Supporting information

A comparative analysis of peptide-delivered antisense antibiotics employing diverse nucleotide mimics

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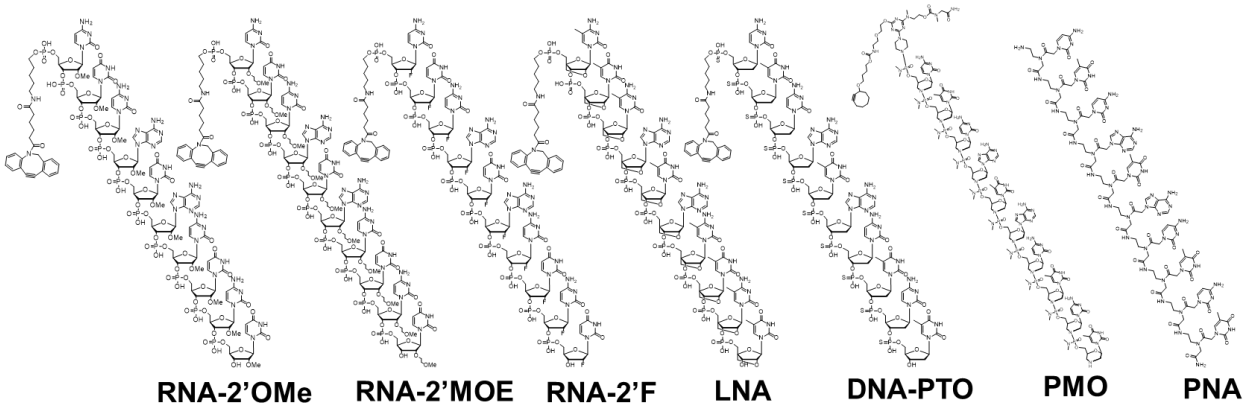

Supplementary Figure S1. Chemical structures of ASOs used in this study.

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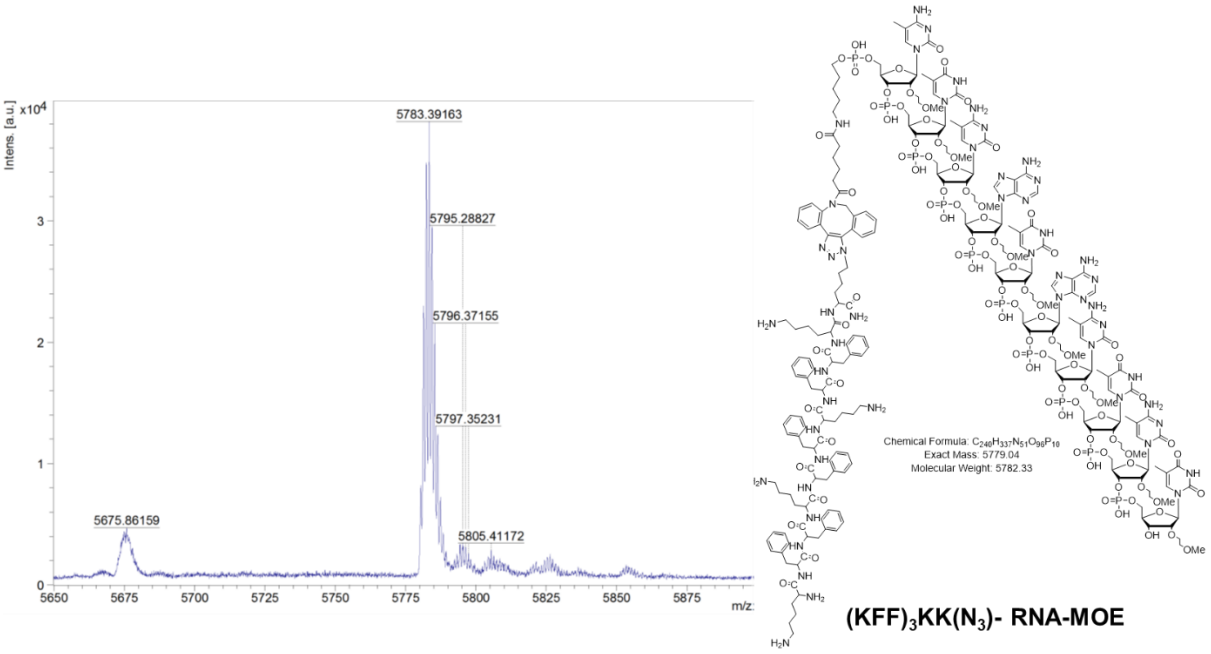
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Molecular Weight: 5782.33
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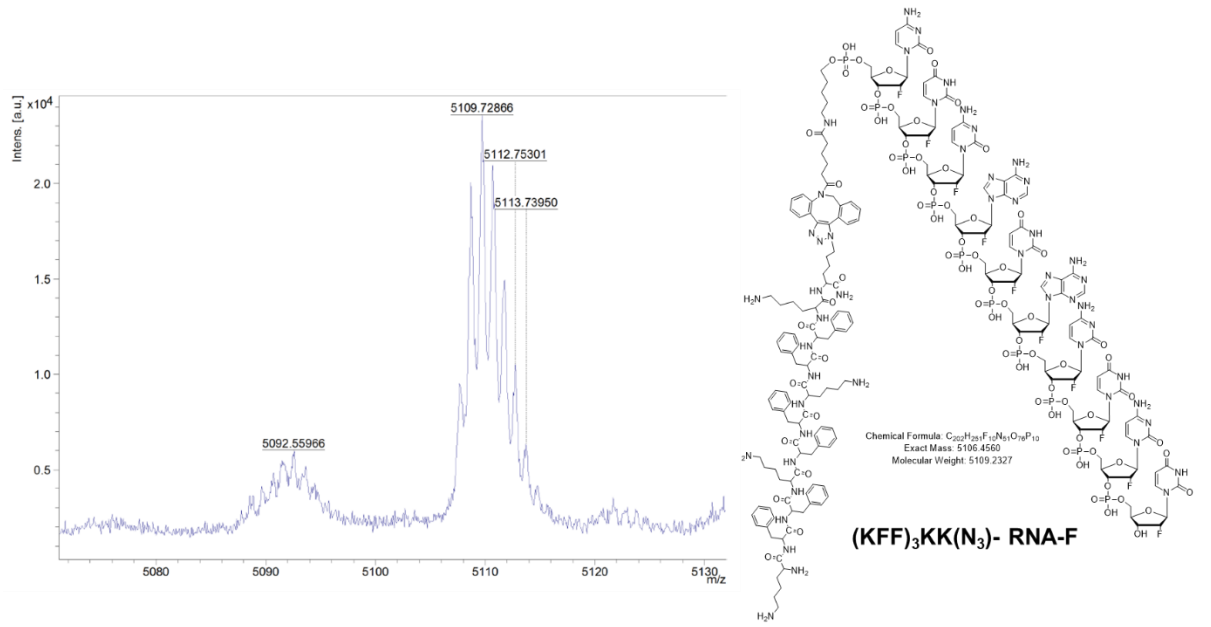
B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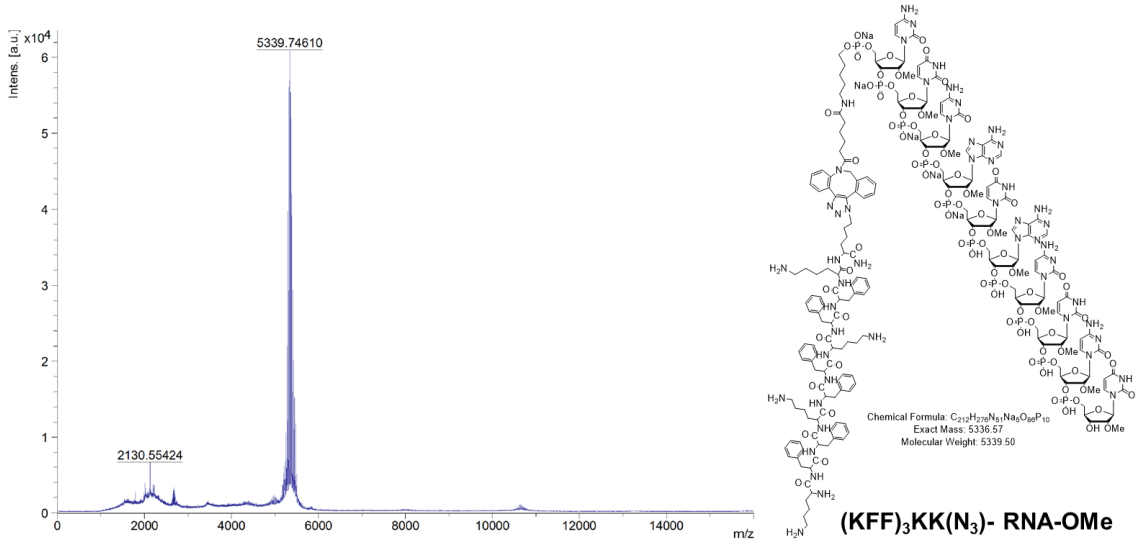
(KFF)3KK(N3)- RNA-F

C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(KFF)3KK(N3)- RNA-OMe

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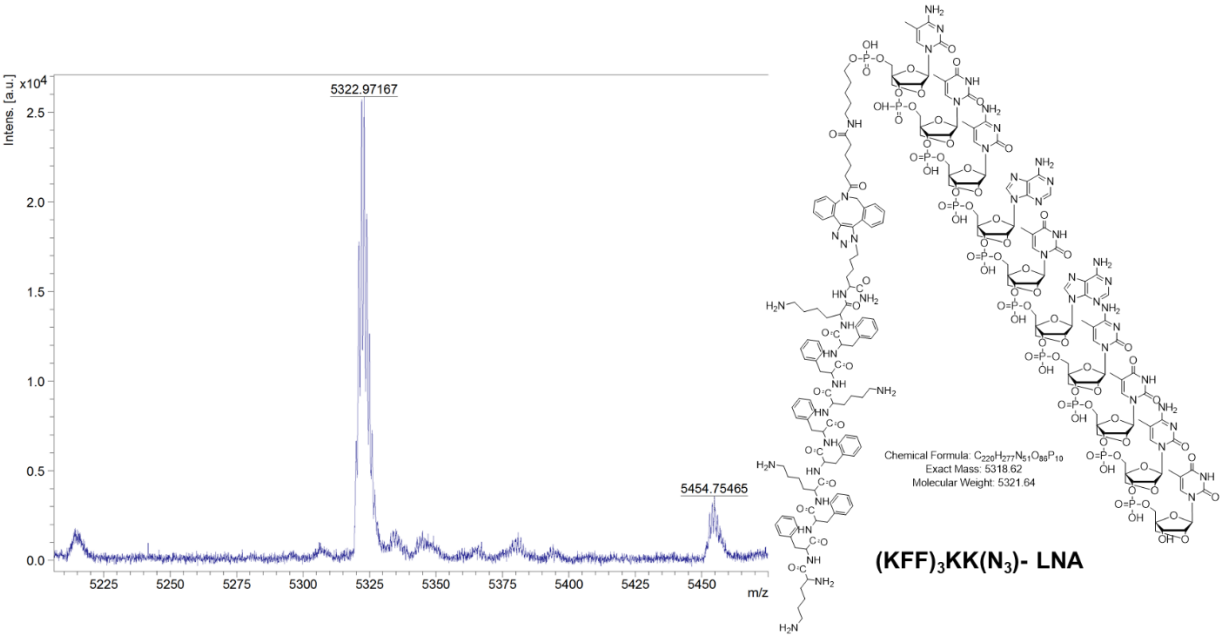
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5425
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m/z
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Molecular Weight: 5321.64
(KFF)3KK(N3)- LNA

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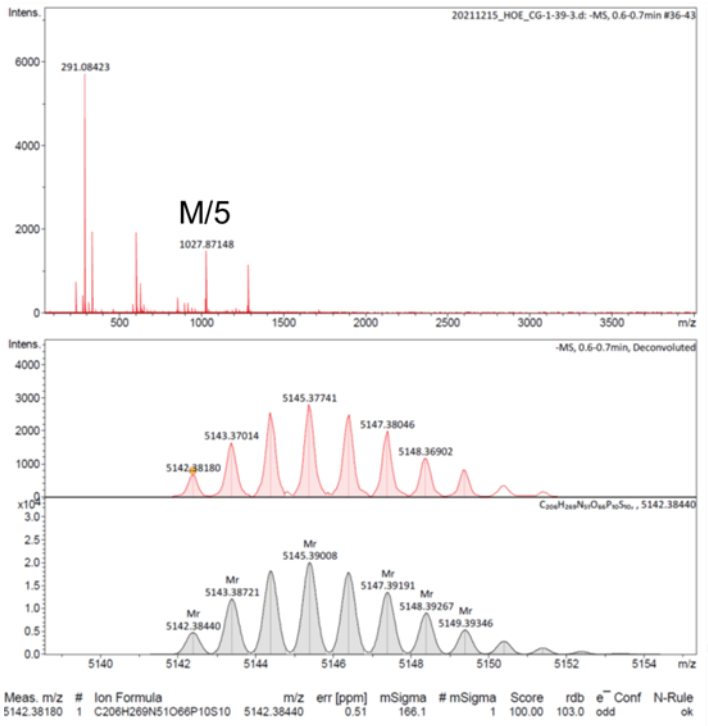
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M/5
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5143.37014
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5148.36902
5142.38180
Mr 5145.39008
Mr 5143.38721
Mr 5147.39191
Mr 5148.39267
Mr 5142.38440
Mr 5149.39346
Meas. m/z # Ion Formula m/z err [ppm] mSigma # mSigma Score rdb e⁻ Conf N-Rule
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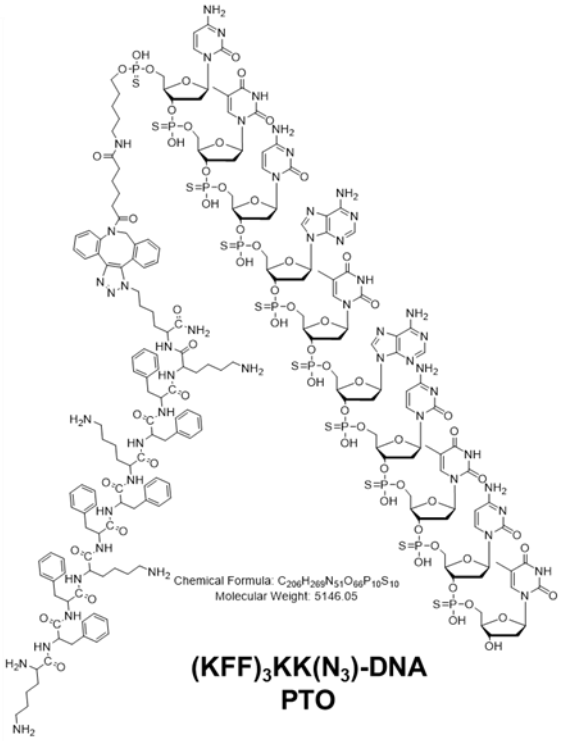
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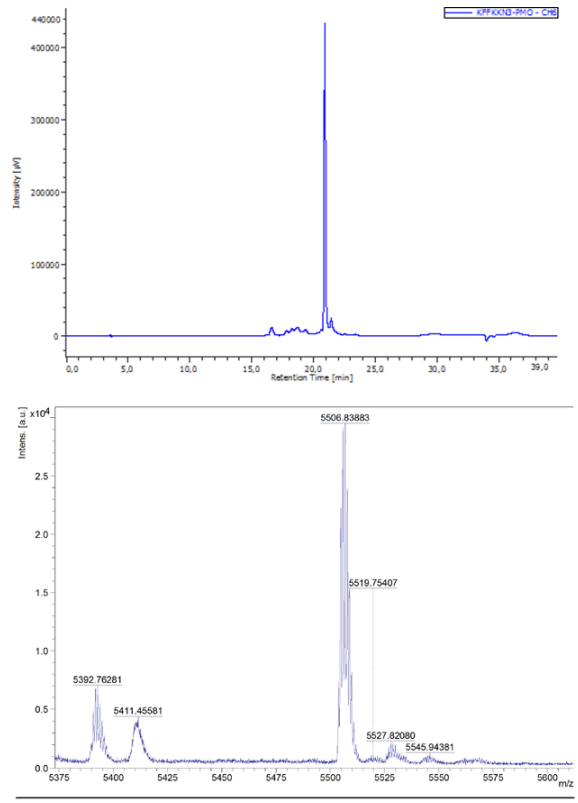
KFF KK(N3)-PMO - CH6
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Retention Time [min]
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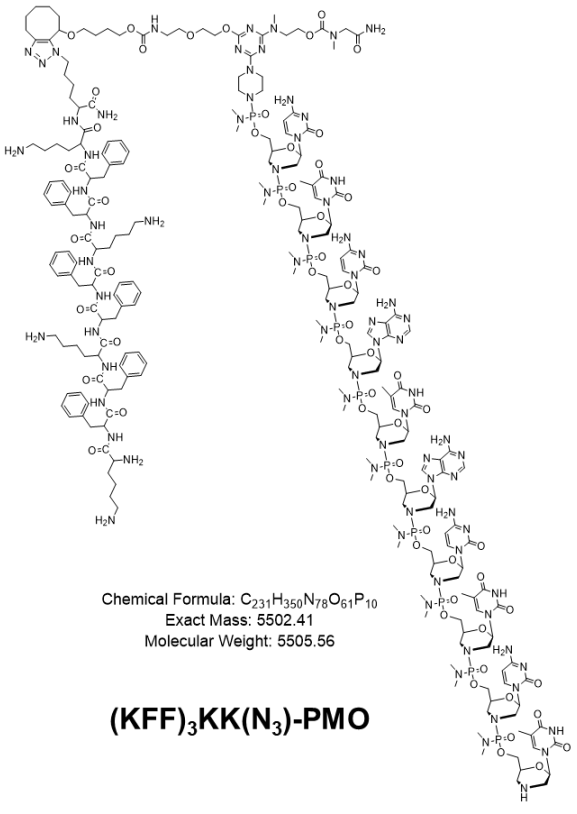
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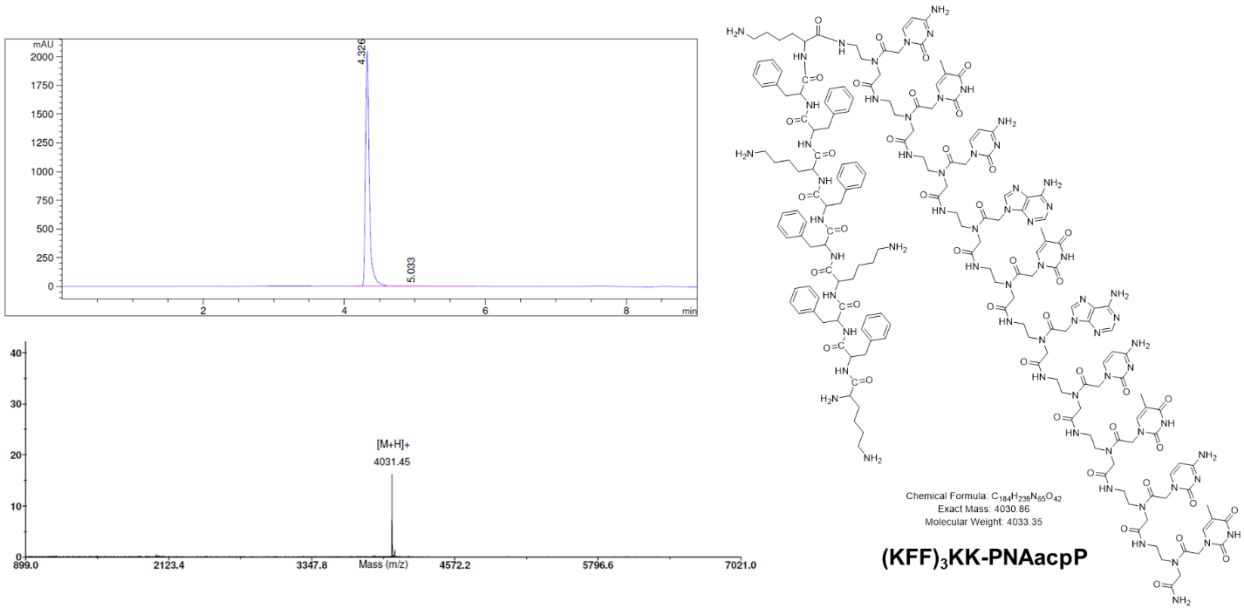
(KFF)3KK(N3)-PMO

G



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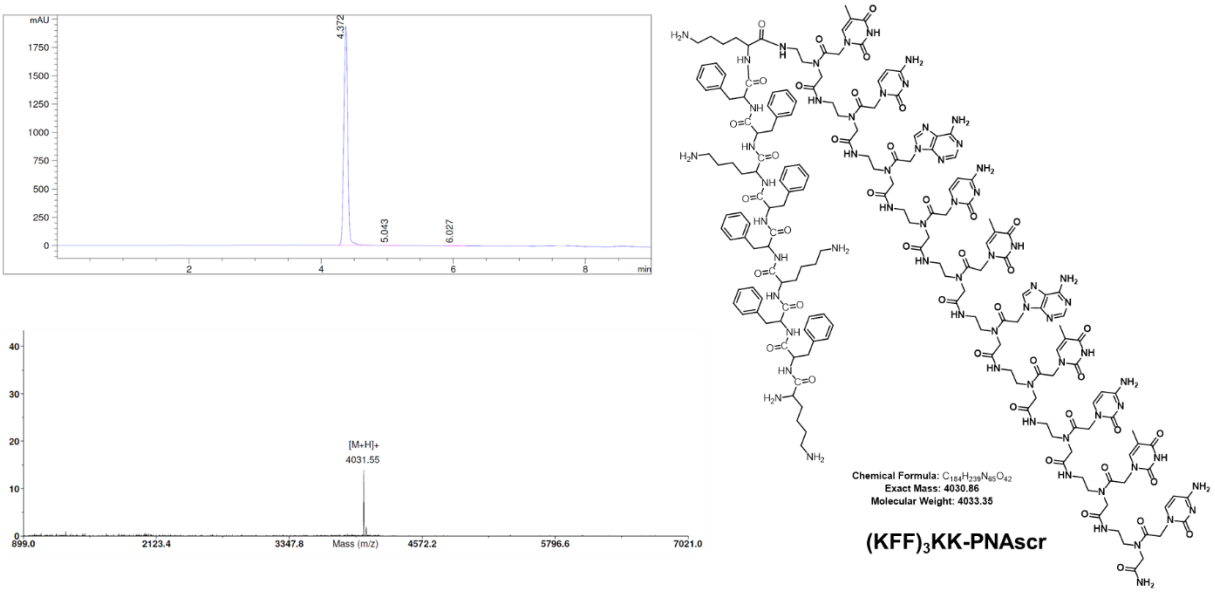
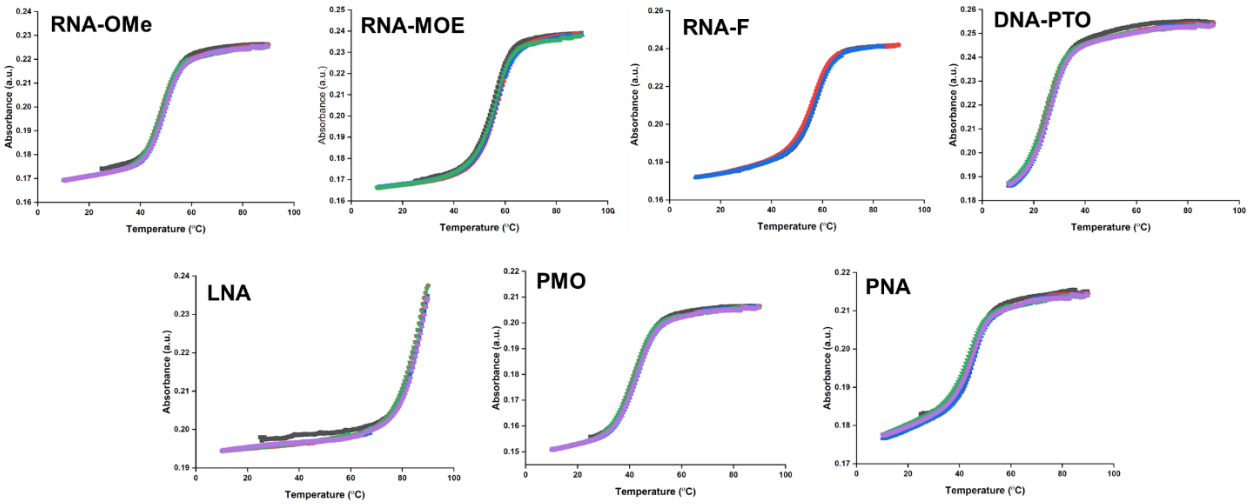


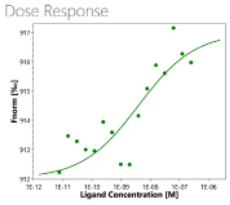
Supplementary Figure S2. Structures and mass spectra of KFF conjugated ASOs used in this study. Structures and mass spectra (MALDI or ESI-MS) of $(KFF)_3KK(N_3)$ conjugated to RNA-MOE (A), RNA-F (B), RNA-OMe (C), LNA (D), DNA-PTO (E) PMO (F), KFF_3K-PNAacpP (G) and KFF_3K-PNAscr. The upper panel of panels F-H show HPLC chromatograms of the PMO and PNA conjugates.



Supplementary Figure S3. Assessment of melting temperatures of each ASO:RNA duplex. Thermal melting curves of ASOs hybridized to target *acpP* RNA (10 nt). At least three cycles of melting and cooling have been performed for all the samples

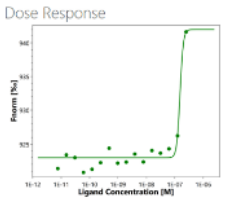
A

RNA OMe



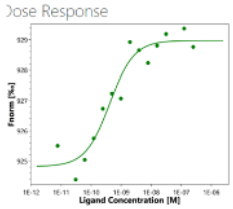
Response Evaluation: On Time 1.5s
Kd model
Unbound 912
Bound 917
EC50 3.94 nM
Hill Coeff. 0.4
Response Amplitude: 5.0
Noise: 0.8
Signal to Noise Ratio: 6.5

RNA F



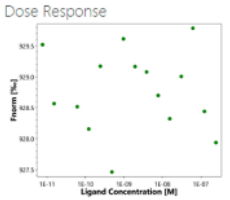
Response Evaluation: On Time 1.5s
Kd model
Unbound 923
Bound 942
EC50 153 nM
Hill Coeff. 7.7
Response Amplitude: 19.0
Noise: 1.1
Signal to Noise Ratio: 17.5

RNA MOE



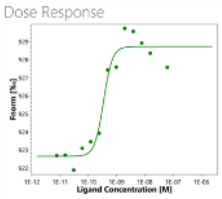
Response Evaluation: On Time 1.5s
Kd model
Unbound 924.8
Bound 929
EC50 413 pM
Hill Coeff. 1.1
Response Amplitude: 4.2
Noise: 0.4
Signal to Noise Ratio: 9.3

DNA PTO



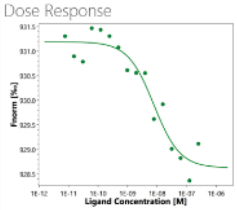
Response Evaluation: On Time 1.5s
Kd model
Unbound N/A
Bound N/A
EC50 N/A
Hill Coeff. N/A
Response Amplitude: N/A
Noise: N/A
Signal to Noise Ratio: N/A

LNA



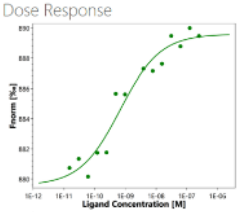
Response Evaluation: On Time 1.5s
Kd model
Unbound 922.6
Bound 928.7
EC50 348 pM
Hill Coeff. 2.8
Response Amplitude: 6.1
Noise: 0.7
Signal to Noise Ratio: 9.2

PMO



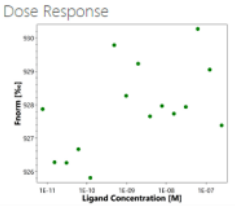
Response Evaluation: On Time 1
Kd model
Unbound 931.2
Bound 928.6
EC50 7.79 nM
Hill Coeff. 1.0
Response Amplitude: 2.6
Noise: 0.3
Signal to Noise Ratio: 8.9

PNA-*acpP*



Response Evaluation: On Time 1.5s
Kd model
Unbound 879.6
Bound 889.6
EC50 675 pM
Hill Coeff. 0.7
Response Amplitude: 10.0
Noise: 0.8
Signal to Noise Ratio: 12.4

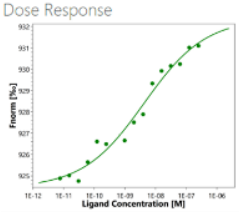
PNA-*scr*



Response Evaluation: On Time 1.5s
Kd model
Unbound N/A
Bound N/A
EC50 N/A
Hill Coeff. N/A
Response Amplitude: N/A
Noise: N/A
Signal to Noise Ratio: N/A

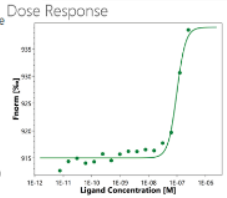
B

RNA OMe



Response Evaluation: On Time
Kd model
Unbound 924.4
Bound 932.4
EC50 4.21 nM
Hill Coeff. 0.4
Response Amplitude: 8.0
Noise: 0.4
Signal to Noise Ratio: 21.4

RNA F



Response Evaluation: On Time 1.5s
Kd model
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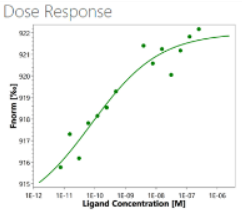
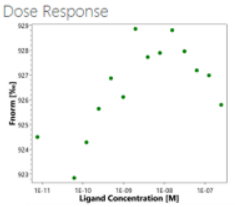
Bound 939
EC50 98.5 nM
Hill Coeff. 2.8
Response Amplitude: 24.0
Noise: 1.1
Signal to Noise Ratio: 21.1

RNA MOE



Response Evaluation: On Tim
Kd model
Unbound 913.6
Bound 922
EC50 77.4 pM
Hill Coeff. 0.4
Response Amplitude: 8.4
Noise: 0.6
Signal to Noise Ratio: 14.7

DNA PTO



Response Evaluation: On Time 1.5s
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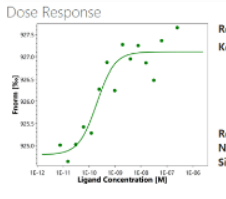
Response Amplitude: N/A
Noise: N/A
Signal to Noise Ratio: N/A

LNA



Response Evaluation: On Time 1.5s
Kd model
Unbound 924.8
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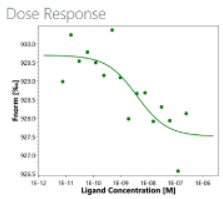
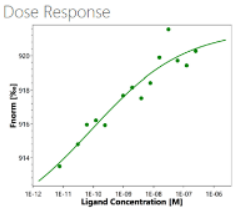
EC50 197 pM
Hill Coeff. 1.2
Response Amplitude: 2.3
Noise: 0.3
Signal to Noise Ratio: 6.7

PMO



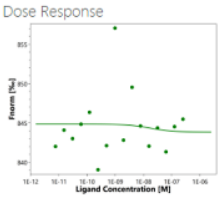
Response Evaluation: On Time 1.5s
Kd model
Unbound 929.7
Bound 927.5
EC50 3.93 nM
Hill Coeff. 0.8
Response Amplitude: 2.2
Noise: 0.6
Signal to Noise Ratio: 3.9

PNA-*acpP*



Response Evaluation: On Time 1.5s
Kd model
Unbound 909.7
Bound 921.6
EC50 84.1 pM
Hill Coeff. 0.3
Response Amplitude: 11.9
Noise: 0.7
Signal to Noise Ratio: 16.5

PNA-*scr*



Response Evaluation: On Time 2.5s
Kd model
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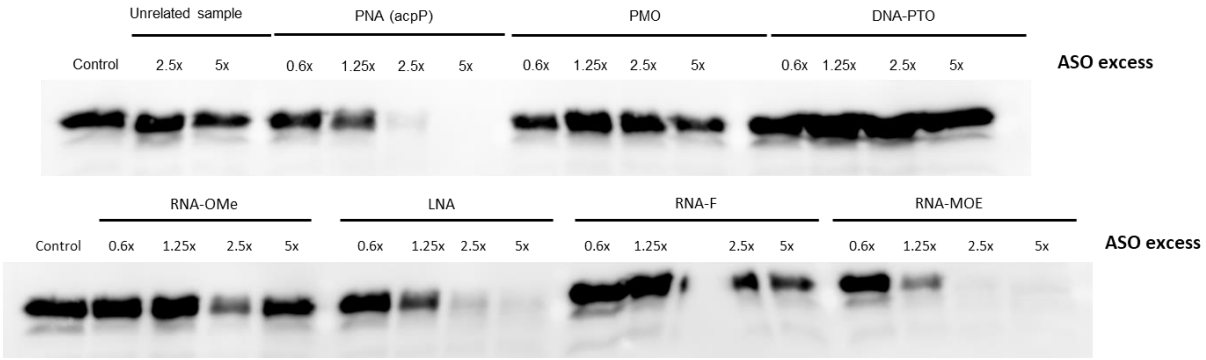
Bound 843.8
Kd 14.5 nM
TargetConc 5 nM
Response Amplitude: 1.0
Noise: 4.1
Signal to Noise Ratio: 0.3

Supplementary Figure S4. Microscale thermophoresis assay. PNA with a scrambled sequence was used as a negative control. Data of two independent experiments (presented in panel A and B) were analyzed to obtain ΔFnorm values using the MO Affinity Analysis software (NanoTemper Technologies Gmbh, Munich, Germany). The fitting for EC_{50} values was done with the Hill equation using the MO Affinity Analysis software. The EC_{50} values presented in Table 1 are the mean ± S.D. of these two independent experiments. Please note that the opposite trend of the binding curve observed for the PMO sample is a common occurrence in MST technology. The 'direction' of the curve is dependent on the specific properties of the measured molecules and does not influence the analysis.

Repeat 1



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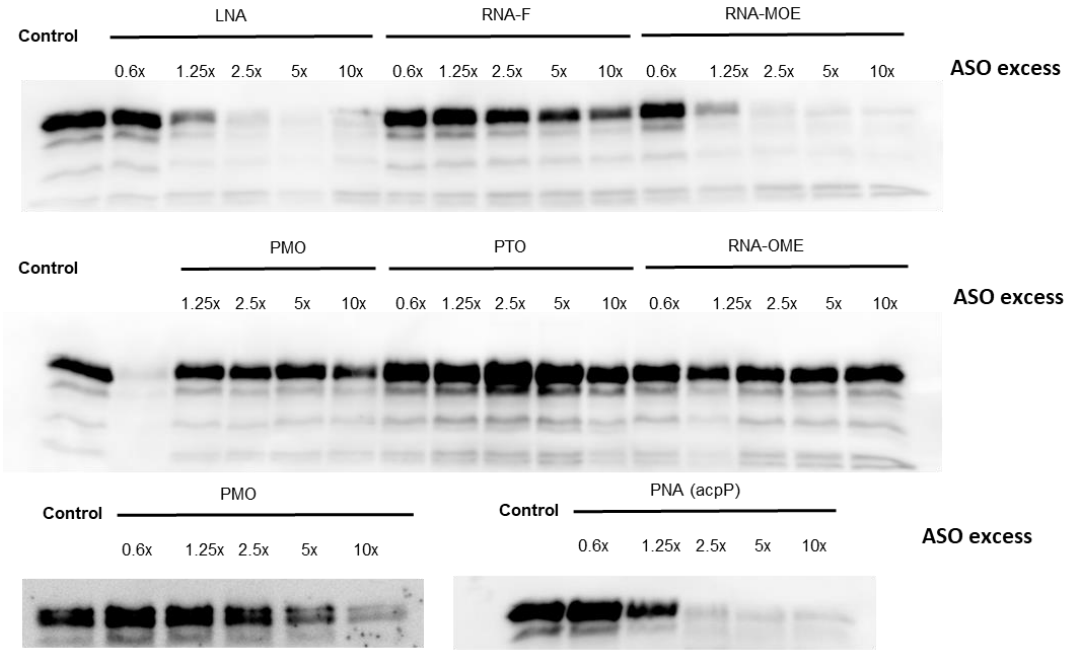
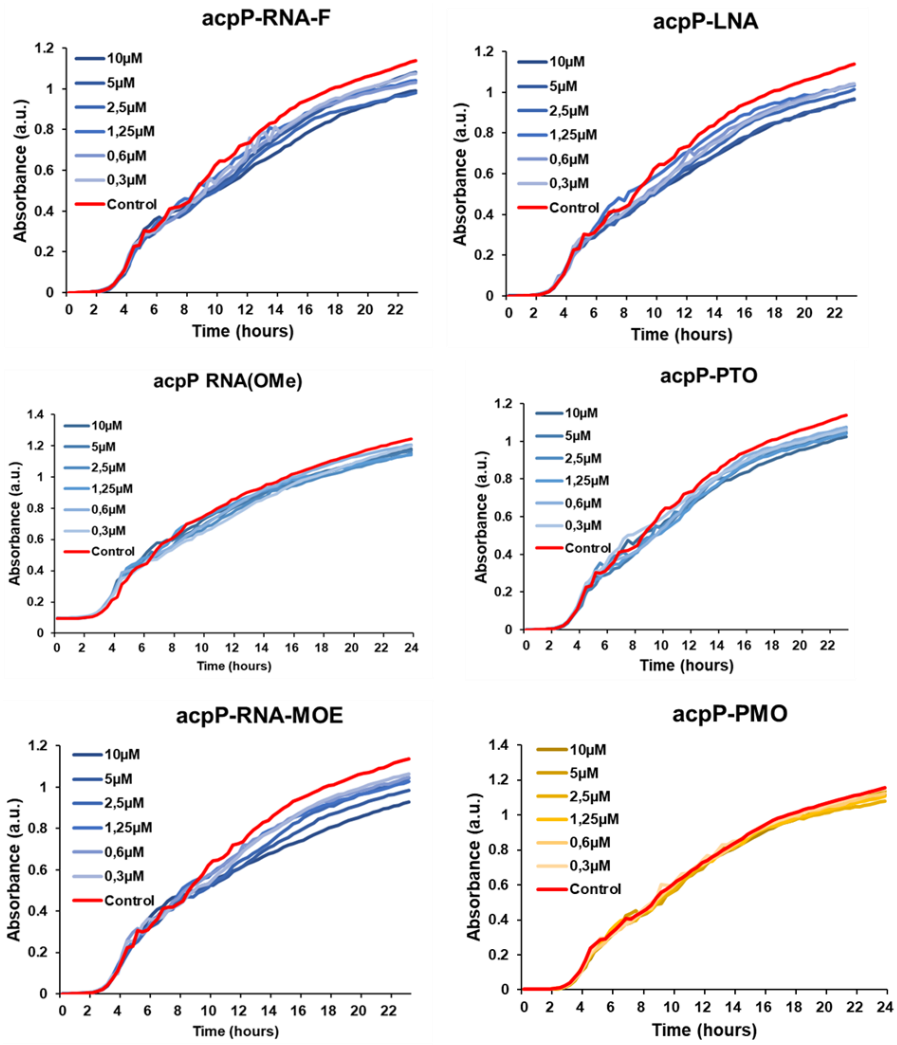


Supplementary Figure S5. *In vitro* translation assay. Western blot analysis of *in vitro* translation reactions using the acpP(1-17)::gfp mRNA as template and different ASO concentrations ranging from 0.625 µM to 10 µM as indicated. Blots were probed with anti-GFP antibody. Results from two independent *in vitro* translation experiments are presented. Control used here is water. In repeat 2, the PMO sample was run a third time to include the 0.6x concentration and this gel was used for the quantitation presented in Figure 3.

A



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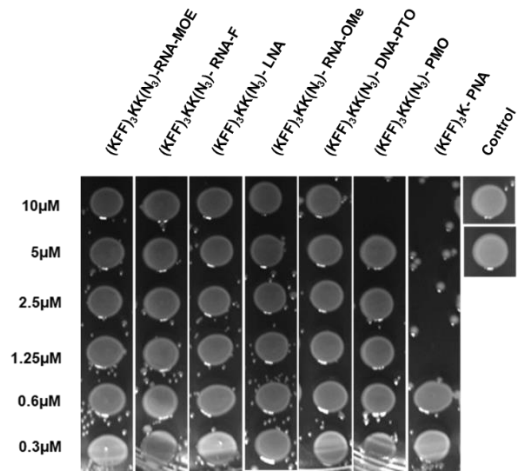
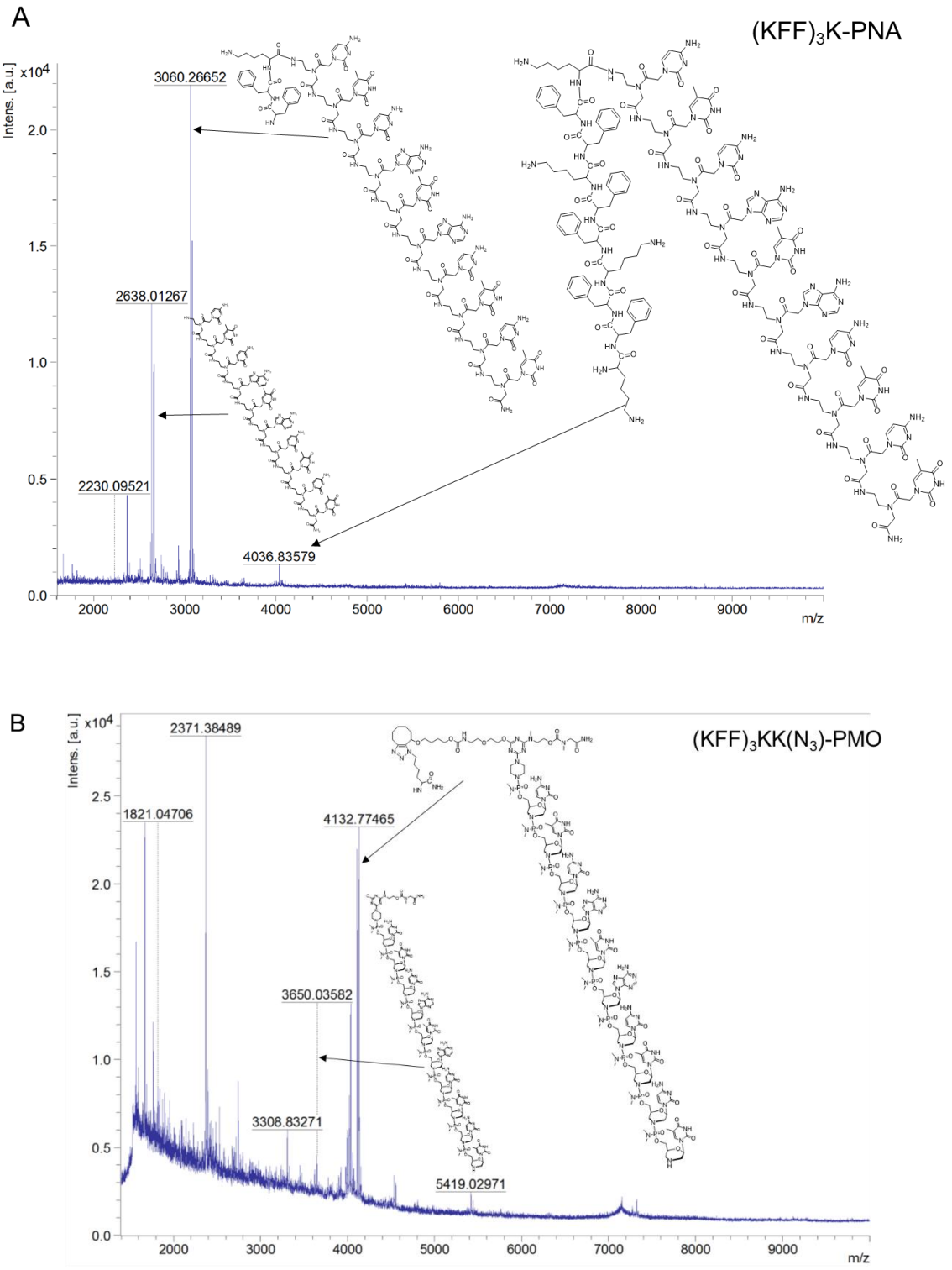


Supplementary Figure S6. Antibacterial assays. A. Growth kinetics of *Salmonella* treated with unconjugated ASOs. Unconjugated PNA is not included here, but was previously shown to be inactive against Salmonella (see *Nucleic Acids Res,* 2021, 49, 4705) **B.** Spotting assay of *Salmonella* treated with KFF-ASO conjugates.



Supplementary Figure S7. Detection of fragments of KFF conjugates of PNA and PMO in the periplasm of Salmonella. MALDI-TOF based detection of fragments of KFF conjugates of PNA (A) and PMO (B) in the periplasm of *Salmonella* 15 minutes post-treatment.

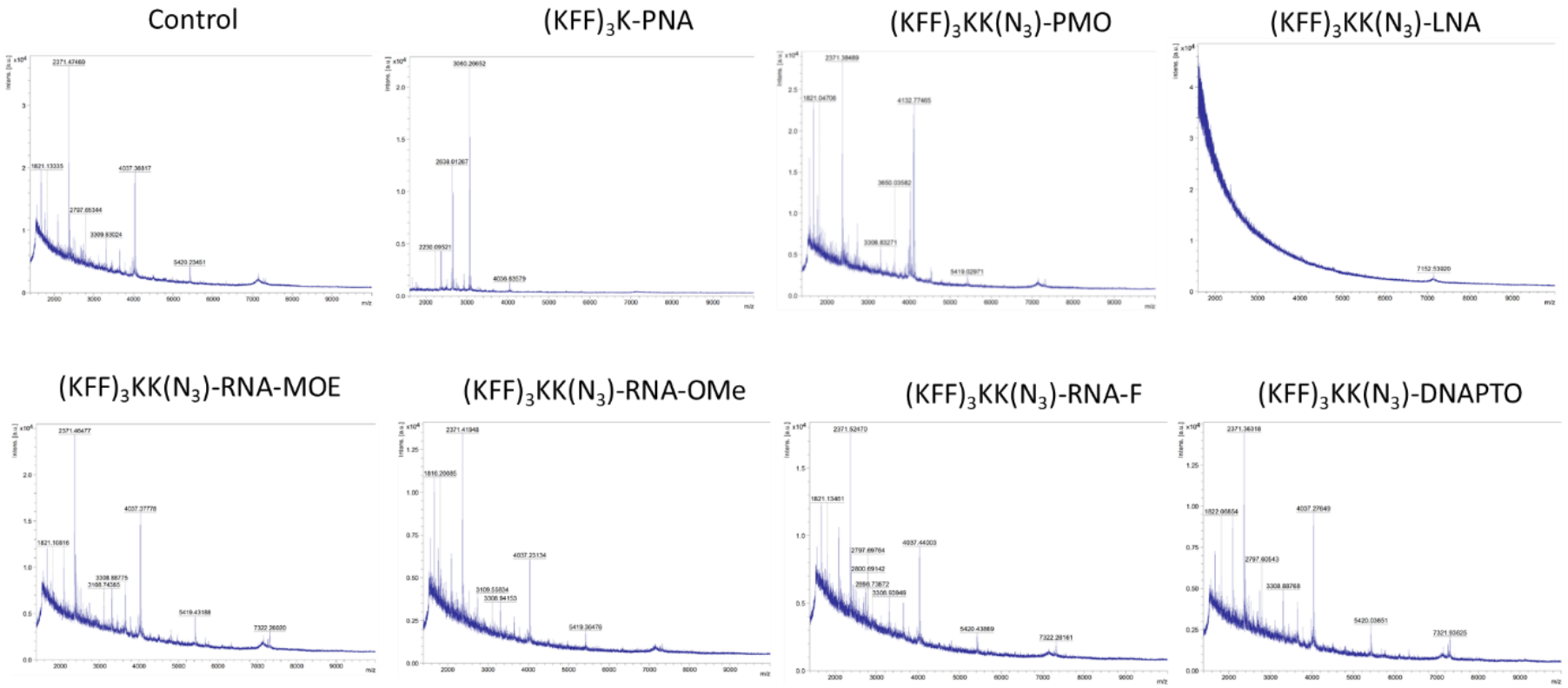


Figure S8: MALDI-TOF spectra of the periplasm of *Salmonella* post-treatment with different KFF-ASO conjugates.

Supplementary Table S1. **Oligonucleotide sequences used for the generation of the AcpP(1-17)::GFP fusion construct.** Table includes internal oligonucleotide number (JVO-), target gene name, and the respective oligonucleotide sequence in 5' to 3' orientation. Fw: forward, rv: reverse. Underlined sequence: T7 promoter sequence, italic sequence: overlap with *gfp*.

Oligo (JVO-)	Gene	Sequence (5´-3´)
19760	*acpP*-fw	TAATACGACTCACTATAGAACCATCGCGAAAGCGAGTT
19761	*acpP*-rv	*TCCAGTGAAAAGTTCTTCTCCTTTGCTAGC*GCCCAGCTGTTCGCCGATAAT
19762	*gfp*-fw	GCTAGCAAAGGAGAAGAACTTTTCAC
19763	*gfp*-rv	TTATTTGTAGAGCTCATCCATGCC