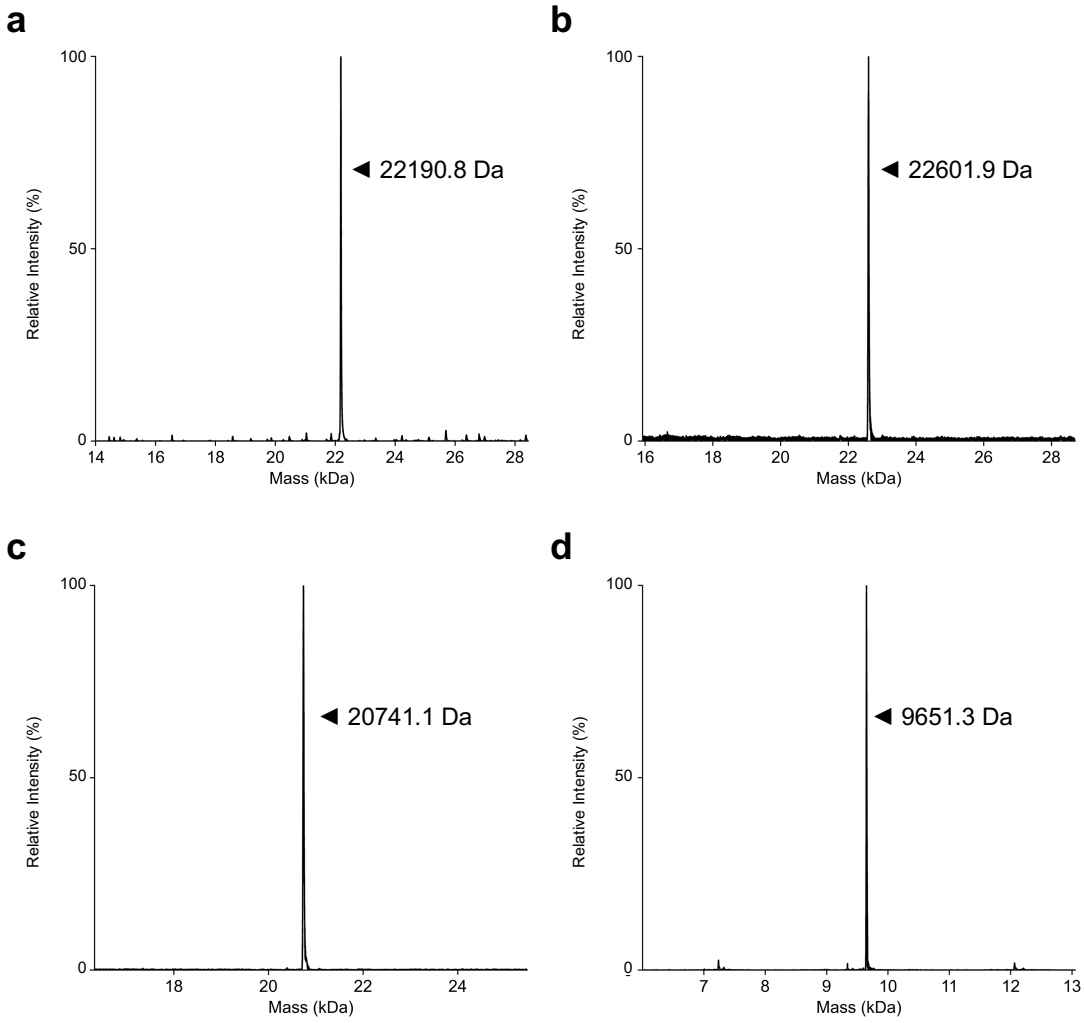

Figure S13. ESI-MS for L-RNA substrates associated with the synthesis of L-27.6t. (a) ESI-MS of p-L-27.6t$_D$. Calculated: 22189.25 Da, observed: 22190.8 Da. (b) ESI-MS of crude Appp-L-27.6t$_D$. Calculated: 22598.4 Da, observed: 22601.9 Da. (c) ESI-MS of L-27.6t$_A$. Calculated: 20741.4 Da, observed: 20741.1 Da. (d) ESI-MS of splint L-27.6t$_S$. Calculated: 9651.7 Da, observed: 9651.3 Da.

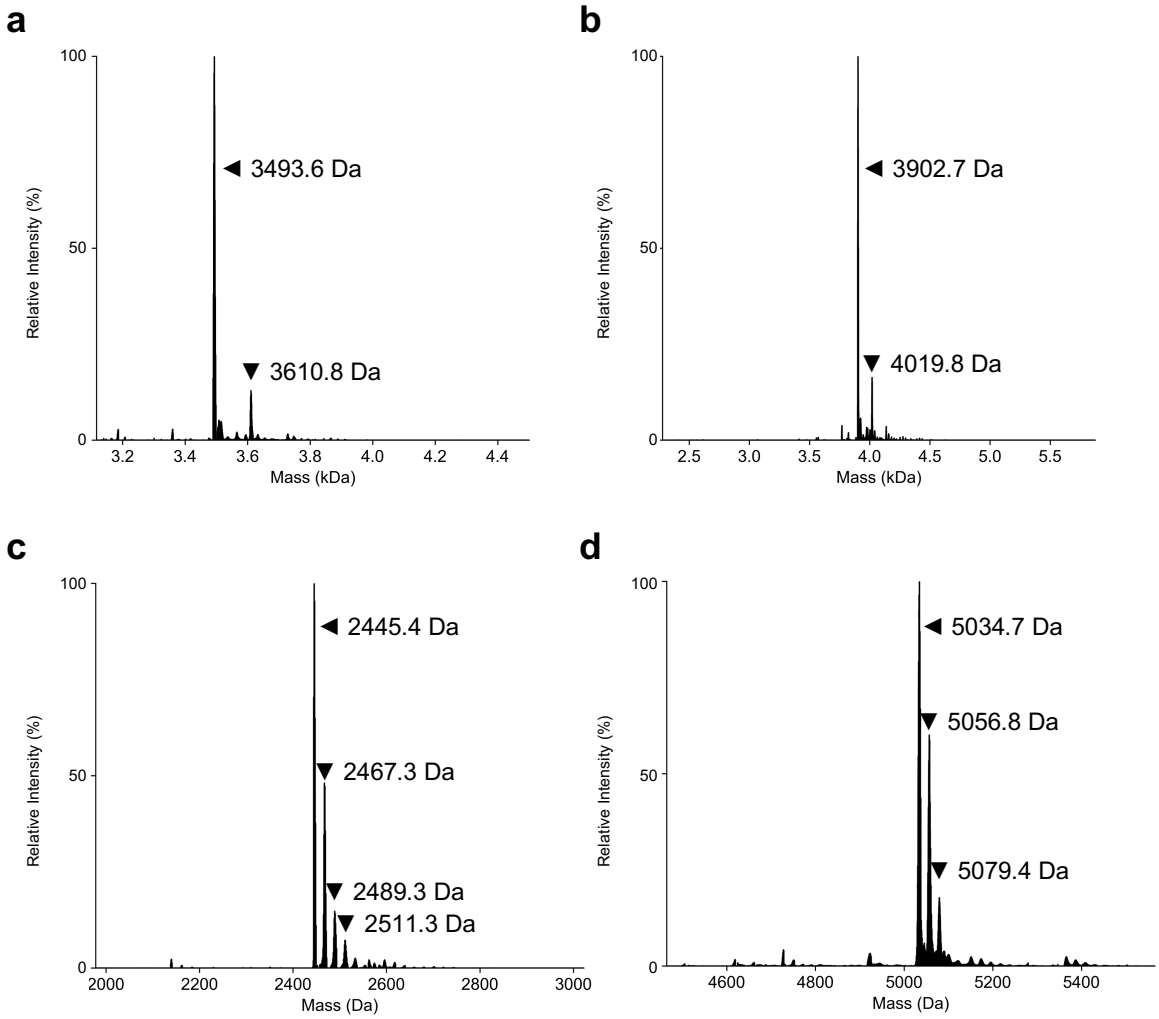

Figure S14. ESI-MS characterization of D-RNA ligation substrates depicted in Figure S9a. (a) ESI-MS of p-D-RNA$_D$. Calculated: 3492.9 Da, observed: 3493.6 Da. (b) ESI-MS of Appp-D-RNA$_D$. Calculated: 3902.0 Da, observed: 3902.7 Da. (c) ESI-MS of D-RNA$_A$. Calculated: 2445.6 Da, observed: 2445.4 Da (plus sodium adducts). (d) ESI-MS of D-RNA$_s$. Calculated: 5034.0 Da, observed: 5034.7 Da (plus sodium adducts).

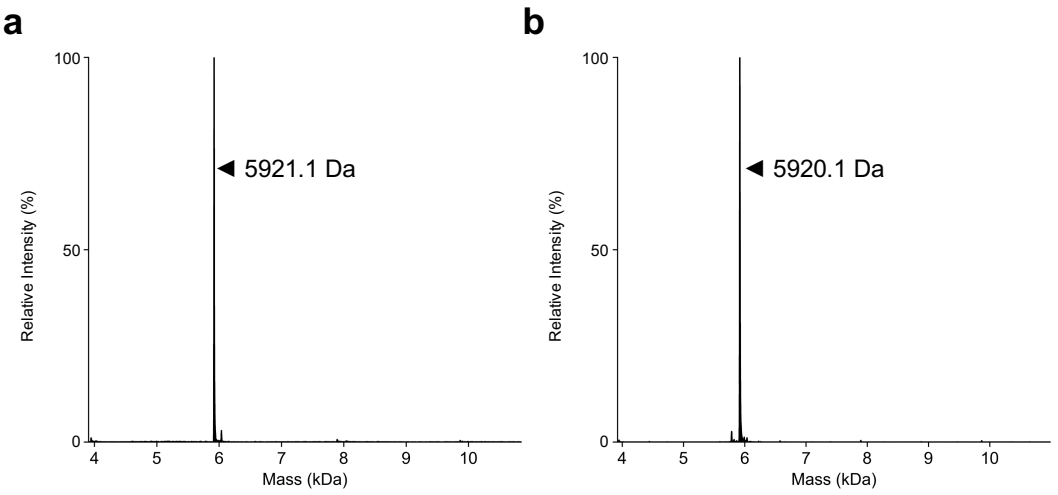

Figure S15. ESI-MS characterization of the D-RNA ligation product (LP) and synthetic D-RNA_{16} used in the RNase A digestion analysis (Figure S9). (a) ESI-MS of D-RNA_{LP}. Calculated: 5920.9 Da, observed: 5921.1 Da. (b) ESI-MS of D-RNA_{16}. Calculated: 5920.9 Da, observed: 5920.1 Da.

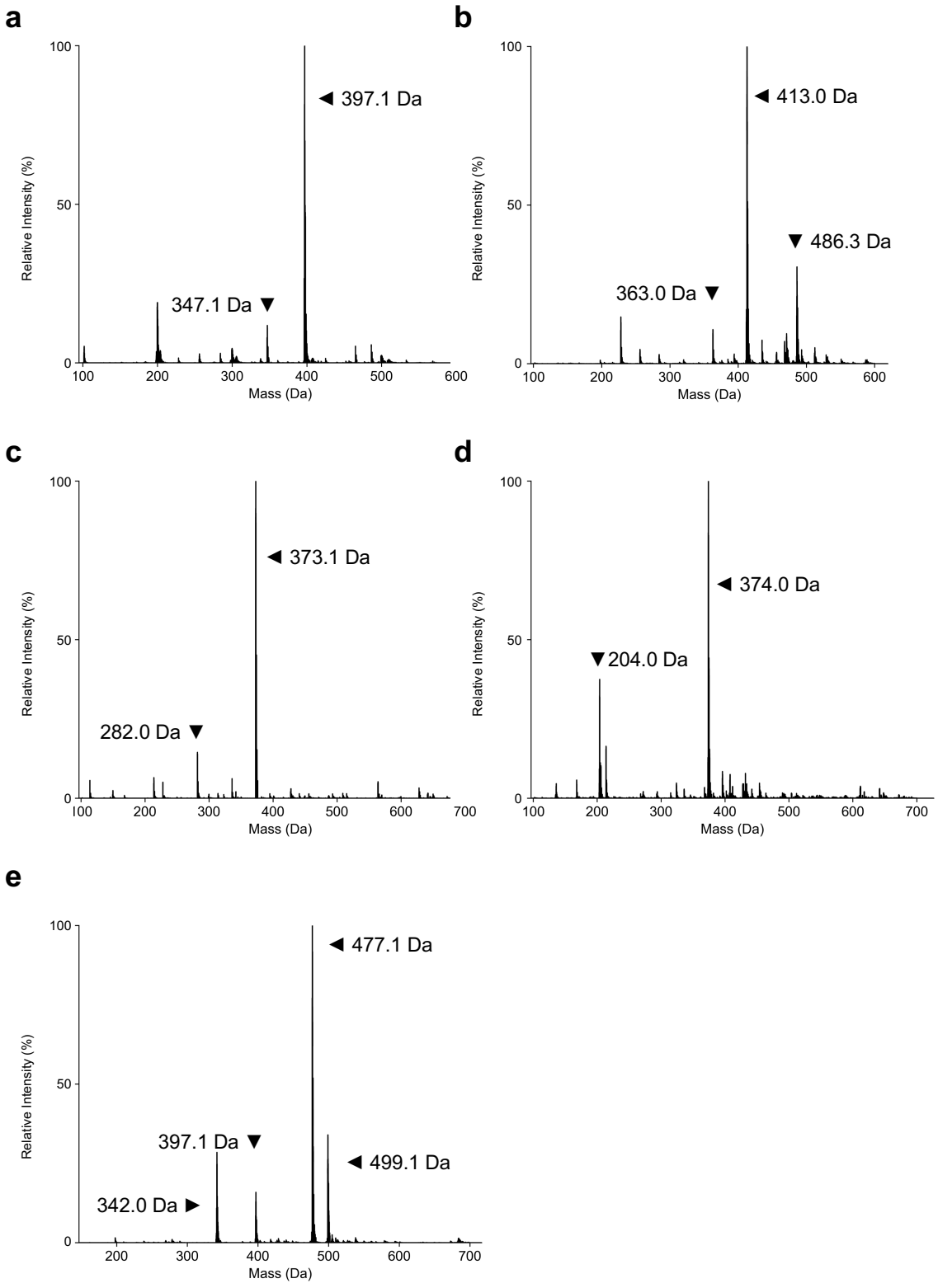
a
100
50
0
Relative Intensity (%)
◄ 397.1 Da
347.1 Da ▼
100 200 300 400 500 600
Mass (Da)
b
100
50
0
Relative Intensity (%)
◄ 413.0 Da
▼ 486.3 Da
363.0 Da ▼
100 200 300 400 500 600
Mass (Da)
c
100
50
0
Relative Intensity (%)
◄ 373.1 Da
282.0 Da ▼
100 200 300 400 500 600 700
Mass (Da)
d
100
50
0
Relative Intensity (%)
◄ 374.0 Da
▼204.0 Da
100 200 300 400 500 600 700
Mass (Da)
e
100
50
0
Relative Intensity (%)
◄ 477.1 Da
397.1 Da ▼
◄ 499.1 Da
342.0 Da ►
200 300 400 500 600 700
Mass (Da)

Figure S16 (previous page). ESI-MS characterization of crude phosphoimidazolides used in the preparation of 5′-N-ylated RNAs. (a) ESI-MS of ImpA. Calculated: 397.1 Da, observed: 397.1 Da. Estimated purity: 77%. (b) ESI-MS of ImpG. Calculated: 413.1 Da, observed: 413.0 Da. Estimated purity: 57%. (c) ESI-MS of ImpC. Calculated: 373.1 Da, observed: 373.1 Da. Estimated purity: 70%. (d) ESI-MS of ImpU. Calculated: 374.1 Da, observed: 374.0 Da. Estimated purity: 65%. (e) ESI-MS of ImppA. Calculated: 477.1 Da, observed: 477.1 Da (plus sodium adduct). Estimated purity: 75%.

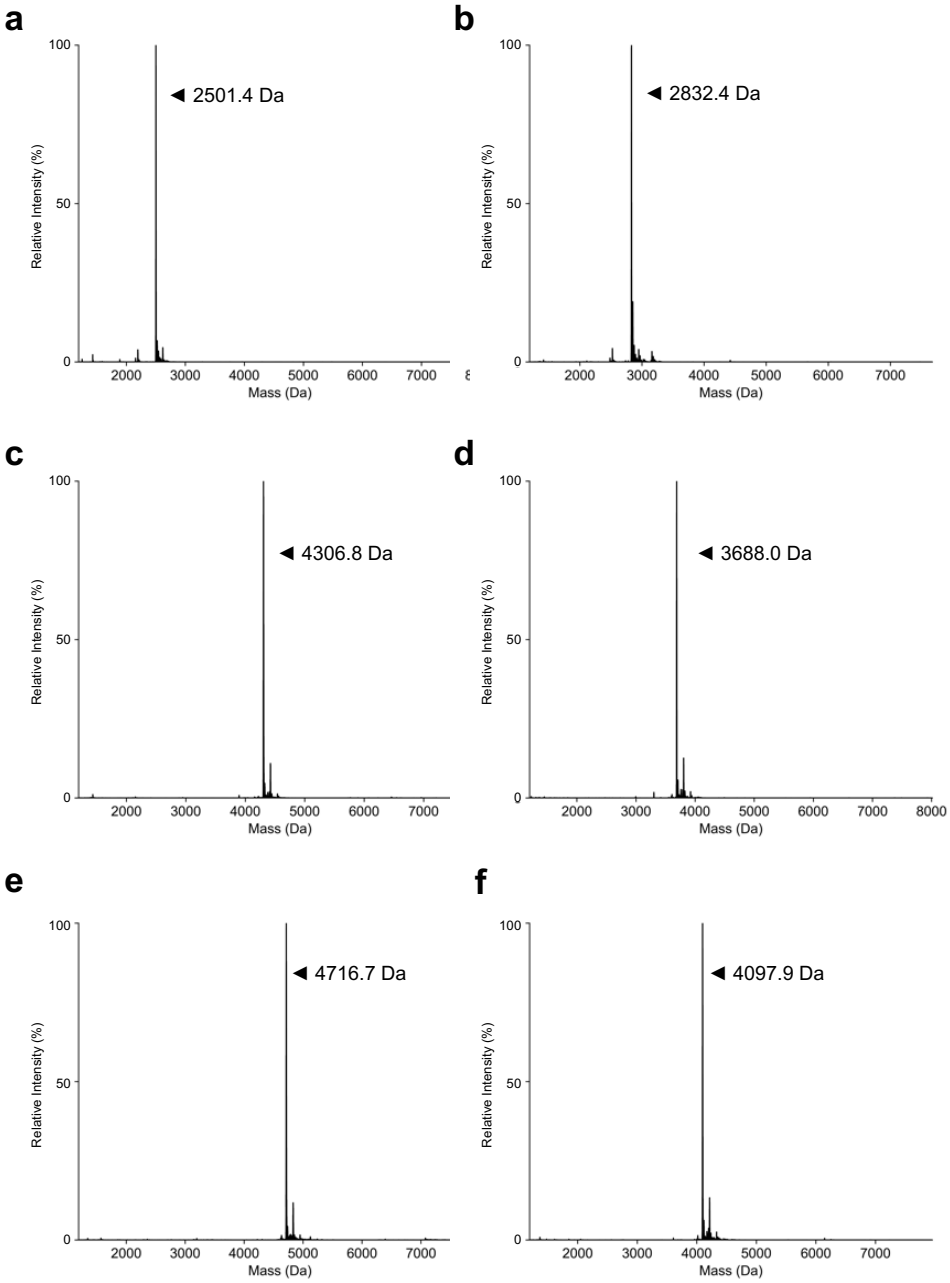

Figure S17. ESI-MS for RNAs associated with the ligation experiments depicted in Figure S8. (a) ESI-MS of L-Acceptor$_{G/A}$. Calculated: 2501.6 Da, observed: 2501.4 Da. (b) ESI-MS of L-Acceptor$_{G/C}$. Calculated: 2831.8 Da, observed: 2832.4 Da. (c) ESI-MS of p-L-Donor$_{G/A}$. Calculated: 4306.3 Da, observed: 4306.8 Da. (d) ESI-MS of p-L-Donor$_{G/C}$. Calculated: 3687.9 Da, observed: 3688.0 Da. (e) ESI-MS of Appp-L-Donor$_{G/A}$. Calculated: 4716.3 Da, observed: 4716.7 Da. (f) ESI-MS of Appp-L-Donor$_{G/C}$. Calculated: 4097.9 Da, observed: 4097.9 Da.

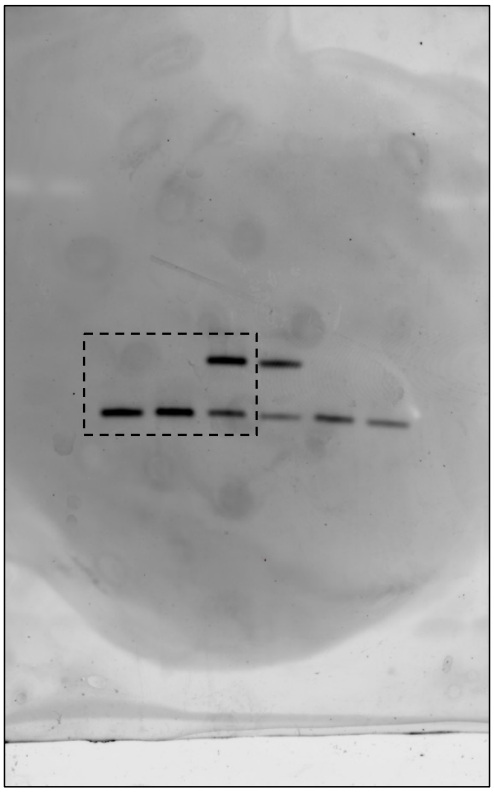

Figure S18. Uncropped gel image for Figure 2b. Frame indicates the cropped region shown in Figure 2b. Unboxed region contains analysis of the same experiment with less amount of RNA loaded on the gel.

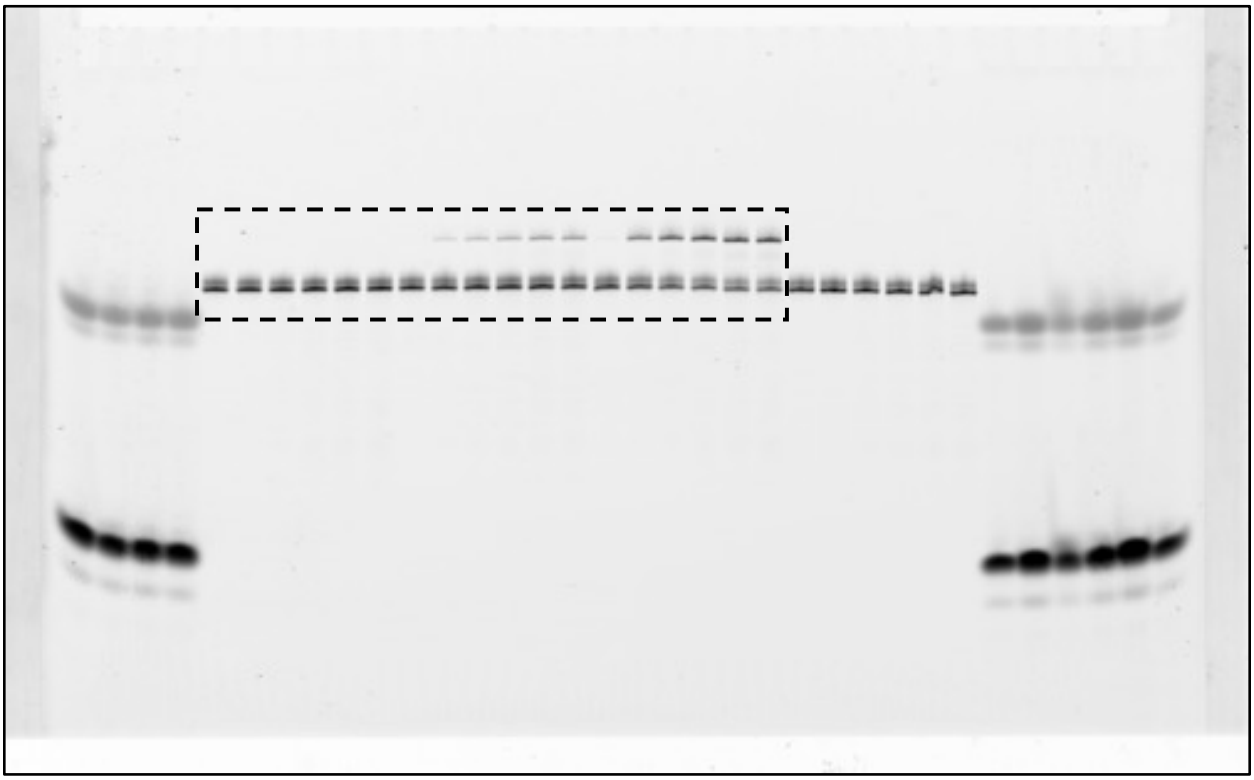

Figure S19. Uncropped gel image for Figure 3a. Frame indicates the cropped region shown in Figure 3a. Unboxed regions contain unrelated experiments.

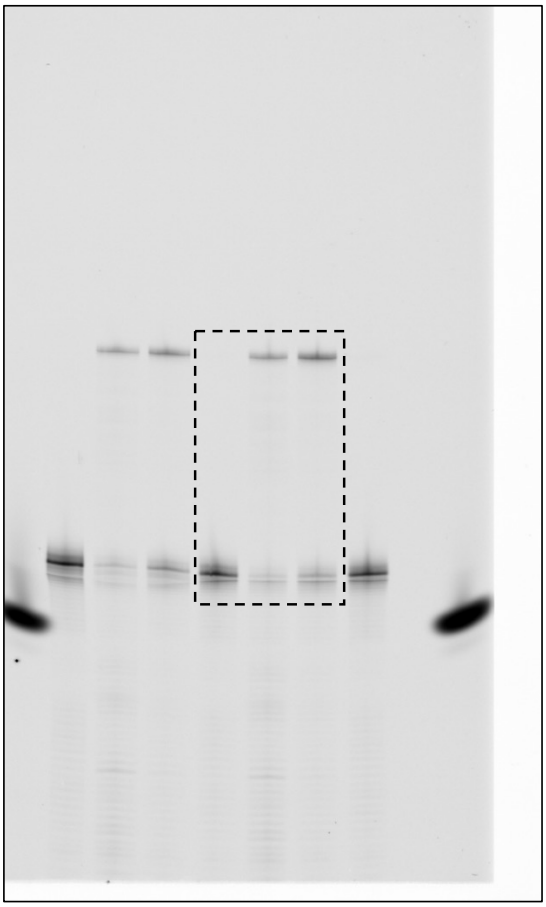

Figure S20. Uncropped gel image for Figure 6b. Frame indicates the cropped region shown in Figure 6b. Unboxed regions contain unrelated experiments.

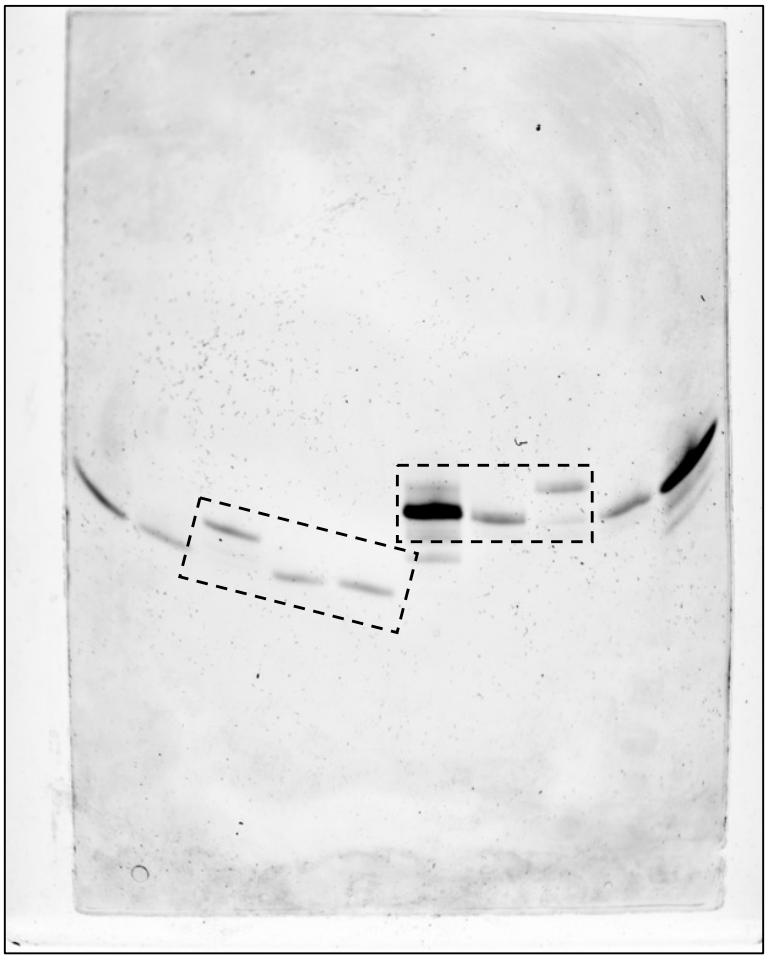

Figure S21. Uncropped gel image for panels in Figure S5a. Frame indicates the cropped regions shown in Figure S5a.

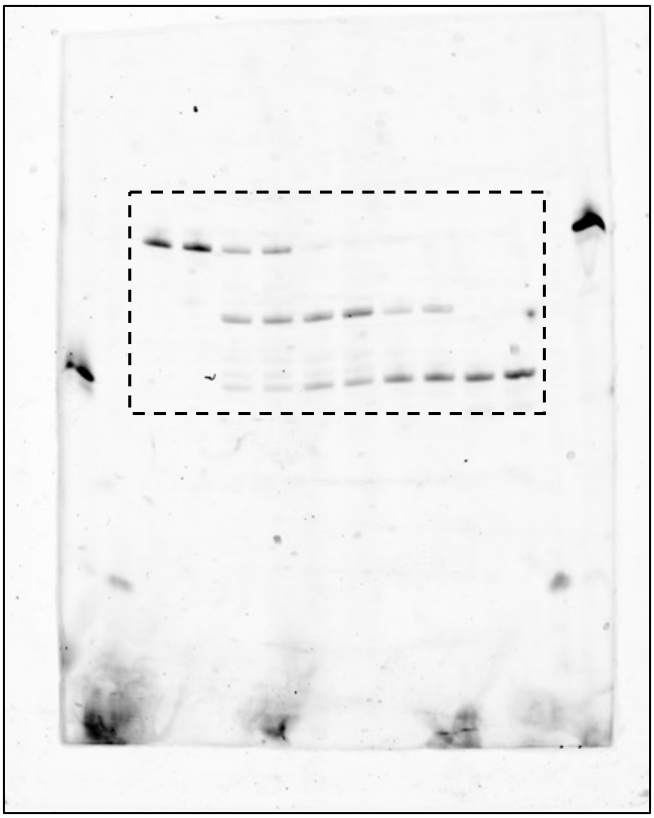

Figure S22. Uncropped gel image for Figure S10b. Frame indicates the cropped regions shown in Figure S10b. Unboxed region contains synthetic controls.

S3. Supplementary Tables.

Table S1. Names and sequences of oligonucleotides used in this work. L-RNA (blue) and D-RNA (black) are indicated by color. /Phos/ = monophosphate; /rNpp/ = N-ylation (N = A,G,C, or U); /rAppp/ = adenosyltriphosphate; /ppp/ = triphosphate; /BioTEG/ = triethylene glycol biotin; /sp18/ = hexaethylene glycol; /AmMC6/ = C6 amino modifier; /6-FAM/ = 6-fluorescein; /Cy5/ = Sulfo-Cy5; /Cy3/ = Sulfo-Cy3.

Sequence Name	Sequence Identity (5'→3')	ESI-MS (Figure)
p-L-RNA$_8$	/Phos/-GACUGGUC-/BioTEG/	S12a
ppp-L-RNA$_8$	/ppp/-GACUGGUC-/BioTEG/	S6b, c
App-L-RNA$_8$	/rApp/-GACUGGUC-/BioTEG/	S1a
Gpp-L-RNA$_8$	/rGpp/-GACUGGUC-/BioTEG/	S3a
Cpp-L-RNA$_8$	/rCpp/-GACUGGUC-/BioTEG/	S3b
Upp-L-RNA$_8$	/rUpp/-GACUGGUC-/BioTEG/	S3c
Appp-L-RNA$_8$	/rAppp/-GACUGGUC-/BioTEG/	S5c
p-L-RNA$_{14}$	/Phos/-GACUGGUCAGUCGC	S12b
ppp-L-RNA$_{14}$	/ppp/-GACUGGUCAGUCGC	S6e
App-L-RNA$_{14}$	/rApp/-GACUGGUCAGUCGC	S1b
Appp-L-RNA$_{14}$	/rAppp/-GACUGGUCAGUCGC	S5e
p-L-RNA$_{30}$	/Phos/-GACUGGUCAGUCGCAGCAGAAUCGCACUGA	S12c
App-L-RNA$_{30}$	/rApp/-GACUGGUCAGUCGCAGCAGAAUCGCACUGA	S1c
p-L-RNA$_{50}$	/Phos/-GACUGGUCAGUCGCAGCAGAAUCGCACUGAAGCACGUGUACGAUGAUAUU	S12d
App-L-RNA$_{50}$	/rApp/-GACUGGUCAGUCGCAGCAGAAUCGCACUGAAGCACGUGUACGAUGAUAUU	S1d
L-Accpetor$_{hp}$	/6-FAM/-GACCAGUCGGAUAGCGCAAGCUAUCC	S11
p-L-27.6t$_D$	/Phos/-GAUAAAAUGCACAUAGGUCGAAAGACCUUAUACAAGAACUGUAUCACCGGAGGGCGAGCACCACC-/sp18/-/AmMC6/-/Cy5/	S13a
Appp-L-27.6t$_D$	/rAppp/-GAUAAAAUGCACAUAGGUCGAAAGACCUUAUACAAGAACUGUAUCACCGGAGGGCGAGCACCACC-/sp18/-/AmMC6/-/Cy5/	S13b
L-27.6t$_A$	GGUGGCGGACGUGUUUCACGUAUAAUCGUGCGGGACACUGACUCGUCAGUGCAUUGAGAAGGAG	S13c
L-27.6t$_S$	CCUAUGUGCAUUUUAUCCUCCUUCUCAAUGC	S13d
p-D-RNA$_D$	/Phos/-GAAGGGAA-/AmMC6/-/Cy3/	S14a
Appp-D-RNA$_D$	/rAppp/-GAAGGGAA-/AmMC6/-/Cy3/	S14b
D-RNA$_A$	GCCUAUCC	S14c
D-RNA$_S$	UUCCCUUCGGAUAGGC	S14d
D-RNA$_{16}$	GCCUAUCCGAAGGGAA-/AmMC6/-/Cy3/	S15b
D-16.12t	GGUCGUCAGUGCAUUGAGAAGGAGGAUAAAAUGCACAUAGGUCGAAAGACCUUAUACAAGAACUGUAUCACCGGAGGGCGACC	N/A
D-27.3t	GGUGGUGGACGUGAUCAUUACGGAUCACUAACUCGUCAGUGCAUUGAGAAGGAGAAUAAAAUGCACAUAGGUCGAAAGACCUUAUACAAGAACUGUAUCACCGGAGGGCGAGCACCACC	N/A
D-27.6t	GGUGGCGGACGUGUUUCACGUAUAAUCGUGCGGGACACUGACUCGUCAGUGCAUUGAGAAGGAGGAUAAAAUGCACAUAGGUCGA	N/A

	AAGACCUUAUACAAGAACUGUAUCACCGGAGGGCGAGCACCAC C	
L-Acceptor$_{G/A}$	CUGCUGCG	S17a
L-Acceptor$_{G/C}$	GCGAUUCUG	S17b
p-L-Donor$_{G/A}$	/Phos/-ACUGACCAGUC-/AmMC6/-/Cy3/	S17c
p-L-Donor$_{G/C}$	/Phos/-CUGCGACUG-/AmMC6/-/Cy3/	S17d
Appp-L-Donor$_{G/A}$	/rAppp/-ACUGACCAGUC-/AmMC6/-/Cy3/	S17e
Appp-L-Donor$_{G/C}$	/rAppp/-CUGCGACUG-/AmMC6/-/Cy3/	S17f

Table S2. DNA oligonucleotides used in cross extension reactions to generate the dsDNA templates for *in vitro* transcription of the indicated D-ribozymes. The T7 promoter sequence is underlined.

Ribozyme	Strand	Sequence (5'→3')
16.12t	Forward	TTCTAATACGACTCACTATAGGTCGTCAGTGCATTGAGAAGGAGGATAAAATGCACATAGGT
	Reverse	GGTCGCCCTCCGGTGATACAGTTCTTGTATAAGGTCTTTCGACCTATGTGCATTTTATCCTCC
27.3t	Forward	TTCTAATACGACTCACTATAGGTGGTGGACGTGATCATTACGGATCACTAACTCGTCAGTGCATTGAGAAGGAGAATAAAATGCAC
	Reverse	GGTGGTGCTCGCCCTCCGGTGATACAGTTCTTGTATAAGGTCTTTCGACCTATGTGCATTTTATTCTCCTTCTCAATGCACTGACG
27.6t	Forward	TTCTAATACGACTCACTATAGGTGGCGGACGTGTTTCACGTATAATCGTGCGGGACACTGACTCGTCAGTGCATTGAGAAGGAGGATAAA
	Reverse	GGTGGTGCTCGCCCTCCGGTGATACAGTTCTTGTATAAGGTCTTTCGACCTATGTGCATTTTATCCTCCTTCTCAATGCACTGACGAGTC

S4. References

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