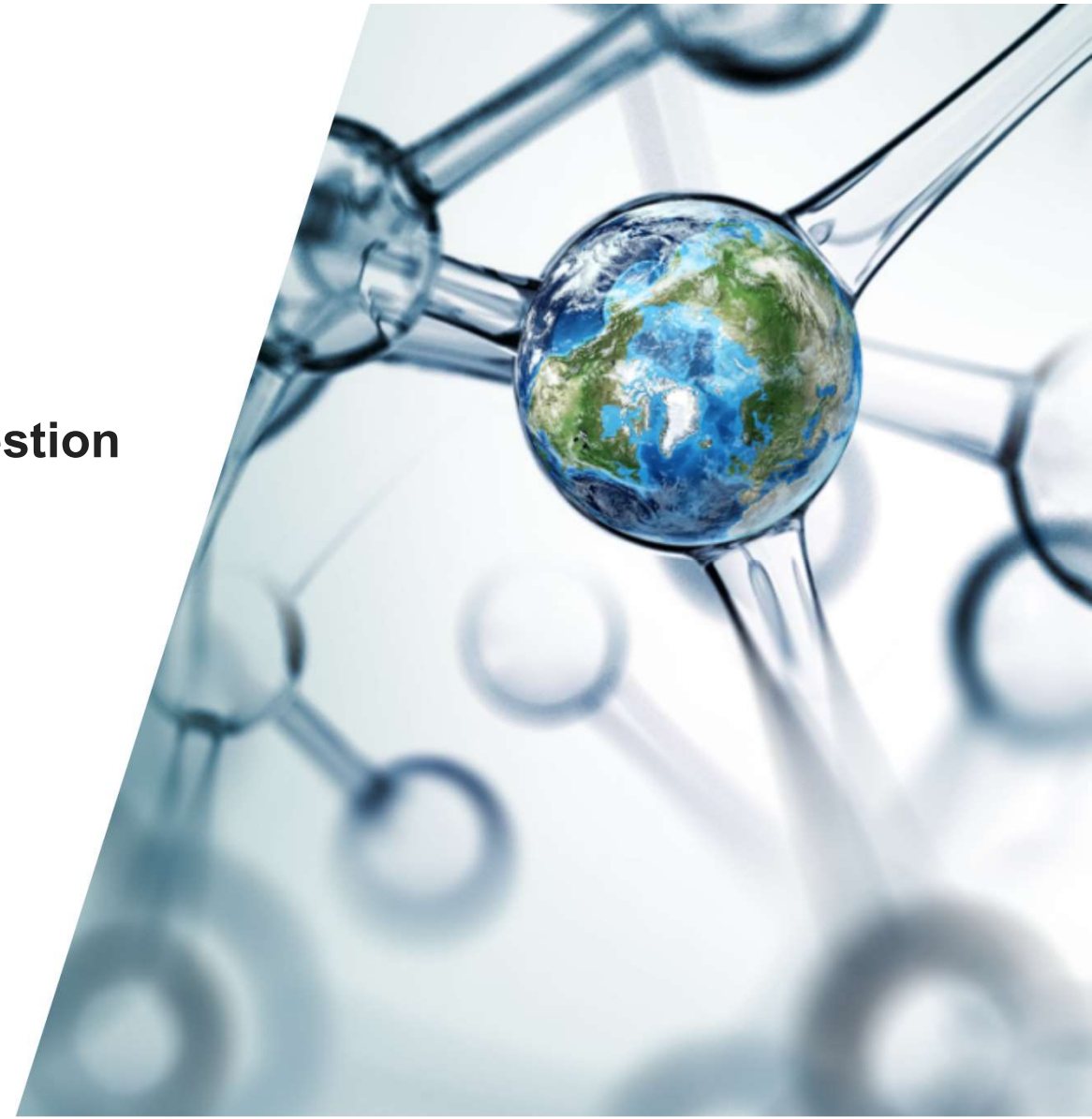
Characterizing 5’ and 3’ mRNA digestion products by LC-HRAM-MS/MS



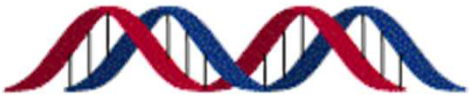
Robert L Ross
Senior Product Application Specialist
4/21/2023

The world leader in serving science

Central Dogma of Molecular Biology

"On Protein Synthesis" Francis Crick, London, 1957

Deoxyribonucleic Acid (DNA)



Ribonucleic Acid (RNA)

mRNA

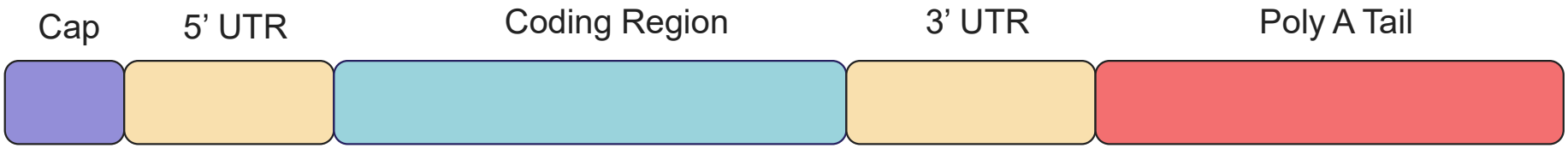
rRNA

tRNA

Protein

Anatomy of a mature mRNA

Carries the message



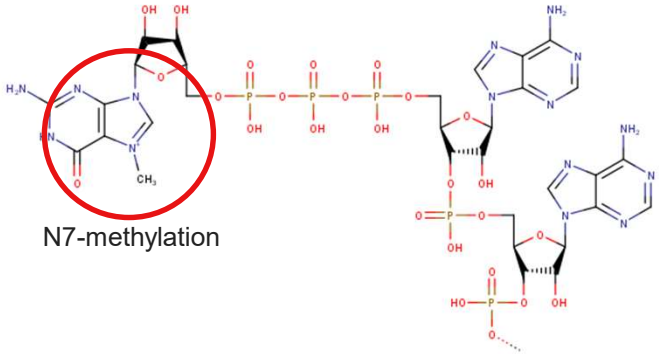
Multifunctional

- Nucleocytoplasmic transport
- Helps stabilization of mRNA against 5' exonucleolytic degradation
- Splicing
- Determinate for eLF-4E, promotes binding to the ribosome for translation
- Assists against 3' exonucleolytic degradation
- Stability of the strand
- Lengths are 100-150 bases

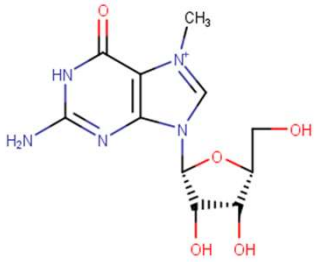
Anatomy of the cap structure

Identification between self and non-self

Cap(0) Structure

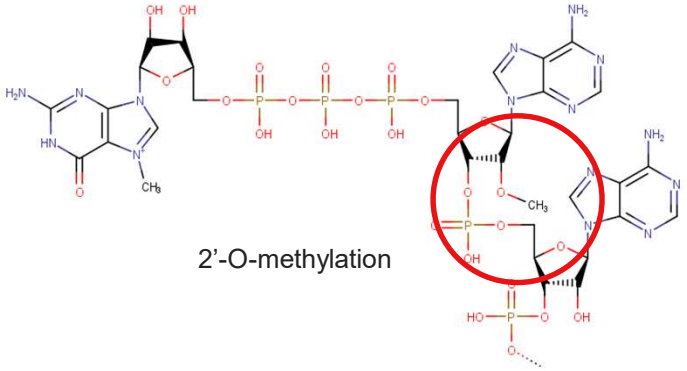


N7-methylation

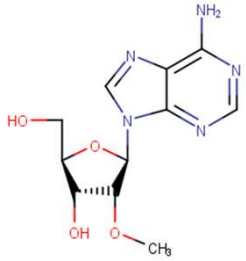


7-methylguanosine

Cap(1) Structure



2'-O-methylation

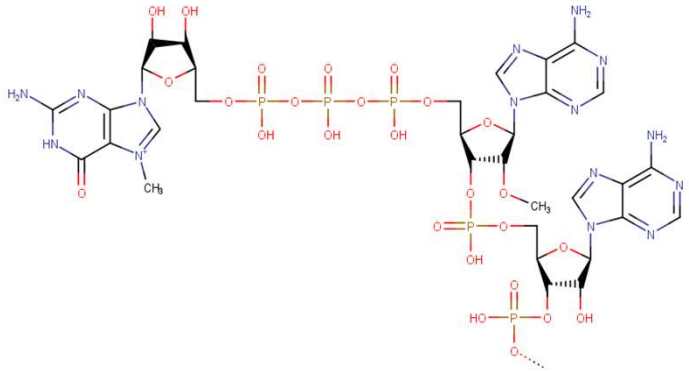


2'-O-methyladenosine

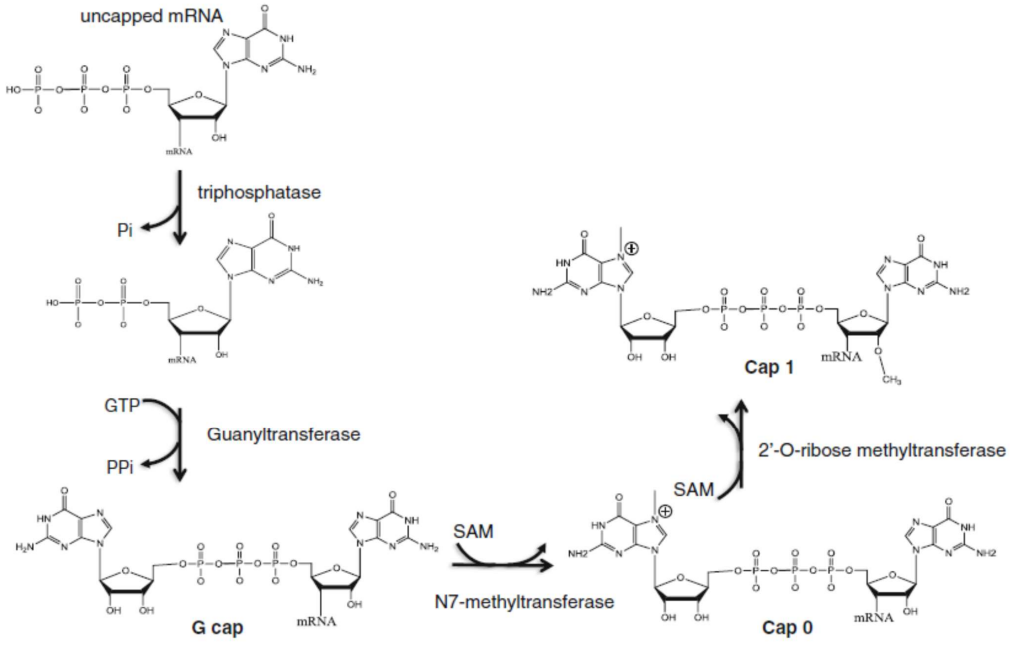
Capping systems

Two modes of capping for synthetic mRNA

TriLink Biotechnologies CleanCap



De novo synthesis



Beverly, M., Dell, A., Parmar, P. et al. Label-free analysis of mRNA capping efficiency using RNase H probes and LC-MS. Anal Bioanal Chem 408, 5021–5030 (2016).

Determination of 5’ capping efficiency

Two approaches

RNase H Digestion

- DNA/RNA chimeric probe
- Complementary to the 5’ end
- Cleavage HIS side chain Mg^{2+}

Beverly, M., et al. Label-free analysis of mRNA capping efficiency using RNase H probes and LC-MS. Anal Bioanal Chem 408, 5021–5030 (2016).

Ribozymes

- Short oligonucleotides
- Designed for 5’ end
- Cleavage coordinated metal ion
- Mg^{2+} or Mn^{2+}

Vlatkovic, I.; et.al., Ribozyme Assays to Quantify the Capping Efficiency of In Vitro-Transcribed mRNA. *Pharmaceutics* **2022**, *14*, 328.

RNase H Digestion

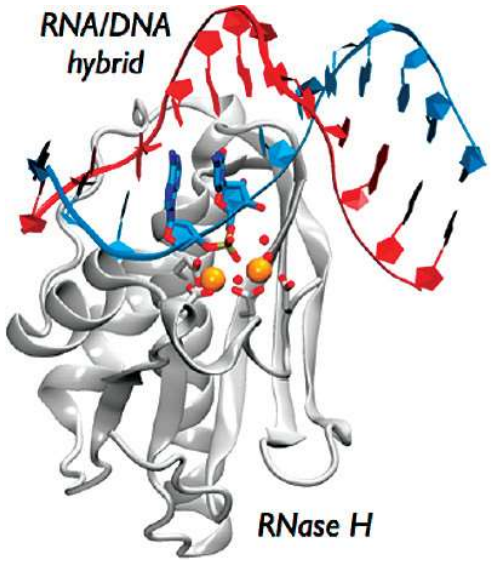
Cleaves the RNA strand in an RNA:DNA duplex

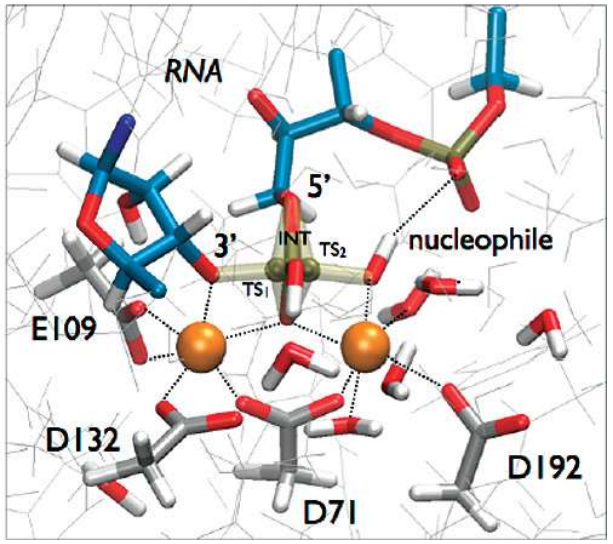
RNA:DNA Duplex

G A U C G A U C G A U C G A U C

C U A G C T A G C T A G C U A G

Minimum of 5 bases





J. Am. Chem. Soc. 2008, 130, 33, 10955-10962

Digestion and purification with RNase H

Biotinylation of probe provides purification

Hybridize probe to transcript

Bind to magnetic bead

Cleave with Rnase H

Isolate cleaved product

Separate oligomer from probe and analyze by LC-MS/MS

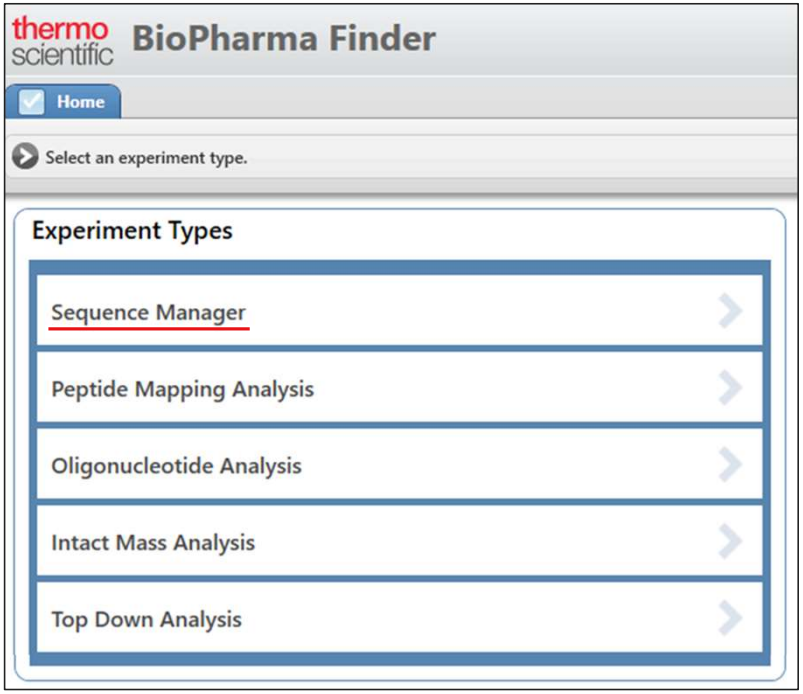
sequence for illustrative purposes only

Data Analysis

BioPharma Finder™

Data processing using BioPharma Finder™

BioPharma Finder™ is an easy-to-use tool for mapping your oligonucleotide data



Sequence Manager

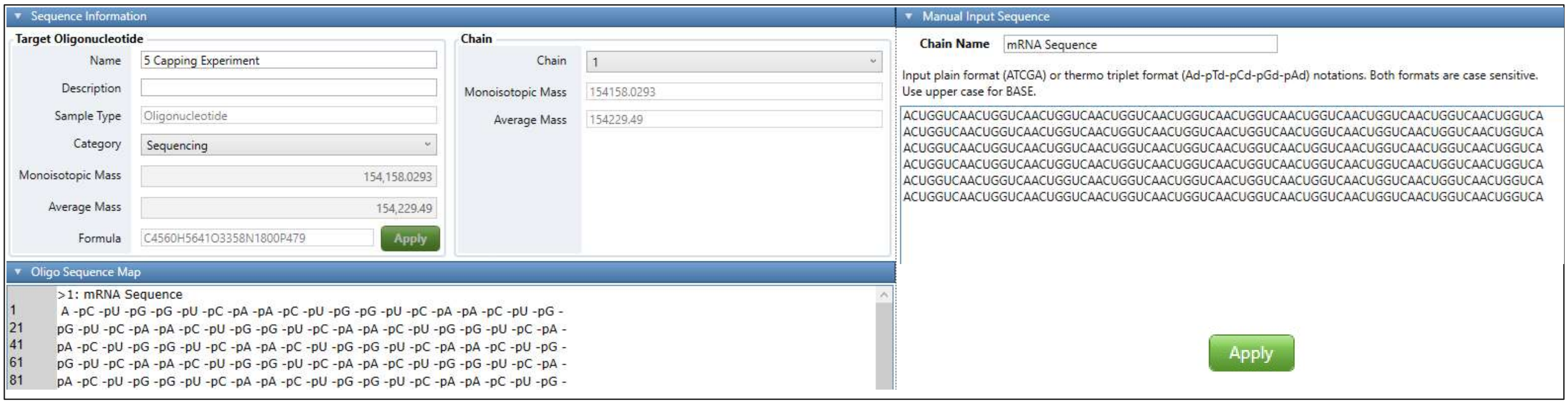
- Enter your mRNA sequence
- Build the modifications
- Add the modifications into the sequence

BioPharma Finder™ Sequence Manager

Input and modify your mRNA sequence

Sequence identifiers

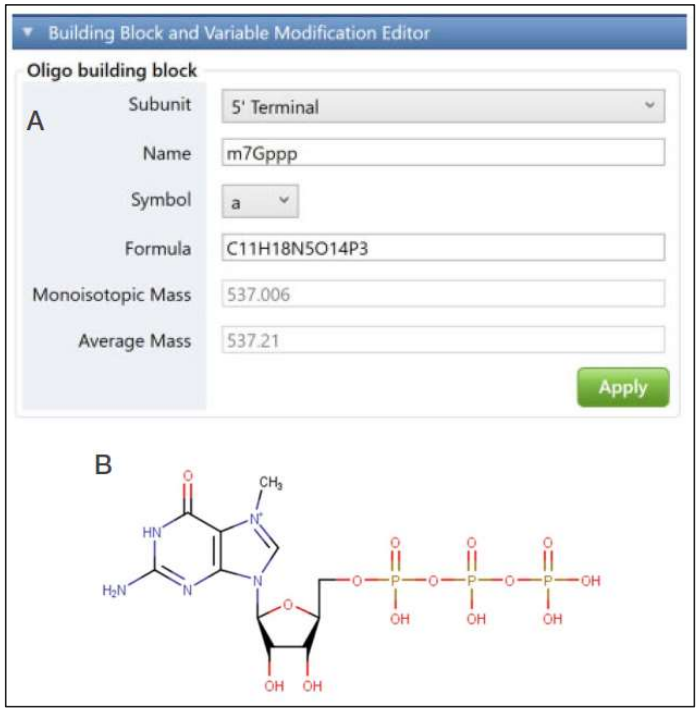
mRNA sequence

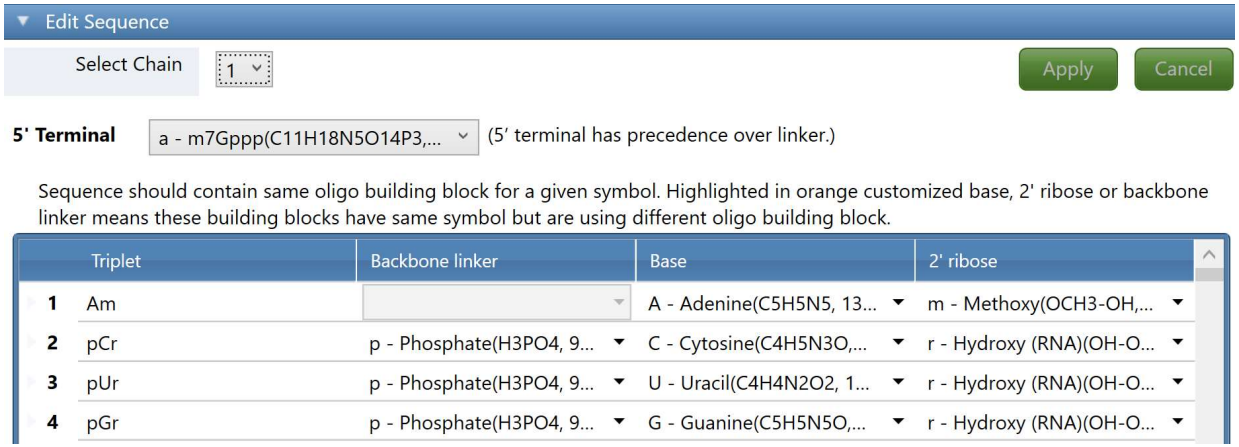


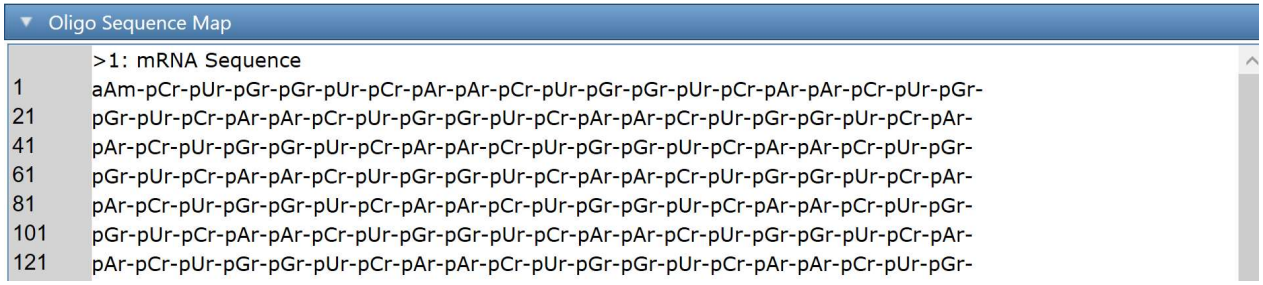
mRNA sequence in triplet format
5'→3'

Building Blocks & Sequence Editor

Build your custom modifications and add them to your sequence at any position

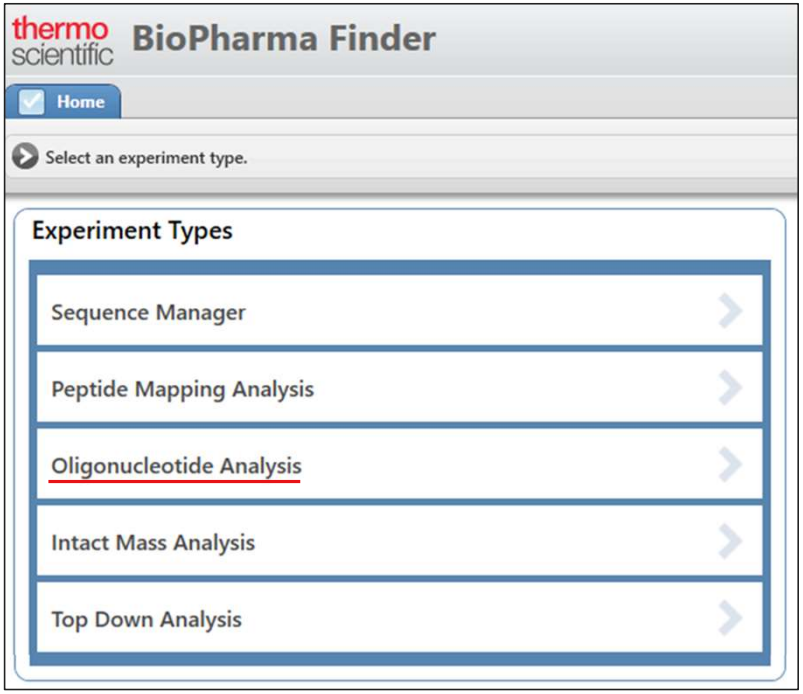






Data processing using BioPharma Finder™

BioPharma Finder™ is an easy-to-use tool for mapping your oligonucleotide data

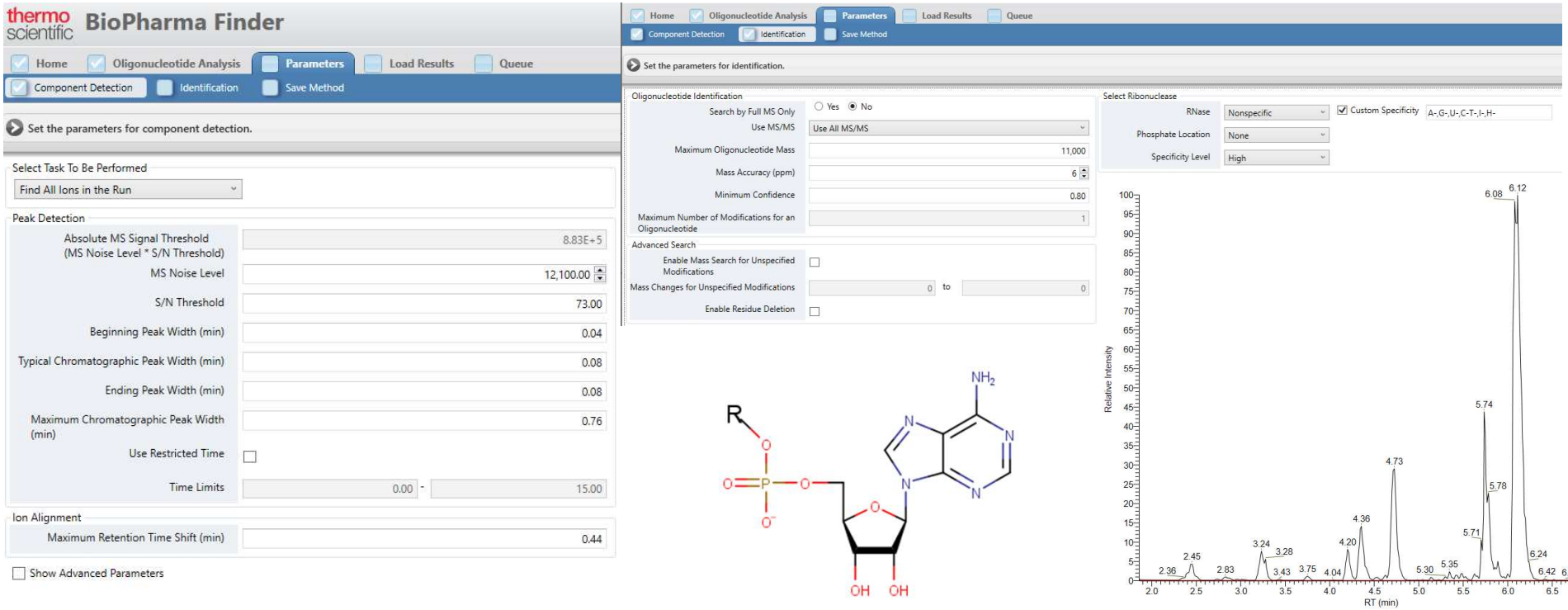


Oligonucleotide Analysis

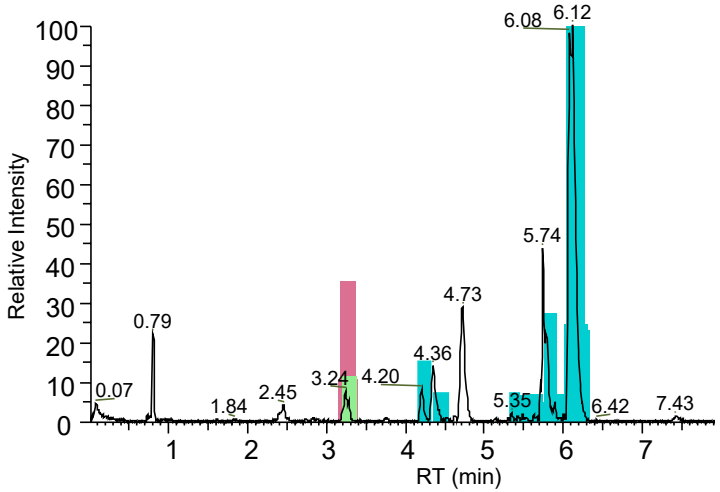
- Name the experiment/choose data file
- Choose the processing method
- Edit the method
  - Detection Parameters
  - Enzyme used

Editing the Method

Choice of detection parameters; nuclease

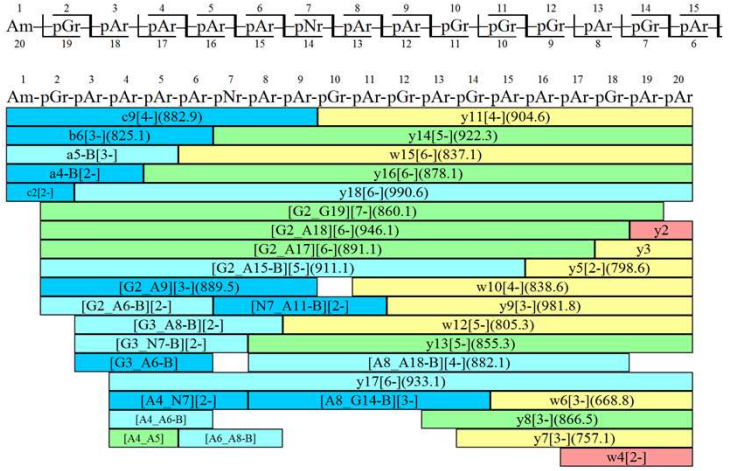


5’ capping analysis results



Chromatogram of RNase H digestion products. Blue highlighted peaks are mapped failure sequences of the probe, as well as the probe itself (6.12 min).

5’ Capped oligonucleotide

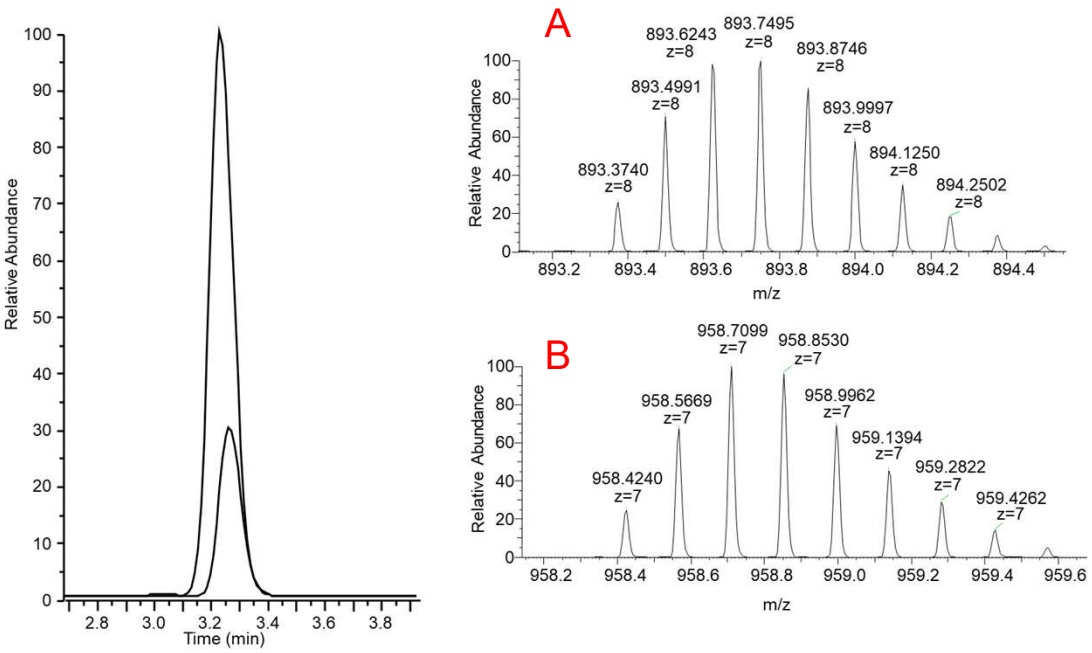


Fragment map of 5’capped oligonucleotide generated through RNase H digestion. The use of MS/MS provides for confidence when assigning oligonucleotide data.

Identification	Oligo Sequence	Mod	Site	Δ ppm	Conf. Score	ID Type	RT	M/Z	Charge St.	Mono Mass Exp.	Theor. Mass	Oligo
3:A1-U25 = 8452.478m	Am-pCm-pUm-pCm-pT...	(3' Biotin-TEG)	(U25)	0.46	100.0	MS2	6.10	767.672	-11	8452.4814	8452.4776	Probe
1:A1-A20 = 7155.0585m	Am-pGr-pGr-pAr-pAr-p...	(m7Gppp)	(A1)	-0.88	100.0	MS2	3.24	893.749	-8	7155.0522	7155.0585	Capped
3:A1-U25 = 8452.478m(Na+)	Am-pCm-pUm-pCm-pT...	Na+, (3' Biotin-TEG)	(U25)	-1.10	100.0	MS2	6.11	769.669	-11	8474.4502	8474.4595	Probe
3:A1-U25 = 8452.478m(Na+)	Am-pCm-pUm-pCm-pT...	Na+, (3' Biotin-TEG)	(U25)	-4.21	100.0	MS2	6.09	846.736	-10	8474.4238	8474.4595	Probe
3:U10-U25 = 5646.0240m	pUm-pUm-pCm-pUm-p...	(3' Biotin-TEG)	(U25)	-1.58	100.0	MS2	5.79	704.995	-8	5646.0151	5646.0240	Probe
1:A1-A20 = 7155.0585m(Na+)	Am-pGr-pGr-pAr-pAr-p...	Na+, (m7Gppp)	(A1)	-1.49	99.9	MS2	3.26	896.496	-8	7177.0298	7177.0405	Capped
3:A1-U25 = 8452.478m	Am-pCm-pUm-pCm-pT...	(3' Biotin-TEG)	(U25)	-0.70	100.0	MS2	6.07	1690.288	-5	8452.4717	8452.4776	Probe
2:A1-A20 = 6716.0294m	Am-pGr-pGr-pAr-pAr-p...	(Phosphate)	(A1)	-1.77	100.0	MS2	3.26	958.710	-7	6716.0176	6716.0294	Uncapped

5’ capping analysis results

Relative quantification of capped vs uncapped



LC-MS data from RNase H digestion. Top five most abundant peaks were used to generate the extracted mass for both capped (A) and uncapped (B) digestion products.

- Highlight accuracy and robustness
- 1:4 ratio of uncapped to capped
- Top 5 most abundant isotopic peaks in the capped and uncapped spectra
- Peak areas of the resulting extracted ion chromatograms
- Theoretical 25%
- Observed 27.9%
- Ratio <3% of theoretical

Characterization of mRNA 3’ poly(A) tail

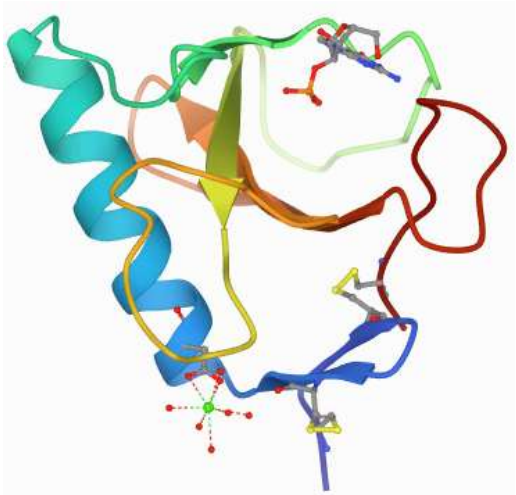
Digestion with RNase T1

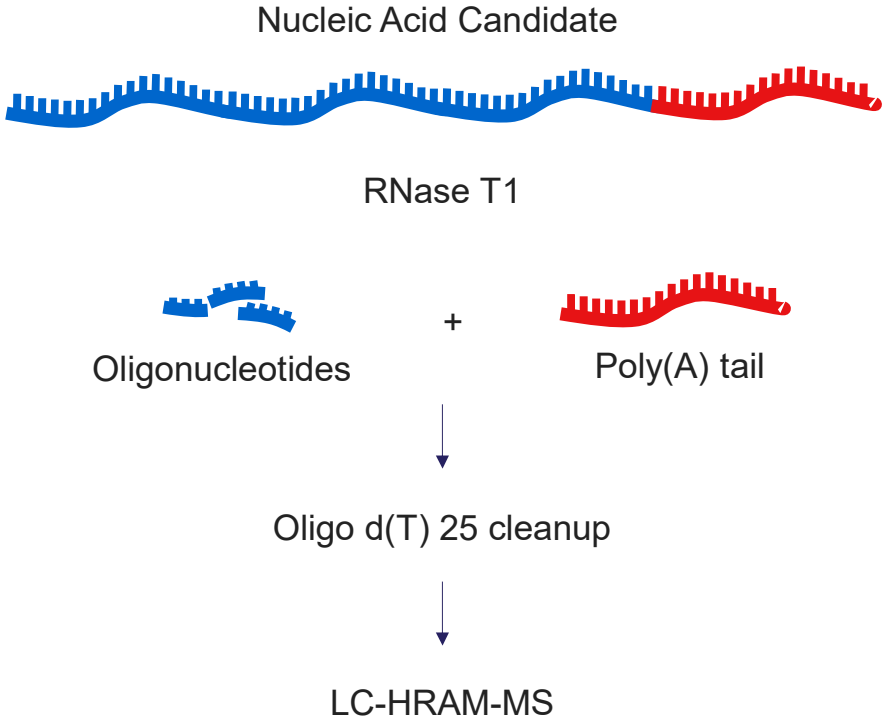
3’ poly(A) tail characterization strategy

Full RNase T1 digest

Ribonuclease T1

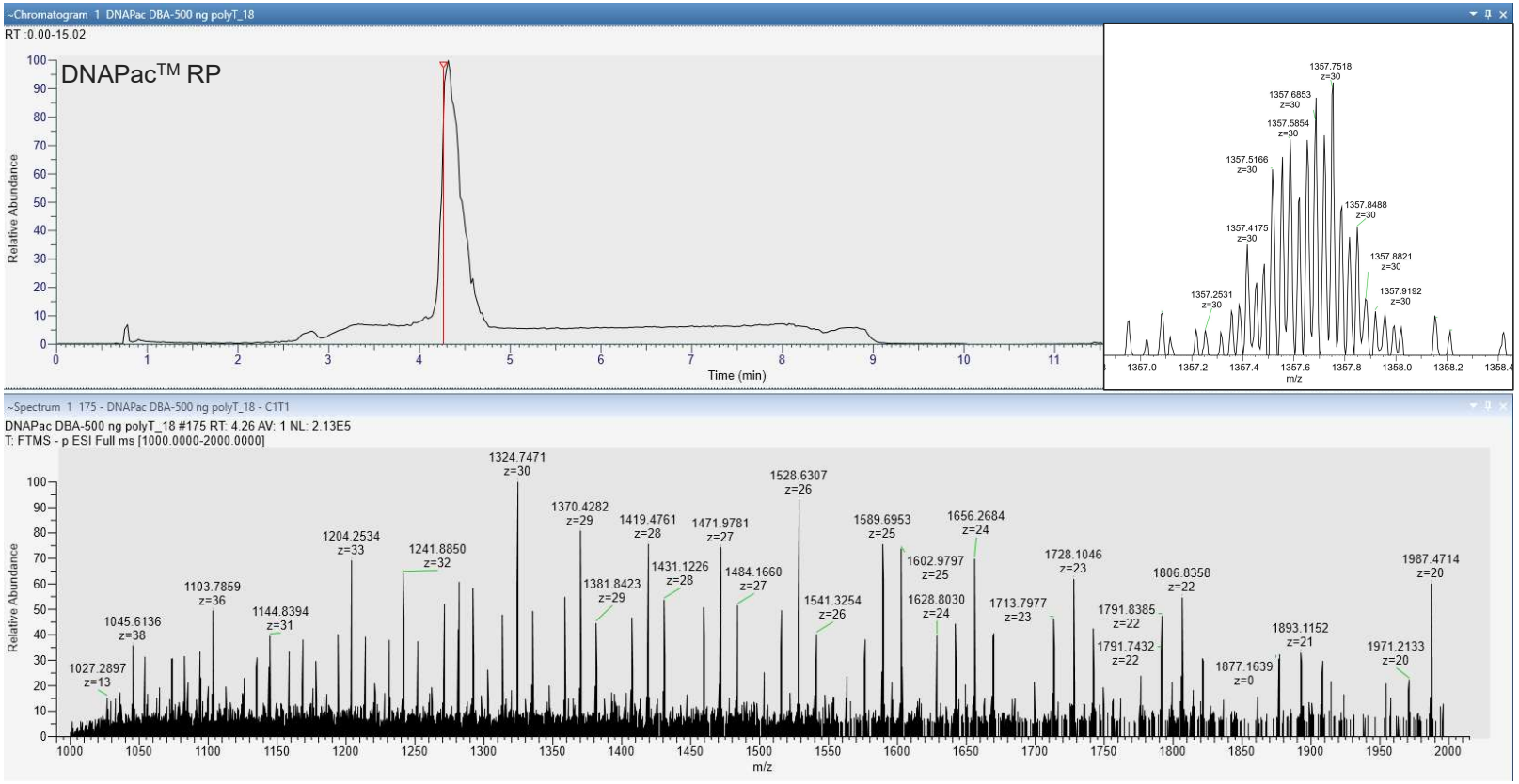
- Small protein 104 residues
- Contains two disulfide bonds
- Cleaves primarily at G in ssRNA





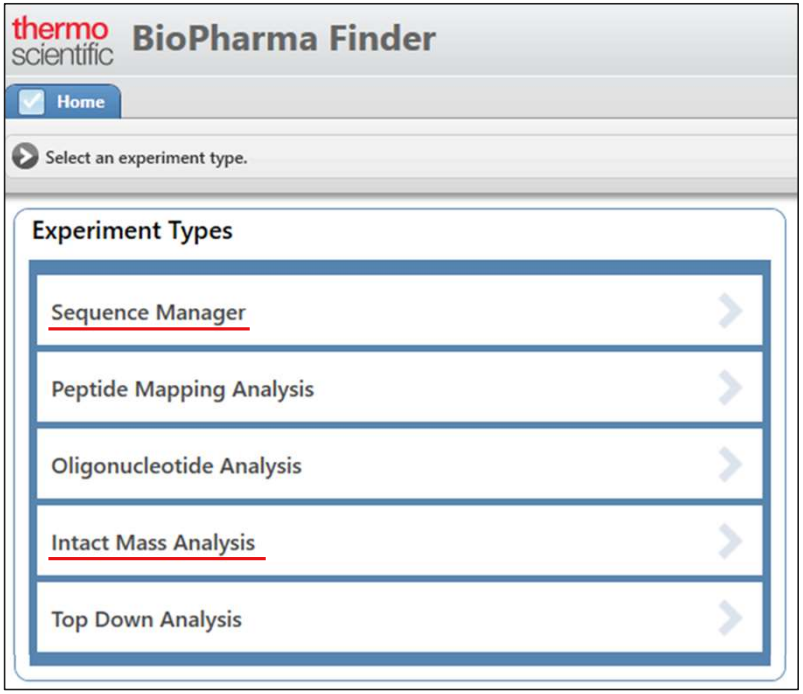
3’ poly(A) tail characterization

Orbitrap Exploris™ 240 data from T1 digestion of 1kb mRNA



3’ characterization using BioPharma Finder™

Deconvolution of the poly(A) pool using isotopically resolved data



Sequence Manager

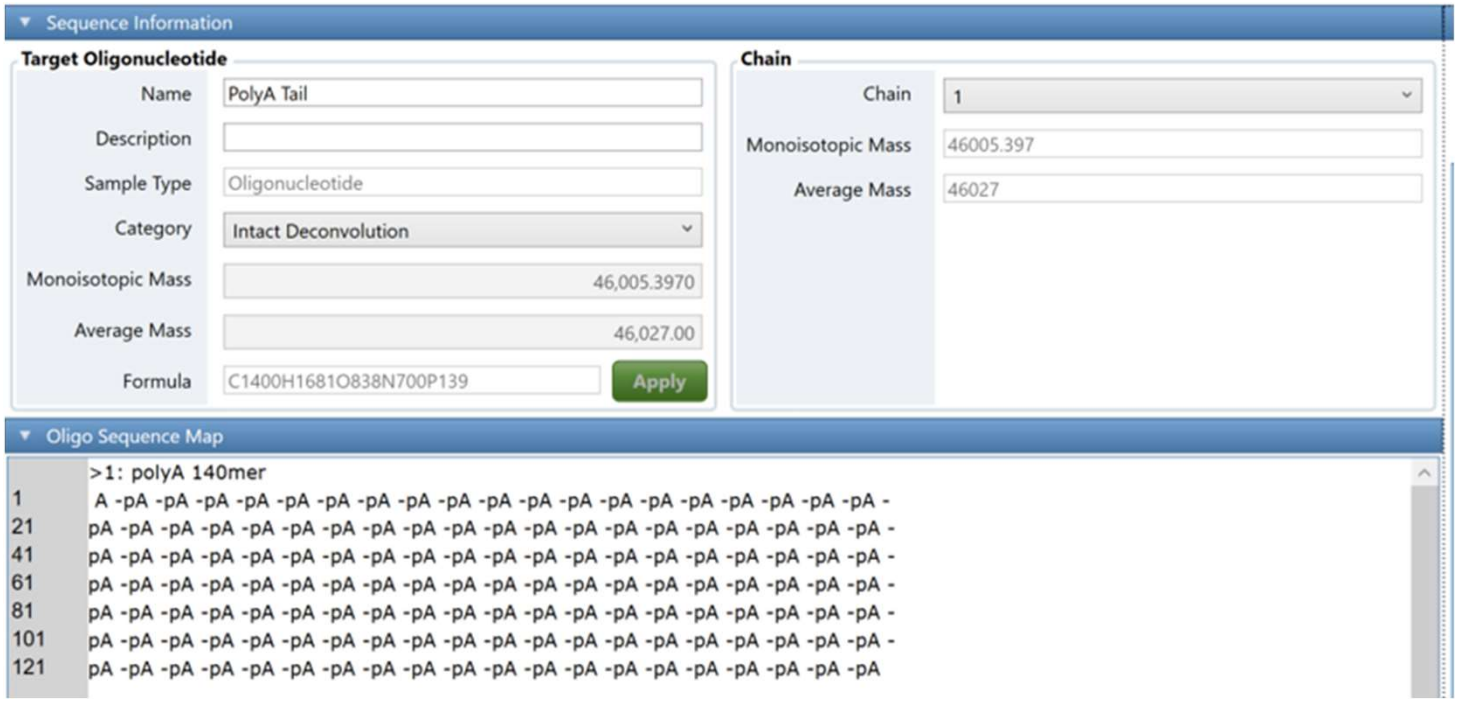
- Enter your poly(A) sequence

Intact Mass Analysis

- Name the experiment/choose data file
- Edit the method - truncation

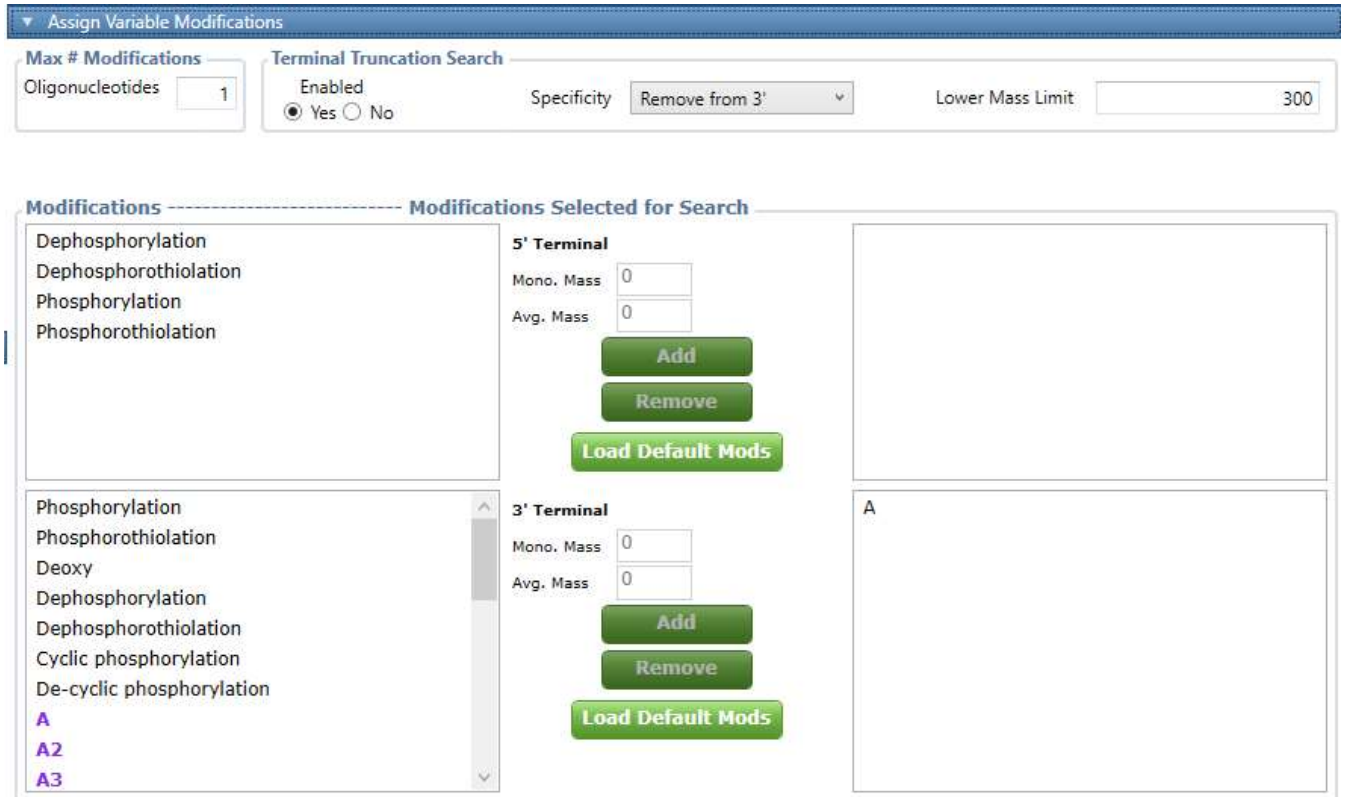
3’ characterization using BioPharma Finder™

A longer sequence provides a guide to measure deconvolution results against



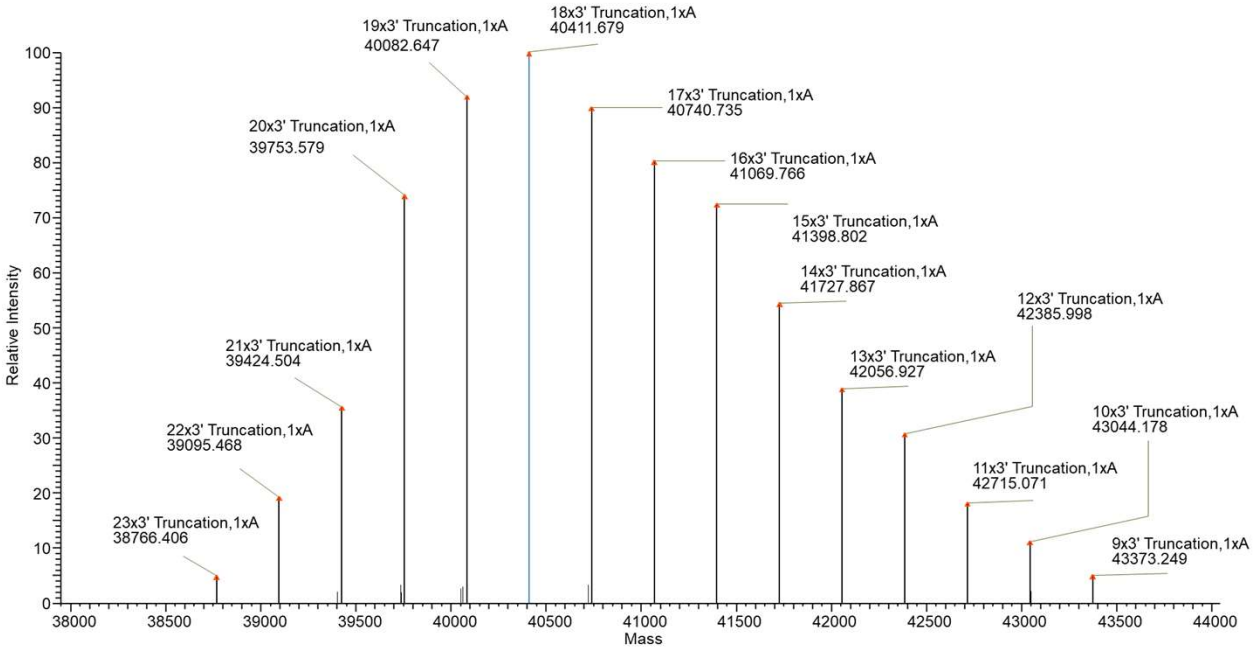
Assign Variable Modifications

Terminal truncation search with 3’ variable modification of adenosine



Xtract deconvolution of mRNA 3’ tail

Intact Mass Analysis

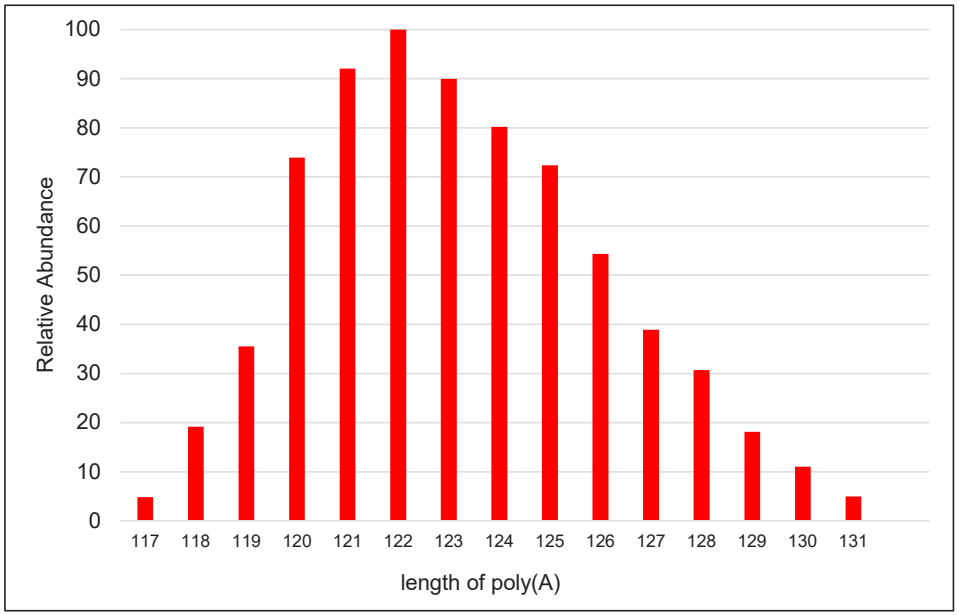


		Modification	Monoisotopic Mass	Theoretical Mass (Da)	Matched Mass Error (ppm)	Relative Abundance
		Aa	=	=	=	> 3
1		9x3'Truncation,1xA	43373.163	43372.977	4.3	5.73
2		22x3'Truncation,1xA	39095.467	39095.295	4.4	18.76
3		21x3'Truncation,1xA	39424.523	39424.347	4.5	31.11
4		20x3'Truncation,1xA	39753.581	39753.400	4.6	66.56
5		19x3'Truncation,1xA	40082.670	40082.452	5.4	93.14
6		18x3'Truncation,1xA	40411.680	40411.505	4.3	100.00
7		17x3'Truncation,1xA	40740.733	40740.557	4.3	89.05
8		16x3'Truncation,1xA	41069.795	41069.610	4.5	82.28
9		15x3'Truncation,1xA	41398.820	41398.662	3.8	72.34
10		14x3'Truncation,1xA	41727.900	41727.715	4.4	54.18
11		13x3'Truncation,1xA	42056.945	42056.767	4.2	37.16
12		12x3'Truncation,1xA	42385.984	42385.820	3.9	31.54
13		11x3'Truncation,1xA	42715.076	42714.872	4.8	20.49
14		10x3'Truncation,1xA	43044.201	43043.925	6.4	9.38

Deconvolution result of poly(A) sample with Biopharma Finder™ 5.0 software. Identification is a measure of the loss of the mass of AMP from a theoretical 140-mer and listed as a truncation

Poly(A) tail length distribution

Deconvoluted monoisotopic mass divided by AMP



Deconvoluted monoisotopic masses are divided by the mass of AMP and plotted against the relative abundance

Mono. Mass	MI/330	Theo. Mass (Da)	Mass Error (ppm)	Rel. Abundance
38766.406	117	38766.242	4.2	4.81
39095.468	118	39095.295	4.4	19.15
39424.504	119	39424.347	4.0	35.51
39753.579	120	39753.400	4.5	73.96
40082.647	121	40082.452	4.9	92.00
40411.679	122	40411.505	4.3	100.00
40740.735	123	40740.557	4.4	89.93
41069.766	124	41069.610	3.8	80.20
41398.802	125	41398.662	3.4	72.38
41727.867	126	41727.715	3.6	54.34
42056.927	127	42056.767	3.8	38.92
42385.998	128	42385.820	4.2	30.66
42715.071	129	42714.872	4.6	18.09
43044.178	130	43043.925	5.9	11.04
43373.249	131	43372.977	6.3	4.97

Summary

- IPRP-LC-HRAM-MS with a BioPharma Finder™ data processing solution provides a robust analytical platform for mRNA characterization
  - RNase H enzymatic digestion workflow for analysis of 5’ end products
    - Duplex design allows control of cleavage site
    - Biotinylated probe for purification
  - RNase T1 digestion for poly(A) tail characterization
    - Robust and well studied enzyme (inexpensive)
    - Cleaves primarily at guanosines
    - Oligo $d(T)_{25}$ purification

https://www.thermofisher.com/vaccines

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END