Absolute quantitative analysis of modified ribonucleosides in tRNA and mRNA using Skyline

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Proteomics Center
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STOWERS INSTITUTE
FOR MEDICAL RESEARCH

Acknowledgement

Skyline

Brian Pratt

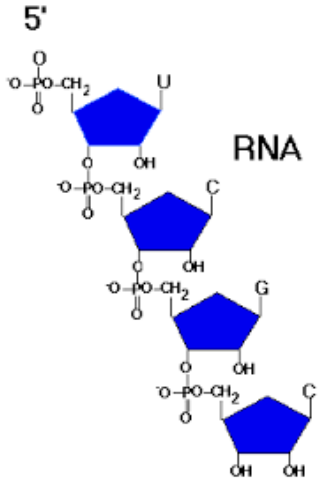
Laurence Florens

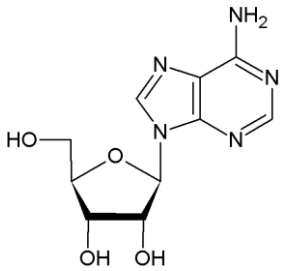
Absolute quantitative analysis of modified ribonucleosides in tRNA and mRNA using Skyline

- Introduction
  - Why study modified ribonucleosides?
- Instrumentation and Methods
  - Nucleoside standards
  - Calibration curves
  - Positional isomers
- Application
  - Targeted analysis of global modification levels in eukaryotic mRNA
- Summary

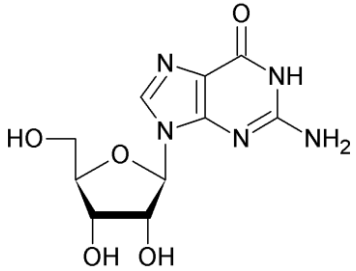
Why?

Epitranscriptomics is a link between epigenomics and proteomics

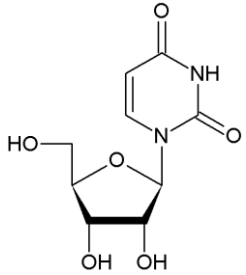




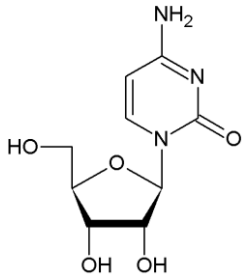
adenosine



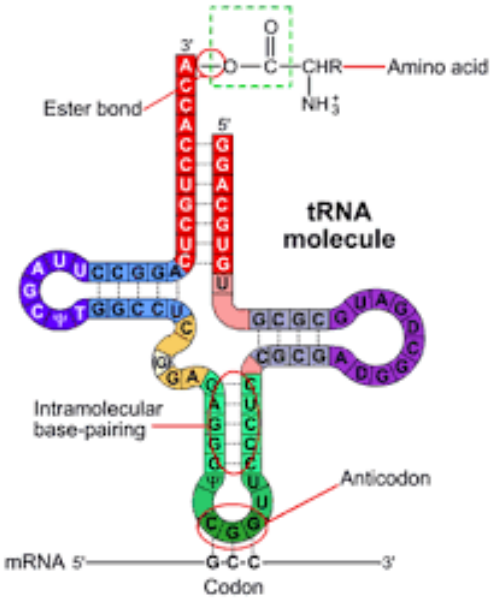
guanosine



uridine

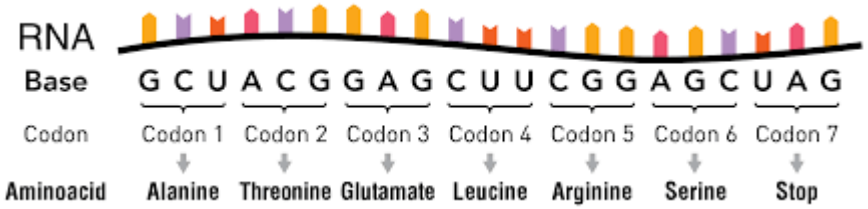


cytidine



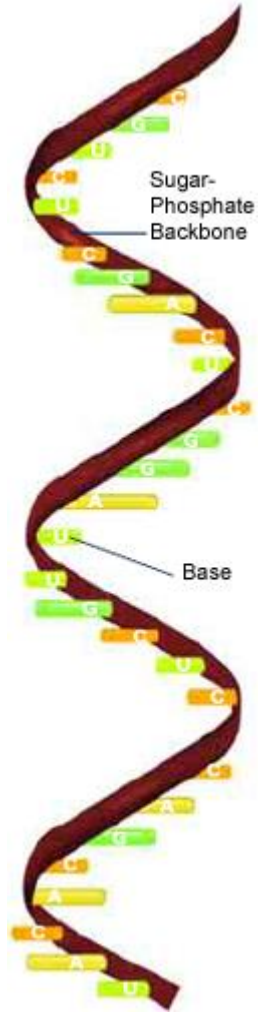
A G U C

genomics



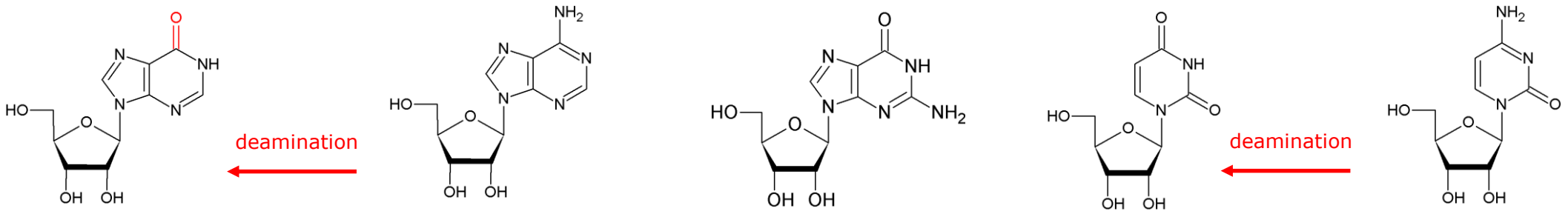
transcriptomics

proteomics



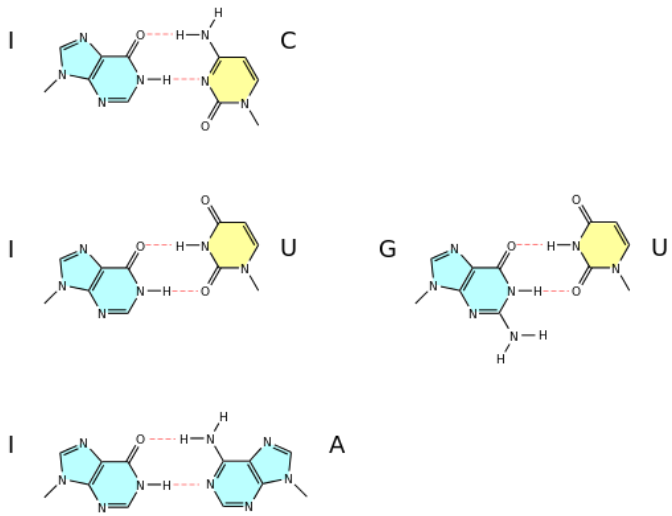
Why?

Crick's Wobble Hypothesis



inosine adenosine guanosine uridine cytidine

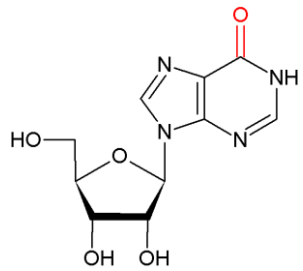
FIRST	SECOND U		SECOND C		SECOND A		SECOND G		THIRD, WOBBLE
U	UUU	Phe	UCU	Ser	UAU	Tyr	UGU	Cys	U
	UUC	Phe	UCC	Ser	UAC	Tyr	UGC	Cys	C
	UUA	Leu	UCA	Ser	UAA	Stop	UGA	Stop	A
	UUG	Leu	UCG	Ser	UAG	Stop	UGG	Trp	G
C	CUU	Leu	CCU	Pro	CAU	His	CGU	Arg	U
	CUC	Leu	CCC	Pro	CAC	His	CGC	Arg	C
	CUA	Leu	CCA	Pro	CAA	Gln	CGA	Arg	A
	CUG	Leu	CCG	Pro	CAG	Gln	CGG	Arg	G
A	AUU	Ile	ACU	Thr	AAU	Asn	AGU	Ser	U
	AUC	Ile	ACC	Thr	AAC	Asn	AGC	Ser	C
	AUA	Ile	ACA	Thr	AAA	Lys	AGA	Arg	A
	AUG	Met	ACG	Thr	AAG	Lys	AGG	Arg	G
G	GUU	Val	GCU	Ala	GAU	Asp	GGU	Gly	U
	GUC	Val	GCC	Ala	GAC	Asp	GGC	Gly	C
	GUA	Val	GCA	Ala	GAA	Glu	GGA	Gly	A
	GUG	Val	GCG	Ala	GAG	Glu	GGG	Gly	G



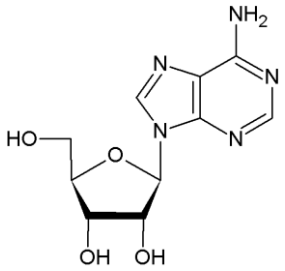
Journal of Molecular Biology, Volume 366, Issue 1, 2007, 1–13

Why?

Epigenetic marks with “writer”, “eraser”, and “reader”



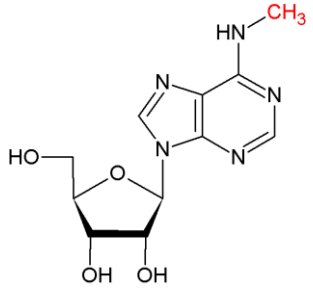
inosine



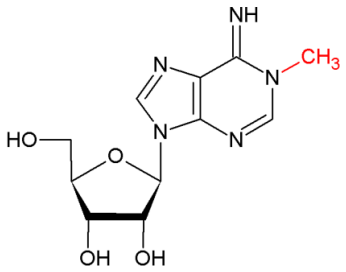
adenosine

demethylation

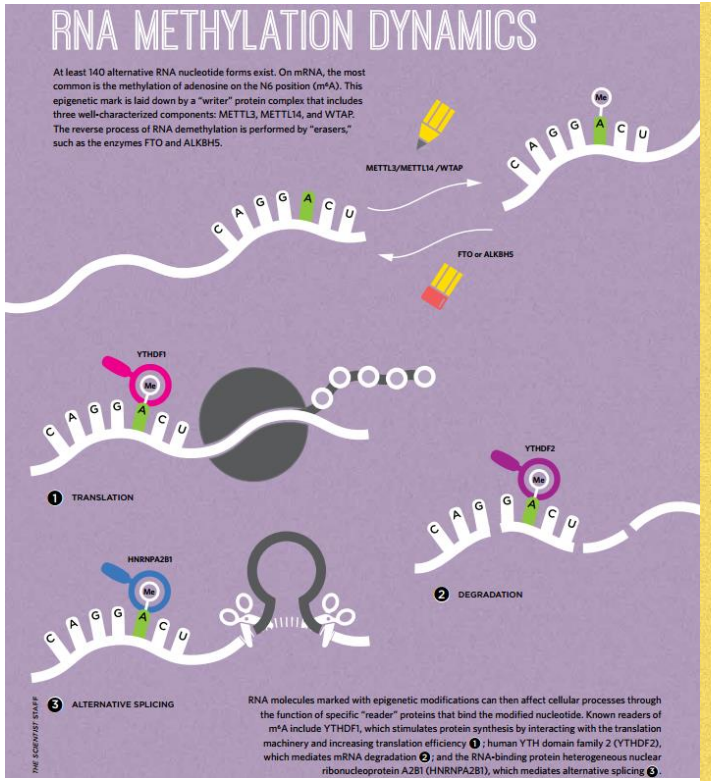
methylation

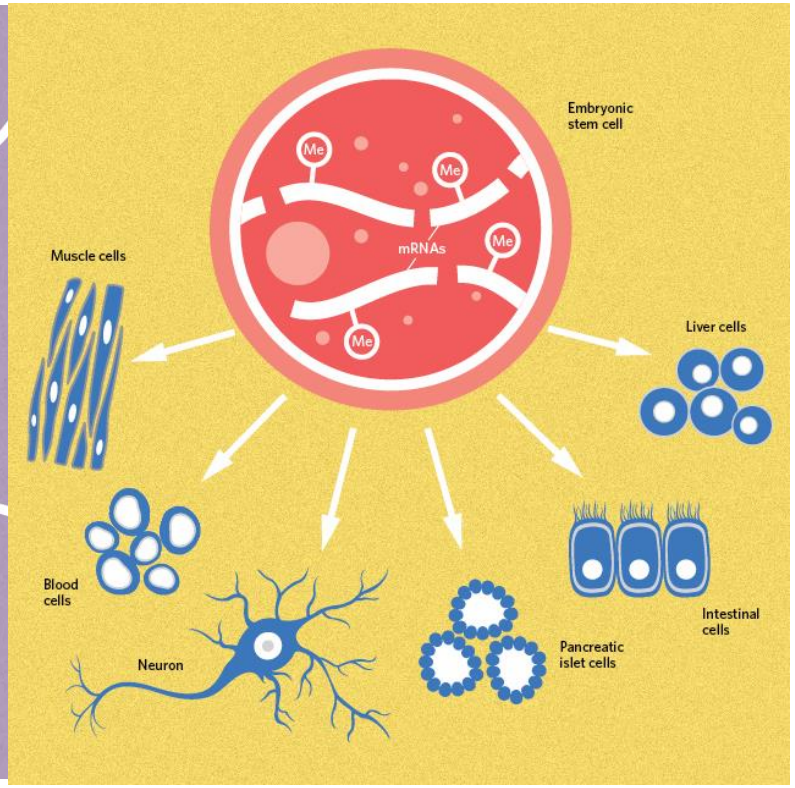


m6A



m1A



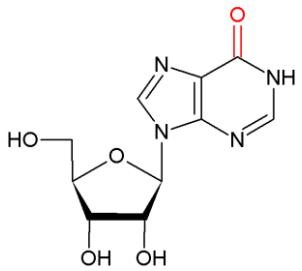


- m1A and m6A are positional isomers that share the same molecular formula, but different arrangement of the atoms in space.

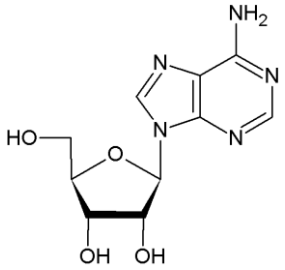
The Scientist. 01.2016

Why?

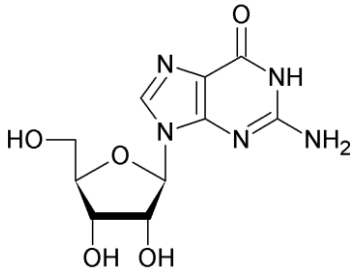
Nuclear RNA capping



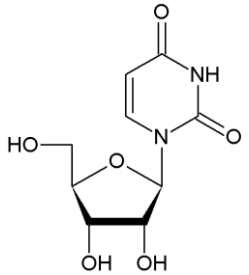
inosine



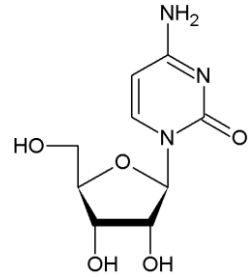
adenosine



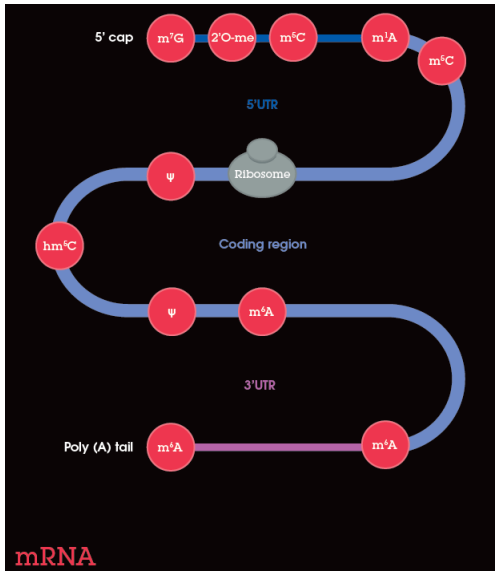
guanosine



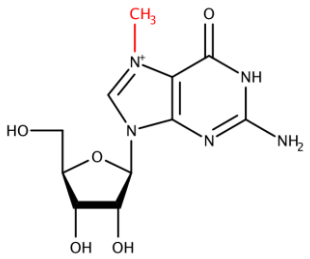
uridine



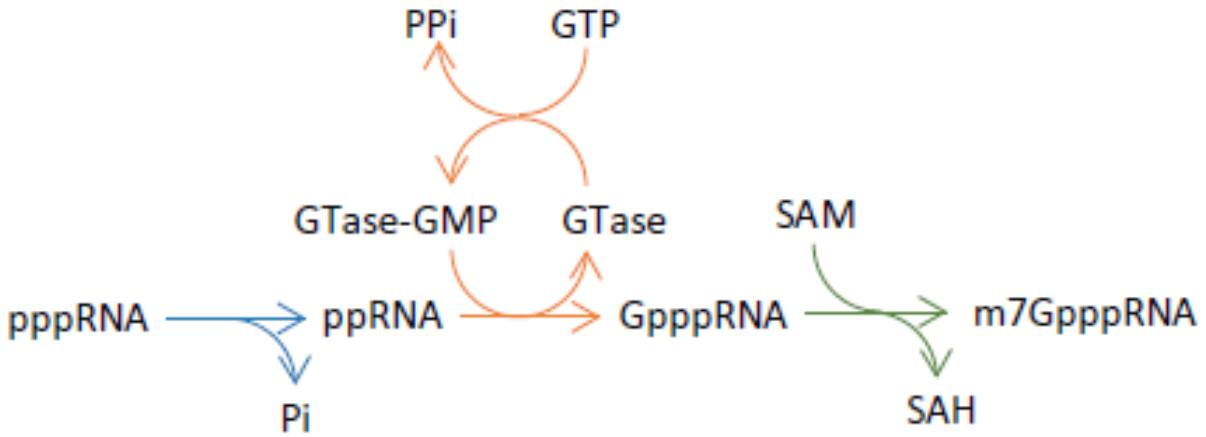
cytidine



methylation

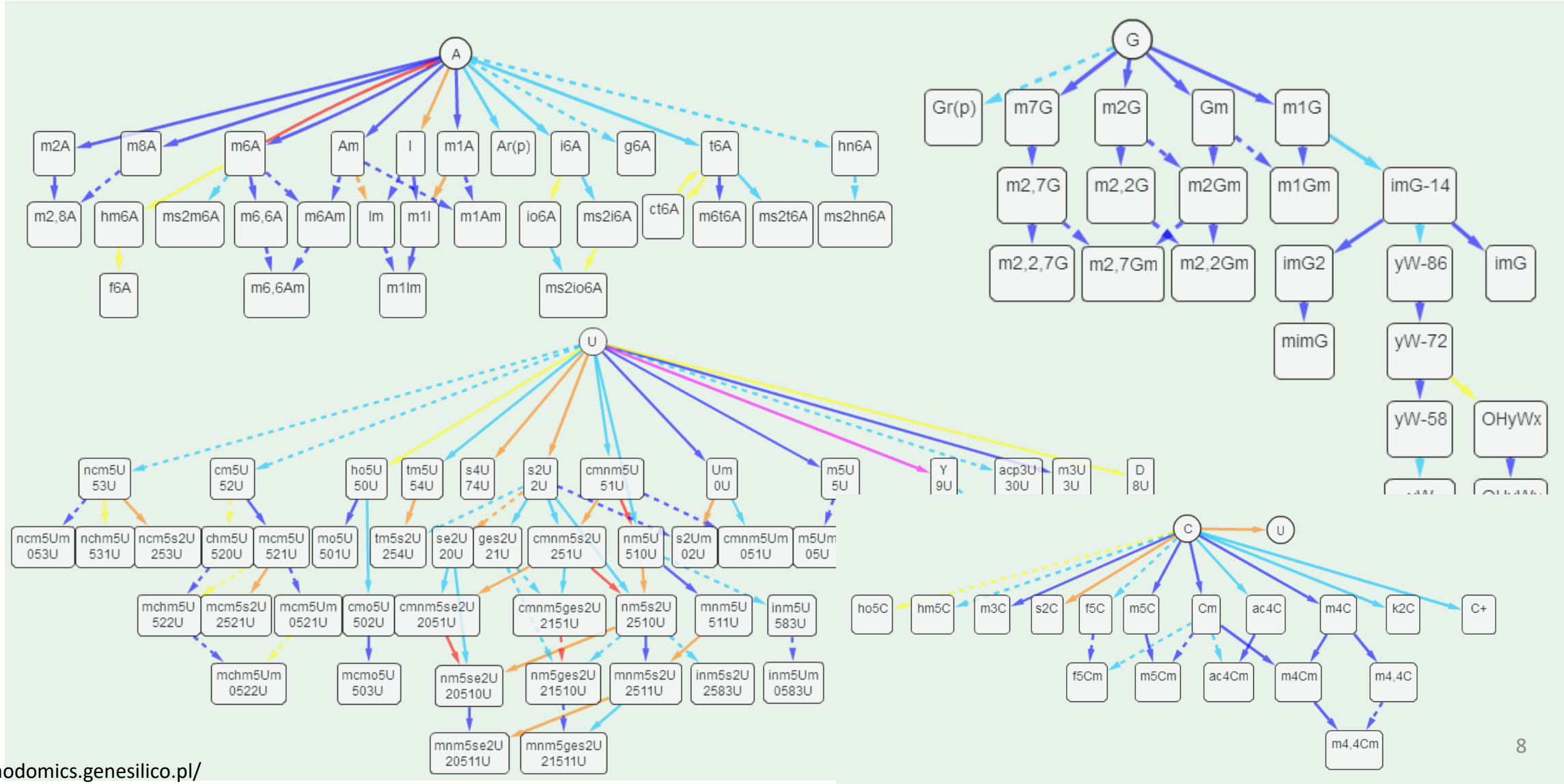


m7G



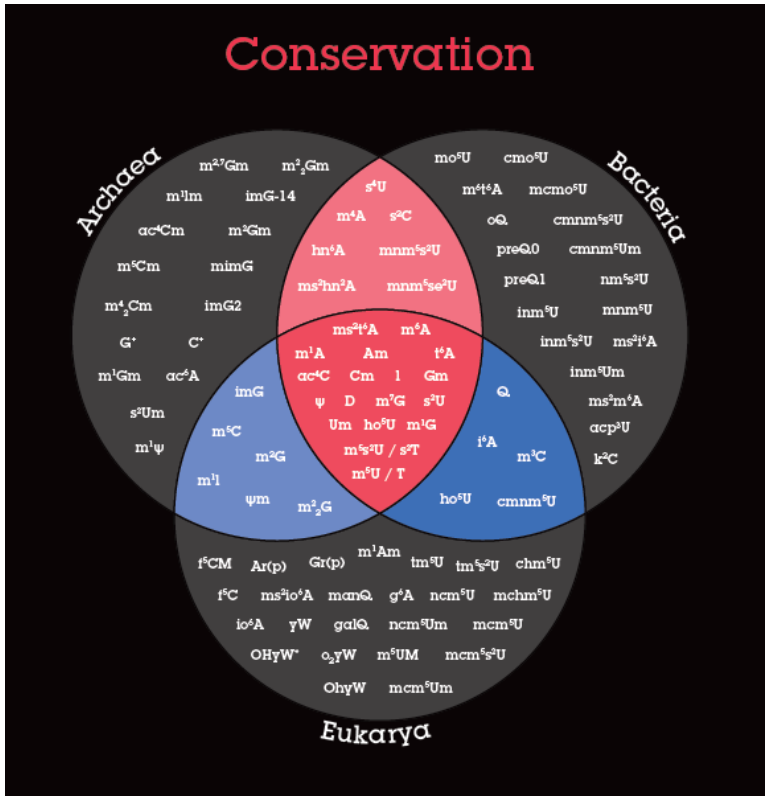
Nucleic Acids Research, Vol. 44, 2016; Abcam

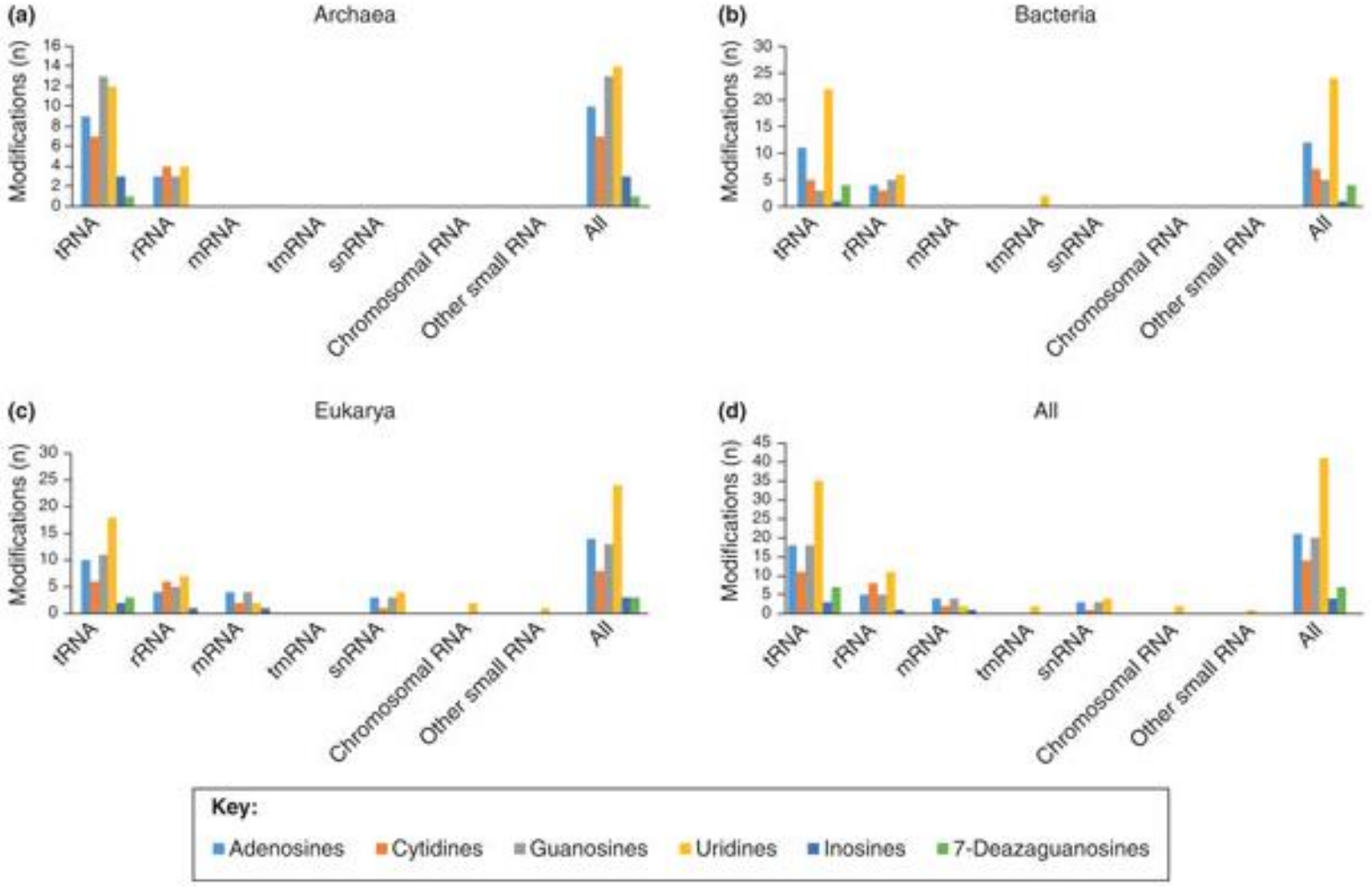
Pathways of RNA Modification



http://modomics.genesilico.pl/

Conservation and types of RNA modifications





Abcam; Genome Biology 2012 13:175

Approaches to Ribonucleosides Analysis

➢Traditional methods:

- 2D thin-layer chromatography
- HPLC with UV-VIS spectrophotometry detection

❖ Caveats:

- Labor- and time-intensive
- Require large RNA quantities
- Are at best semi-quantitative
- Are not sensitive enough for low-abundance modifications
- Are modification-specific and cannot quantitate all RNA modifications at once
- May require radioactive labeling

➢Sequencing-based techniques:

- Immuno-capturing and massively parallel sequencing
- Biochemical identification of A-to-I RNA editing sites by the inosine chemical erasing method
- RNA cytosine methylation analysis by bisulfite sequencing.
- Profiling of ribose methylations in RNA by high-throughput sequencing
- Transcriptome-wide mapping of pseudouridines

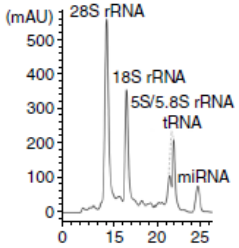
❖ Caveats:

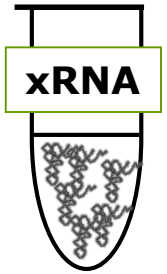
- Relatively poor quantitative capacity
- Application restricted to specific PTMs

LC-MS/MS Approaches to Ribonucleosides Analysis

A Platform for Discovery and Quantification of Modified Ribonucleosides in RNA (Dedon et al. 2015)

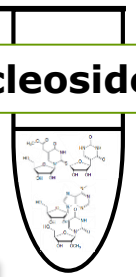
❶ RNA Purification





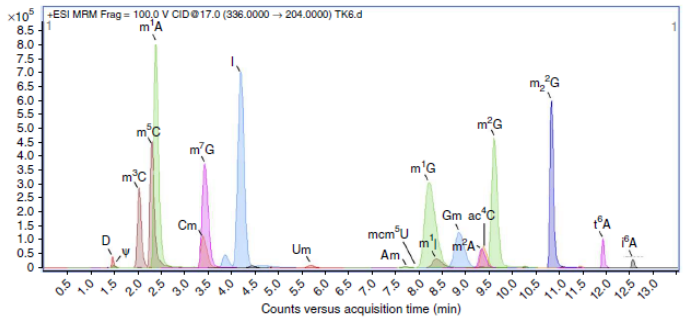
❷ RNA Hydrolysis

Mononucleoside Mixture

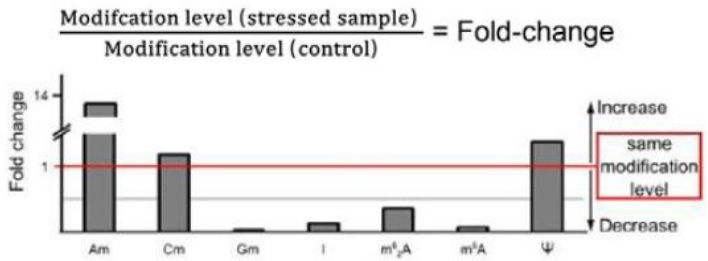


❸ HPLC Resolution

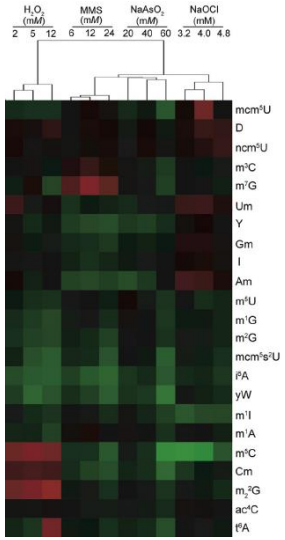
❹ High Accuracy Mass Spectrometry



❺ MS Quantitation



❻ Data Analysis



Methods in Enzymology, Volume 560, 2015

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  - Why study modified ribonucleosides?
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  - Positional isomers
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  - Targeted analysis of global modification levels in eukaryotic mRNA
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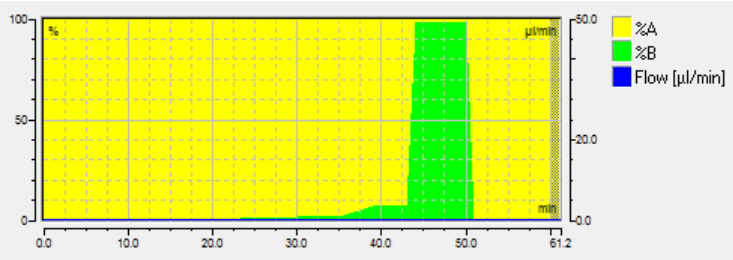
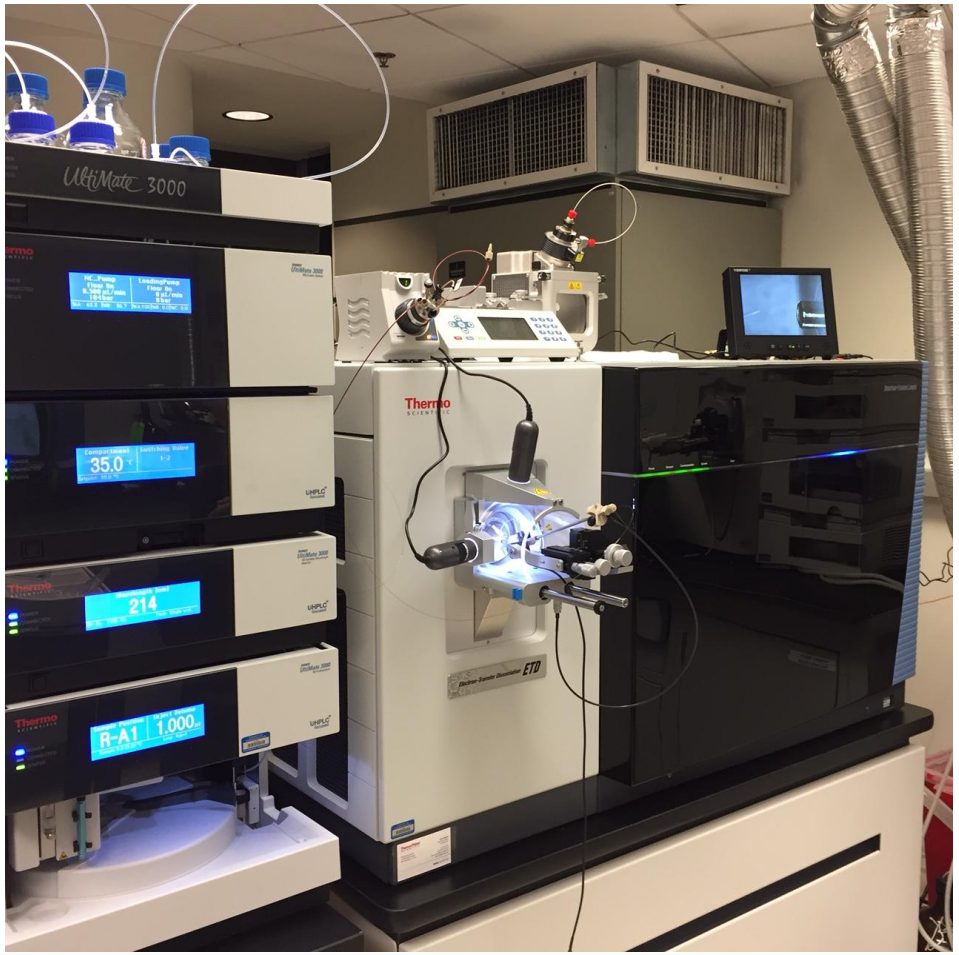
Stowers Proteomics Center

HPLC–MS^n approach

UltiMate 3000 RSLCnano

- ❖ Hypersil Gold aQ HPLC column
- 3 µm particle size; 175Å
- 20 cm x 75µ ID
- Flow rate: 0.3ul/min
- Buffer A: 0.1% formic acid v/v
- Buffer B: 80% acetonitrile with 0.1% formic acid v/v



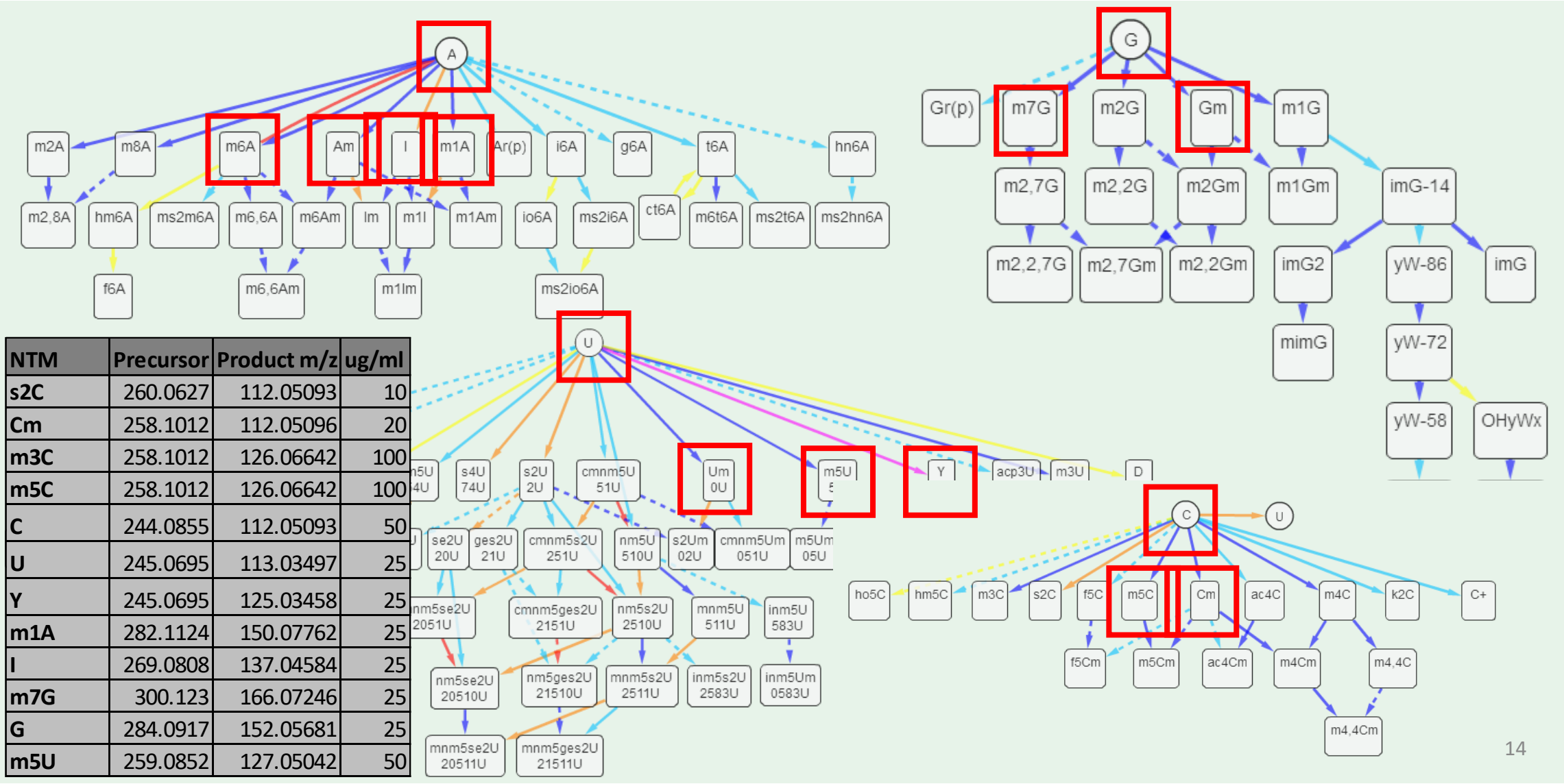


Lumos™ Tribrid™ Mass Spectrometer

- ❖ Positive ion mode
- Scan range 50-400 m/z
- MS OT @ 60,000
- $ddMS^2$ OT @ 30,000; 40% HCD
- $ddMS^3$ IT @ 50% HCD

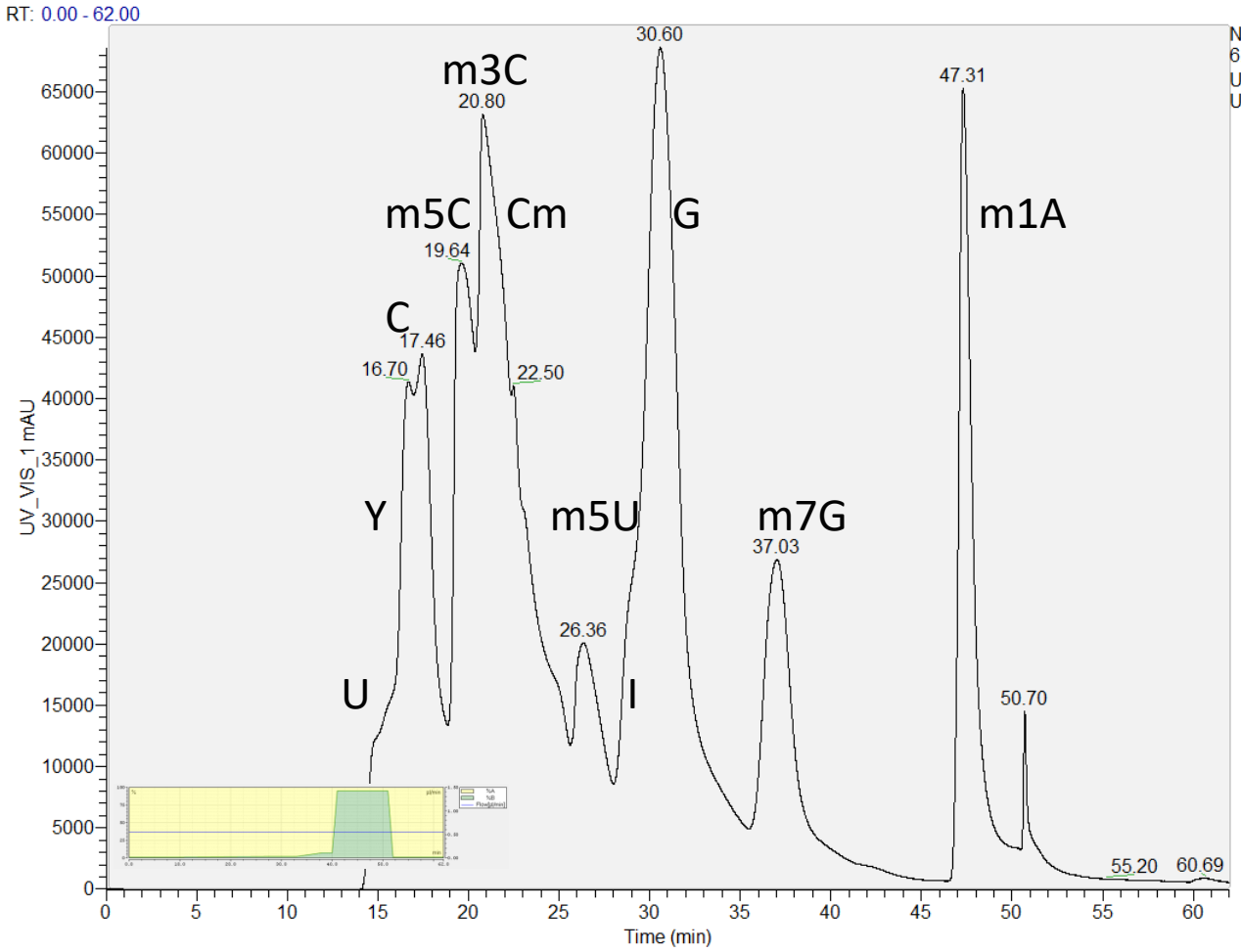
Skyline

Analysis of ribonucleoside standards

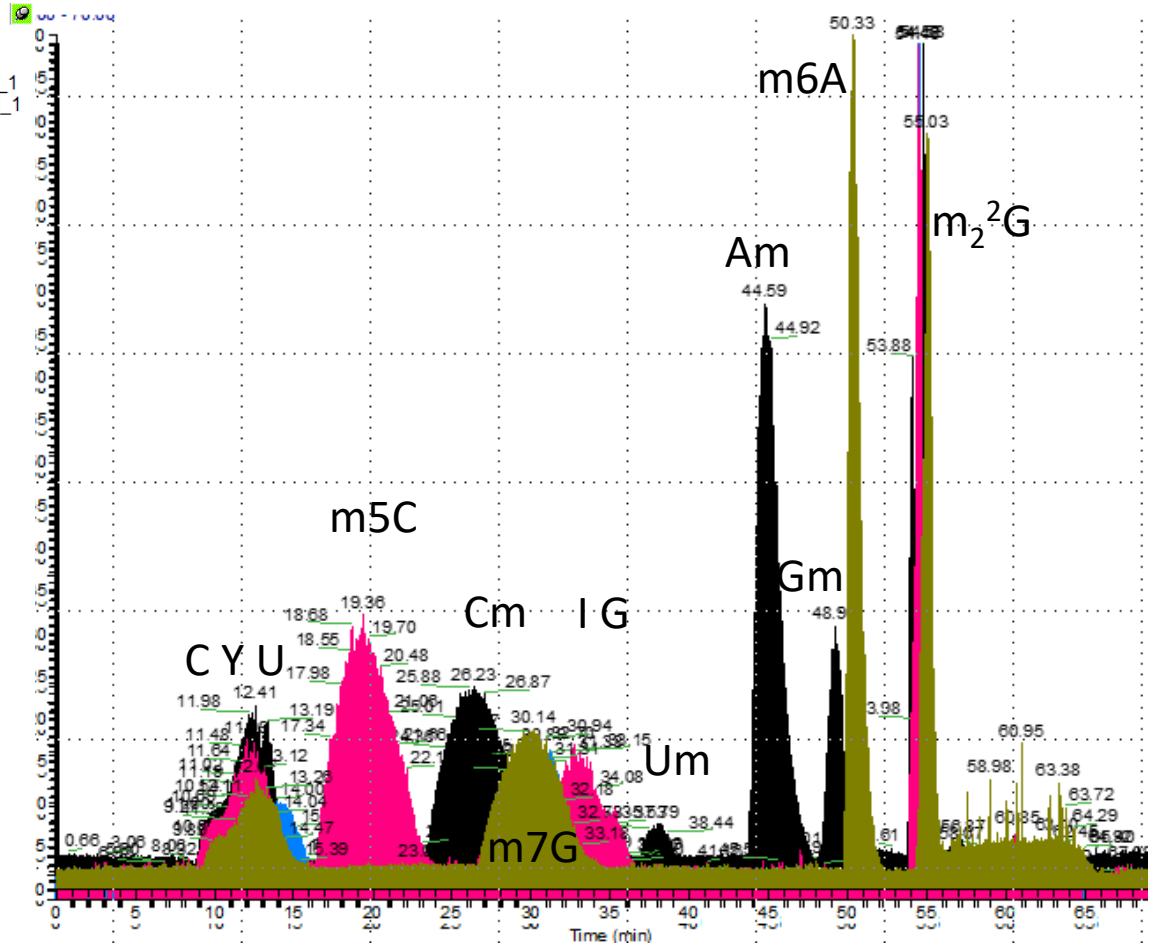


NTM	Precursor	Product m/z	ug/ml
s2C	260.0627	112.05093	10
Cm	258.1012	112.05096	20
m3C	258.1012	126.06642	100
m5C	258.1012	126.06642	100
C	244.0855	112.05093	50
U	245.0695	113.03497	25
Y	245.0695	125.03458	25
m1A	282.1124	150.07762	25
I	269.0808	137.04584	25
m7G	300.123	166.07246	25
G	284.0917	152.05681	25
m5U	259.0852	127.05042	50

Analysis of ribonucleoside standards



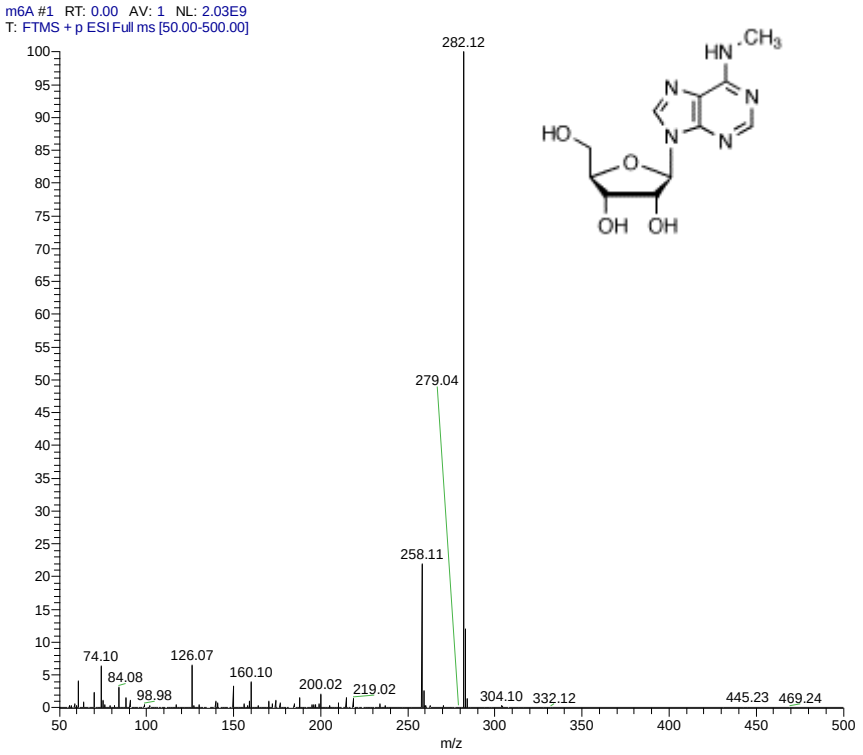
Nucleotide Test Mix

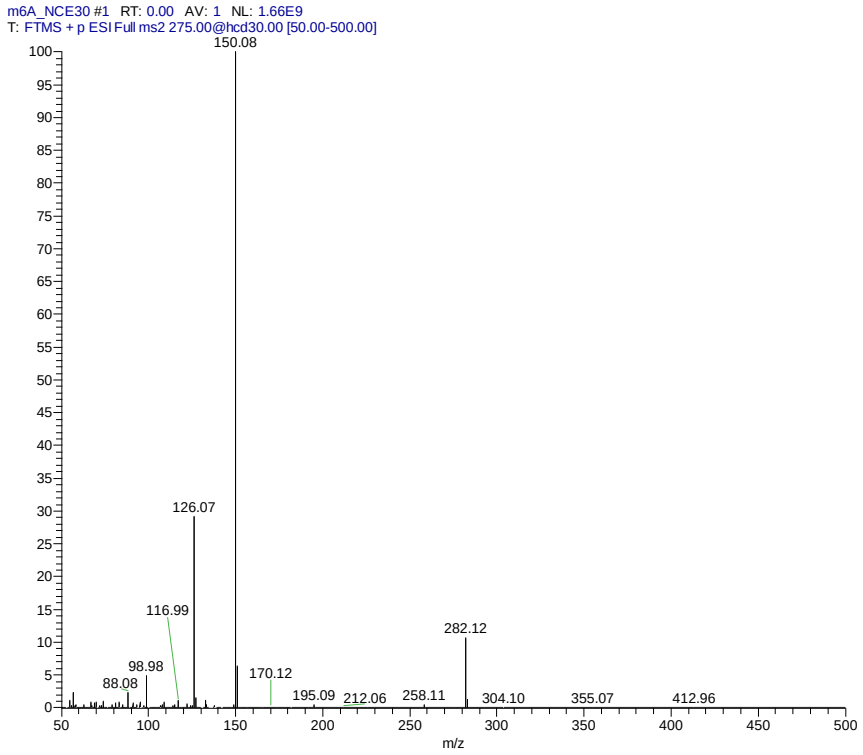


RN standards

Analysis of ribonucleoside standards

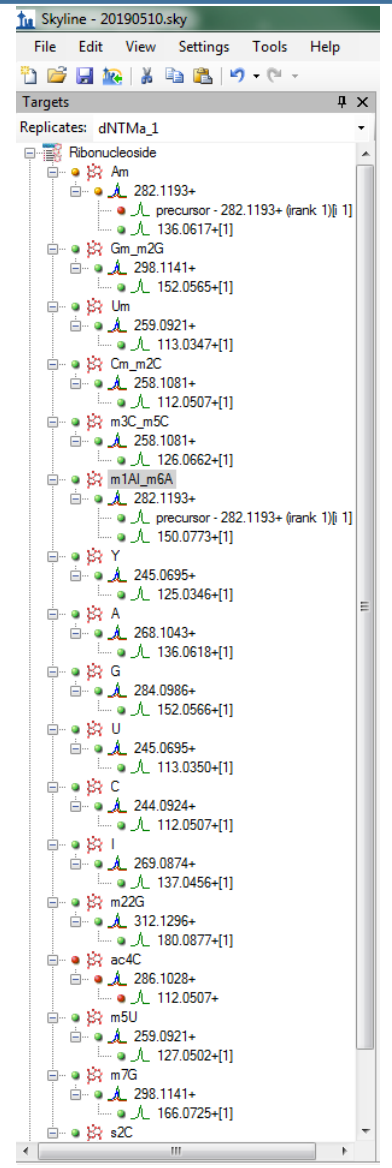
From raw data to Skyline





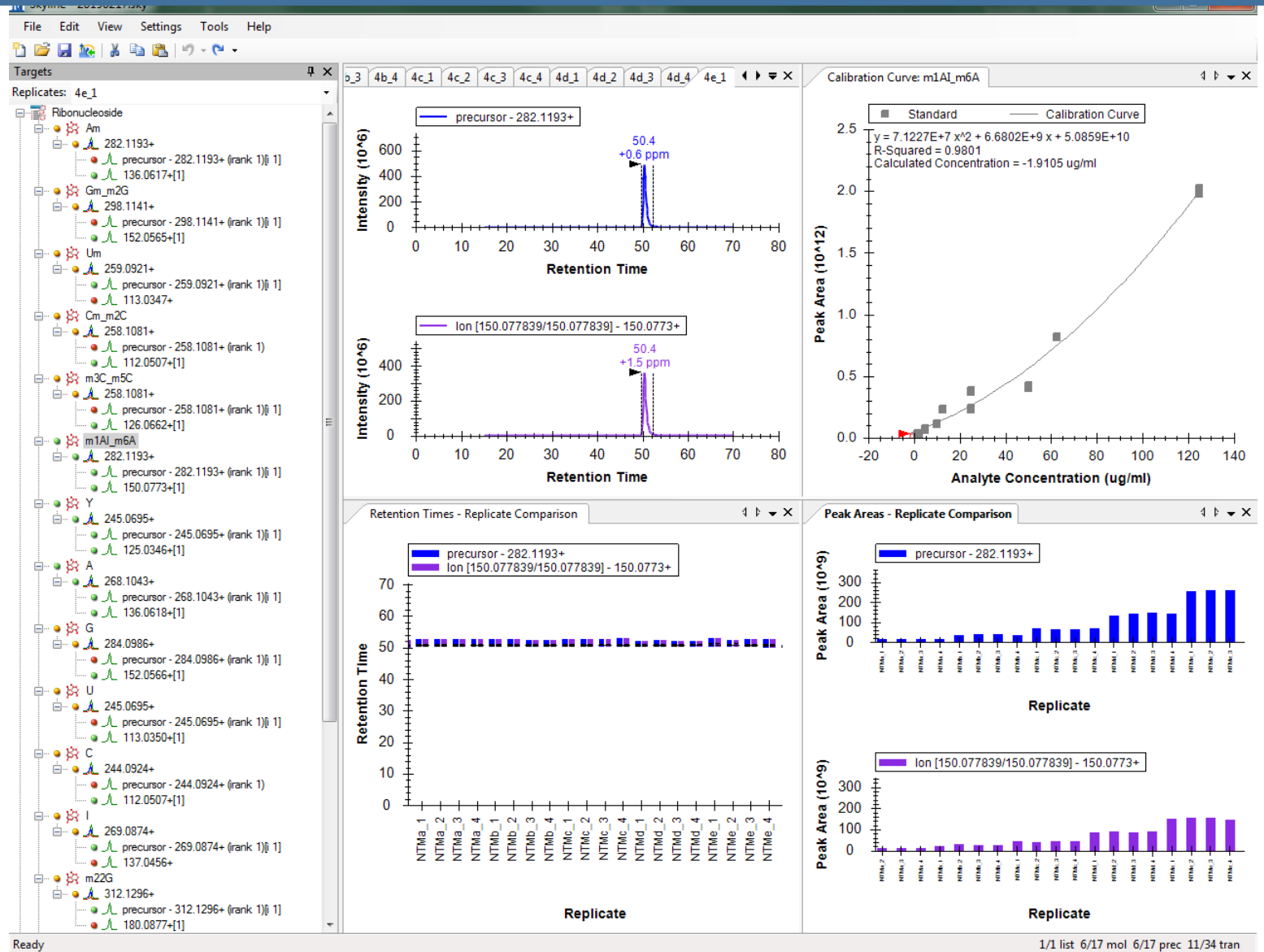
Precursor m/z

Product m/z



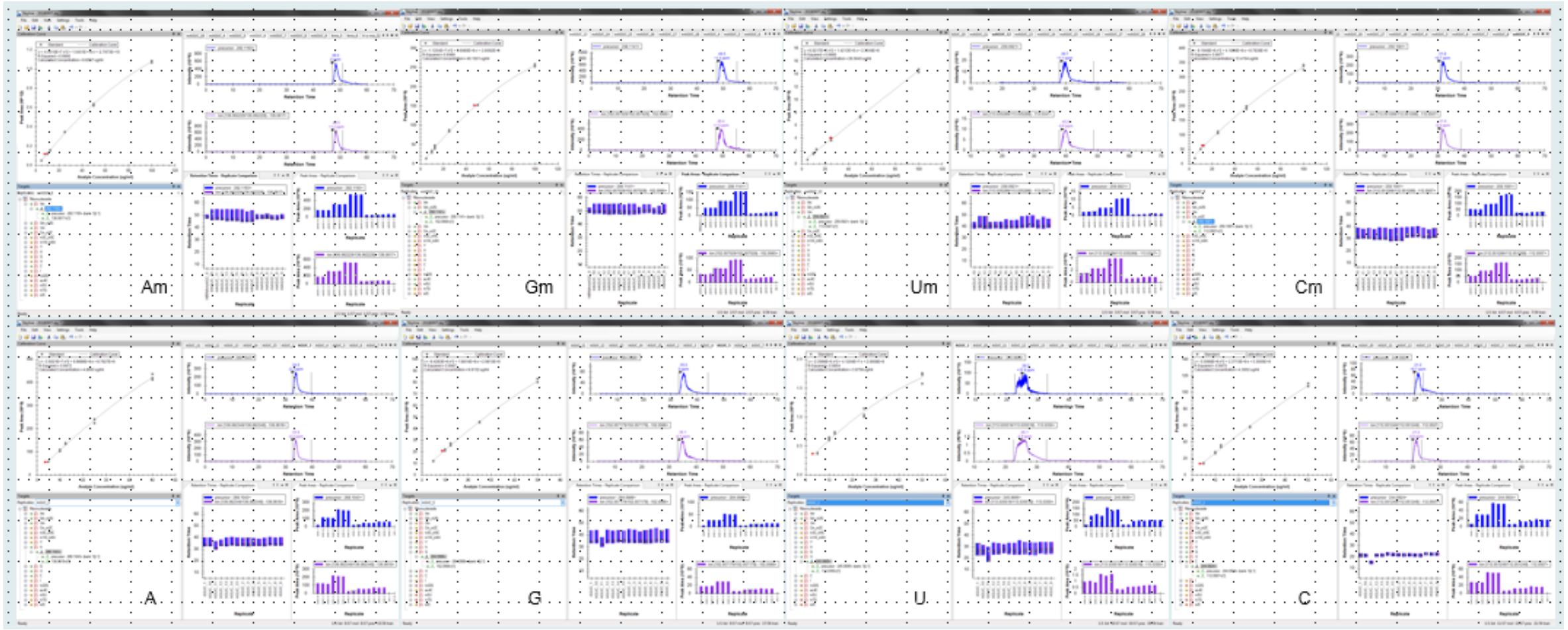
Analysis of ribonucleoside standards

Generating calibration curves



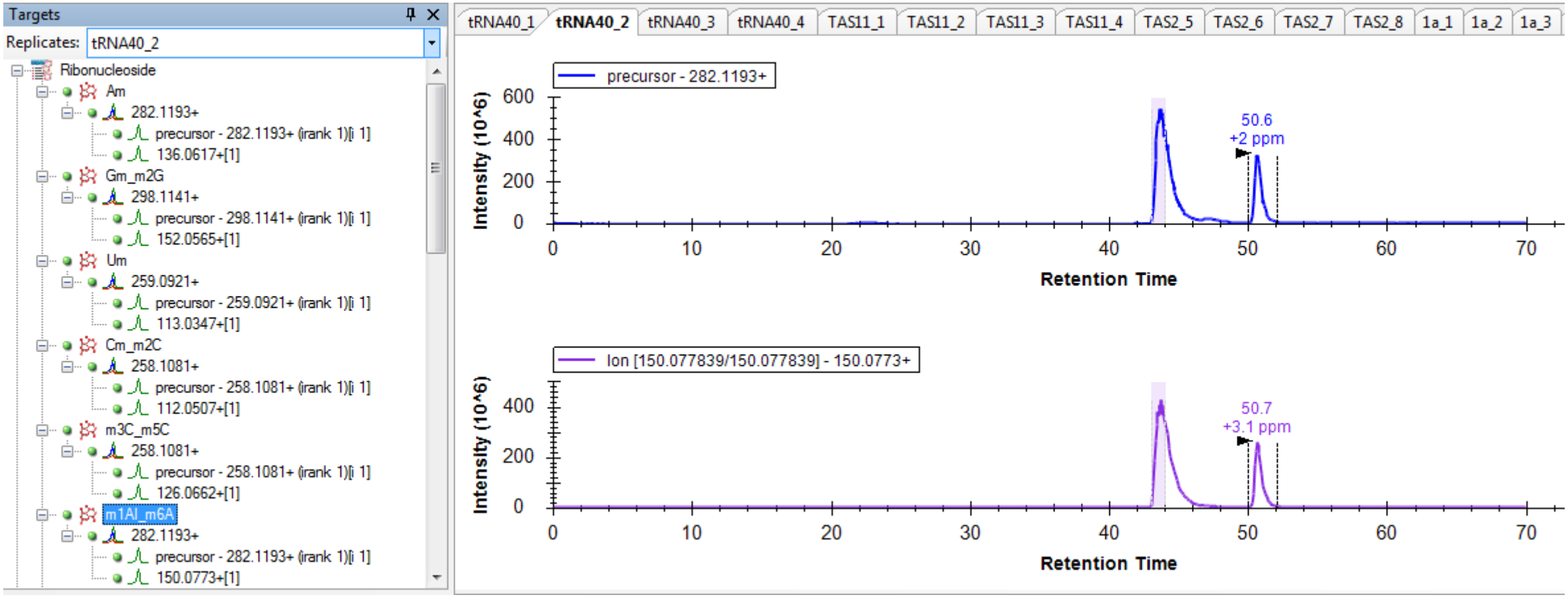
Analysis of ribonucleoside standards

More calibration curves



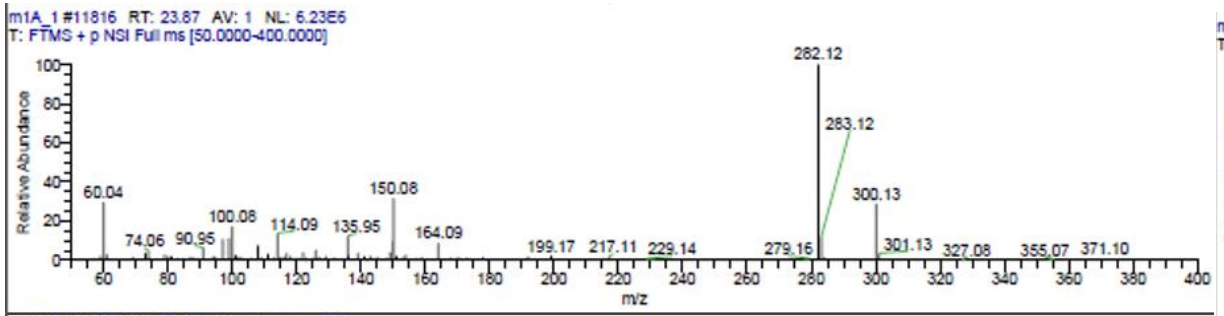
Analysis of ribonucleoside standards

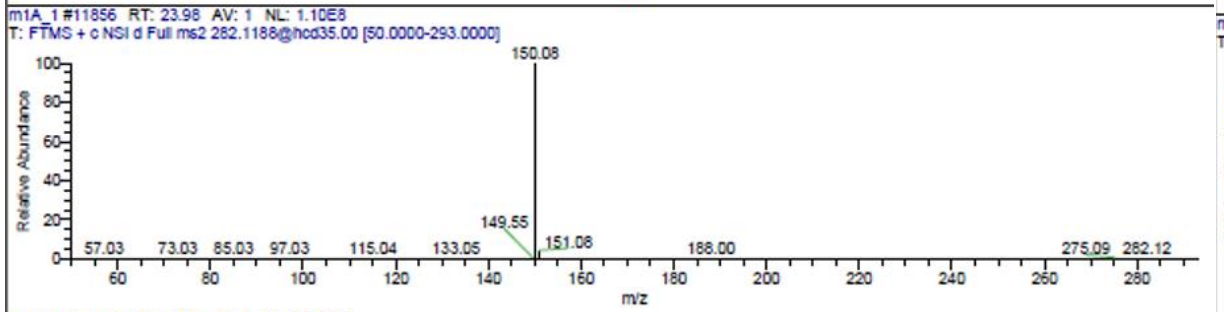
Positional isomers

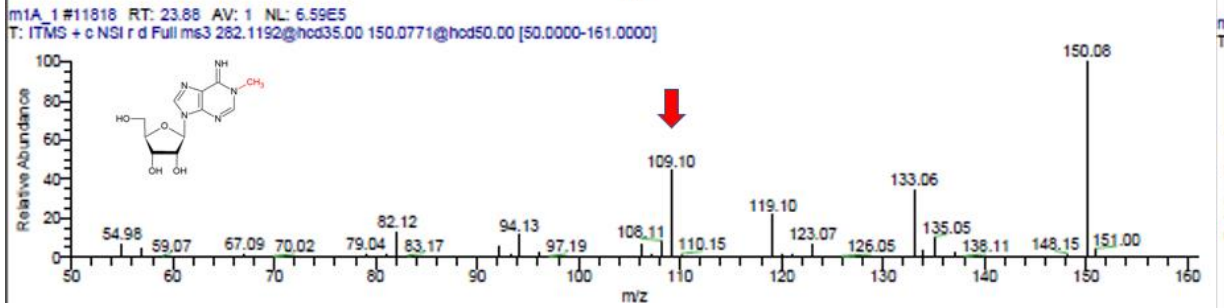


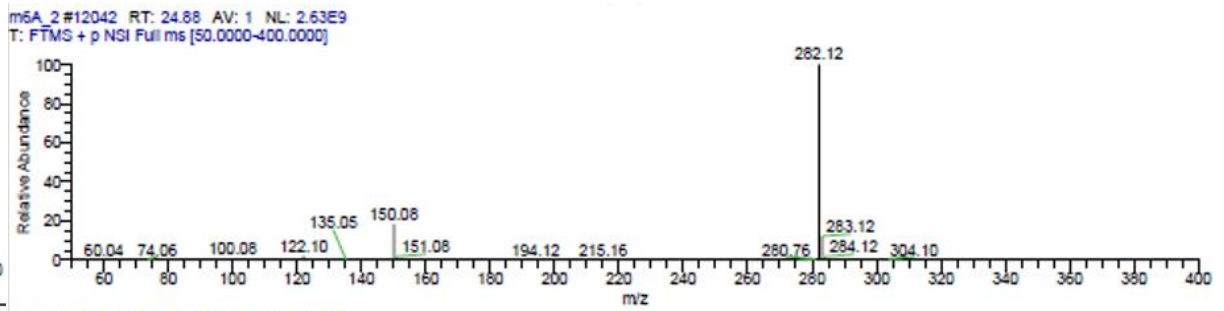
Analysis of ribonucleoside standards

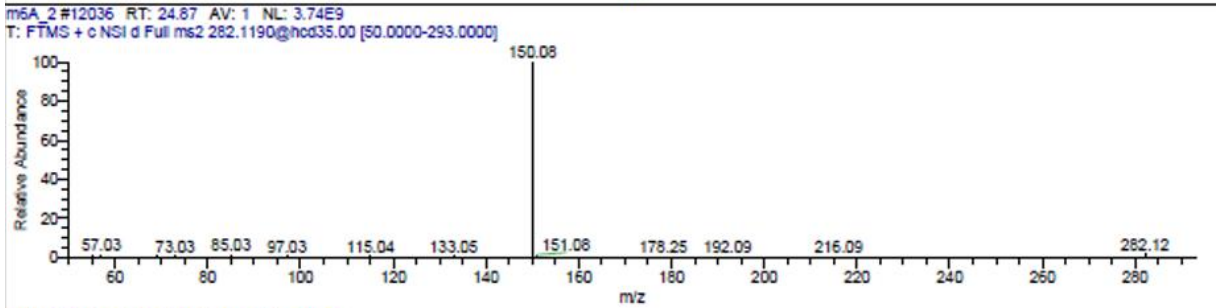
Positional isomers – distinct MS^3 ion pair

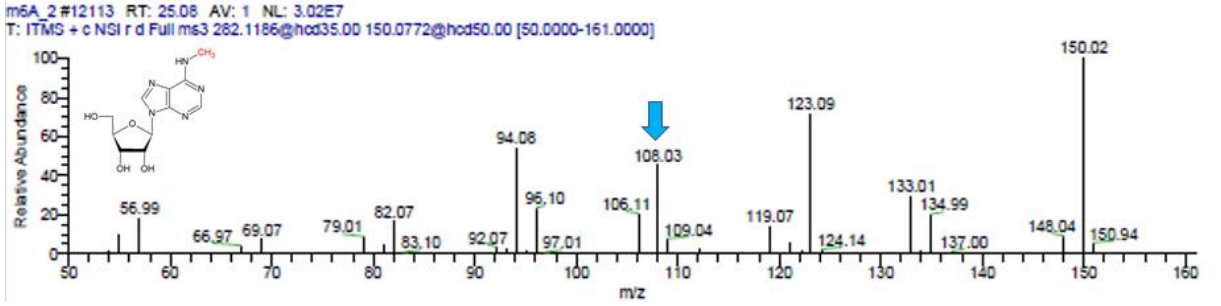






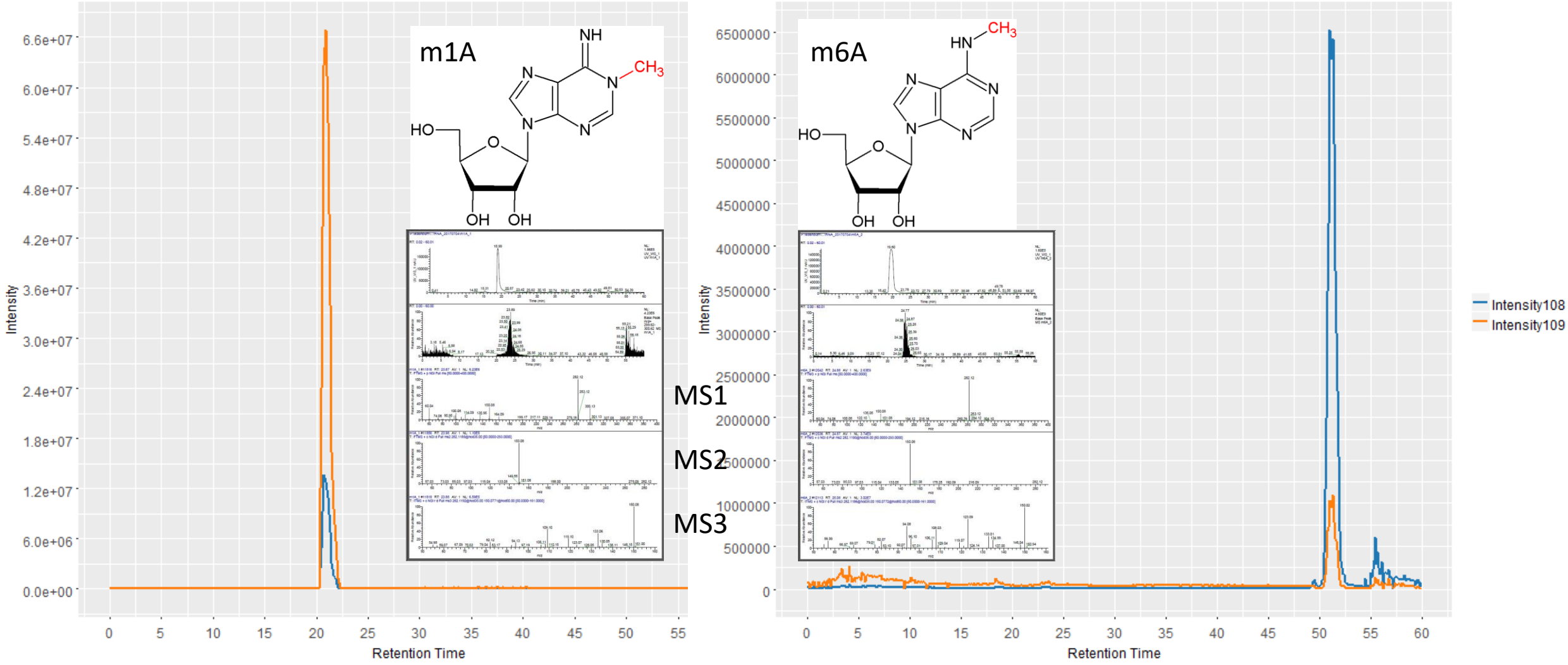






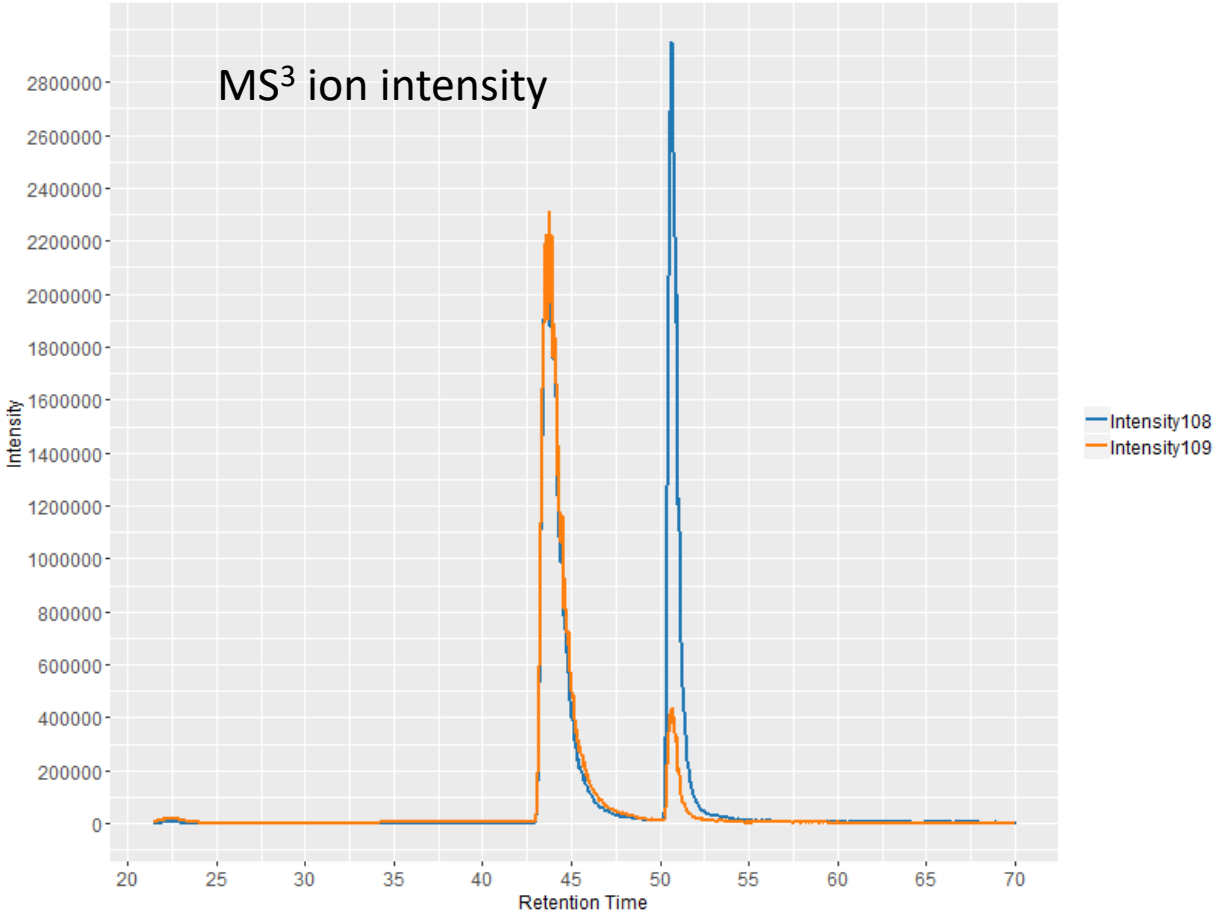
Analysis of ribonucleoside standards

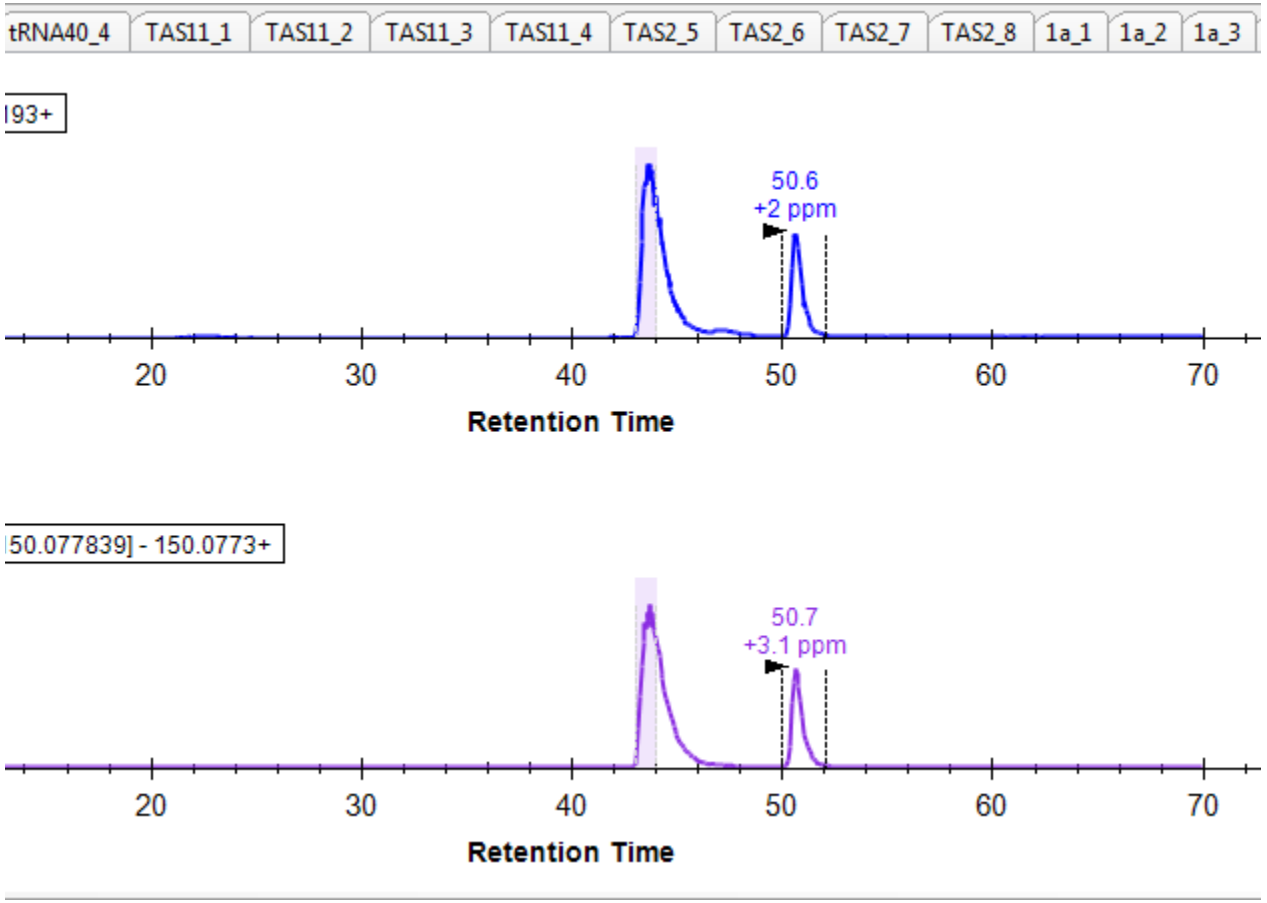
Positional isomers – RawConverter and in-house R script



Analysis of ribonucleoside standards

Positional isomers – positive identification requires MS^3 data

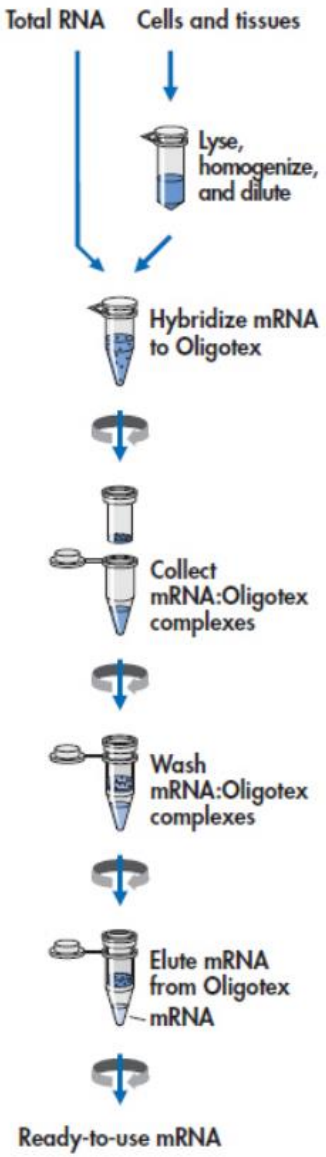




Absolute quantitative analysis of modified ribonucleosides in tRNA and mRNA using Skyline

- Introduction
  - Why study modified ribonucleosides?
- Instrumentation and Methods
  - Nucleoside standards
  - Calibration curves
  - Positional isomers
- Application
  - Targeted analysis of global modification levels in eukaryotic mRNA
- Summary

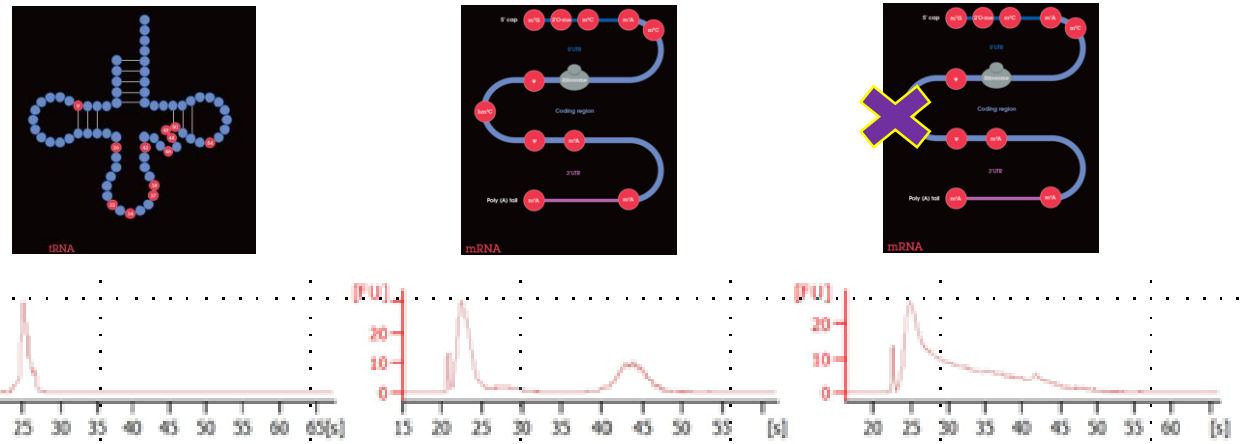
Targeted analysis of global modification levels in eukaryotic mRNA



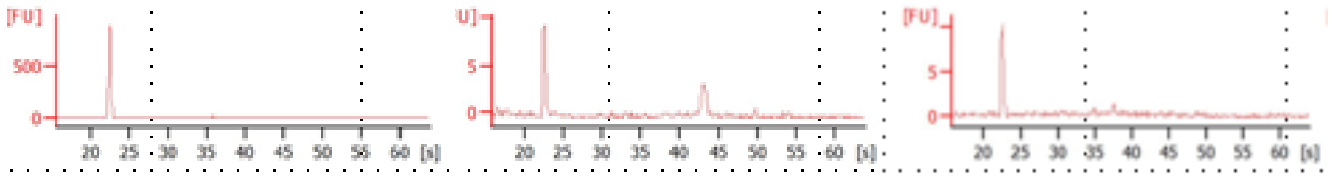
E. coli tRNA
(Positive Control)

WT mRNA

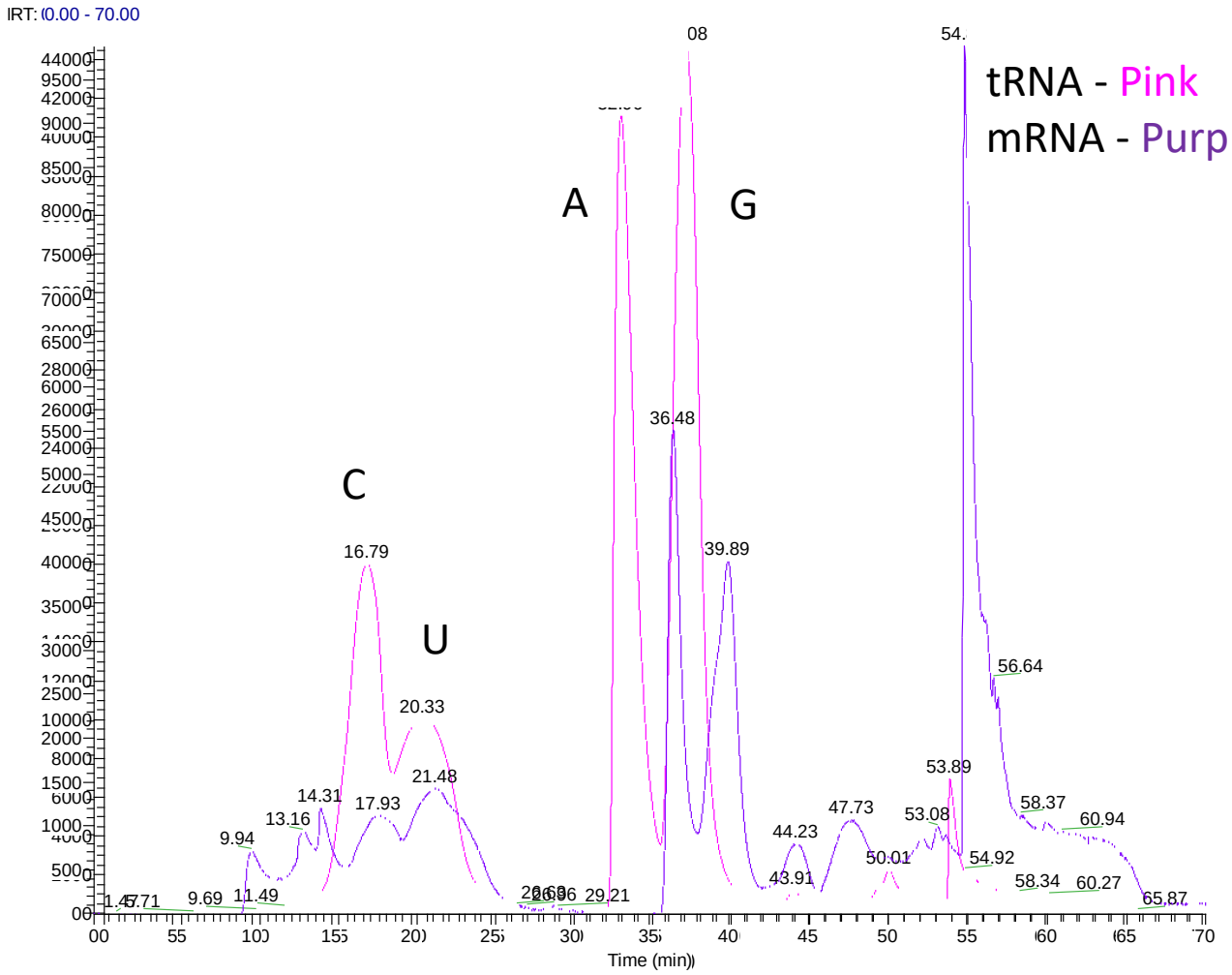
KD mRNA

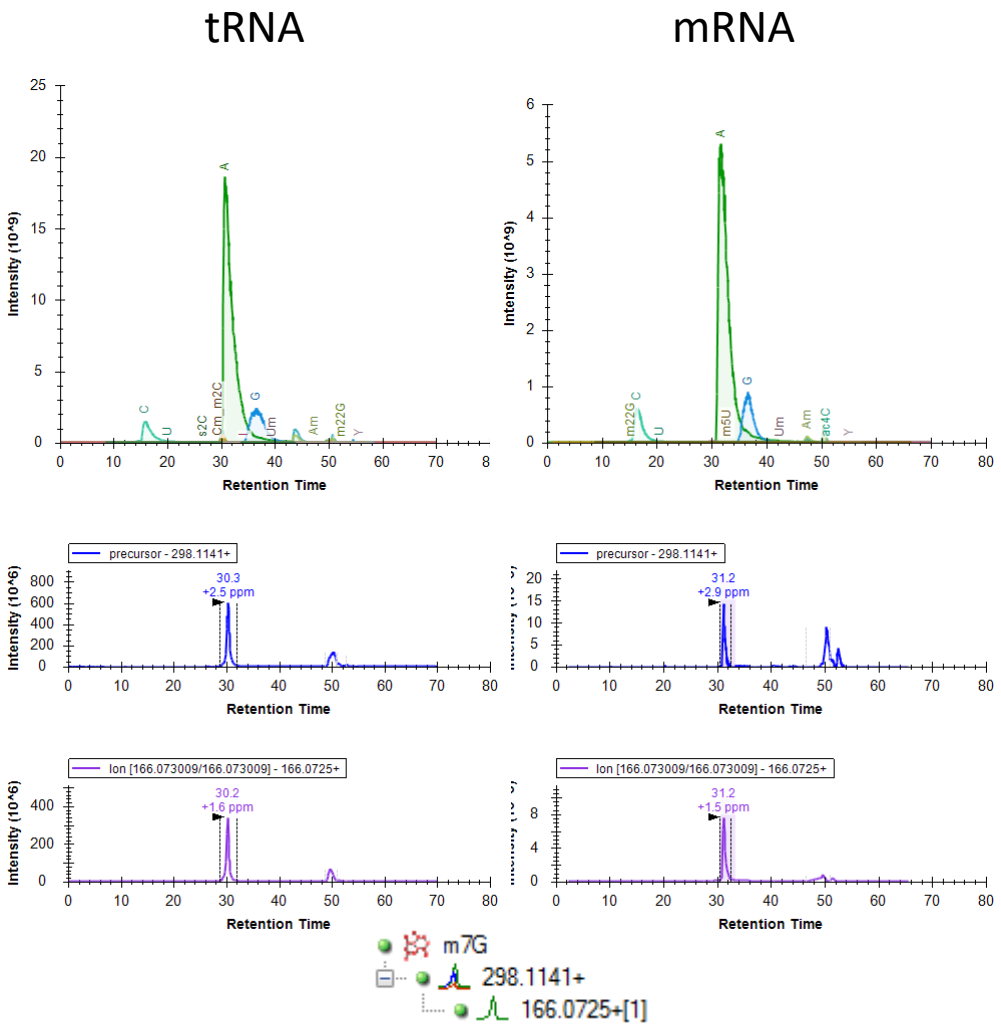


Benzonase, Phosphodiesterase I,
Alkaline phosphatase, 37 °C for 3 h



Targeted analysis of global modification levels in eukaryotic mRNA





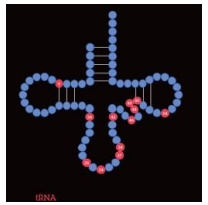
Targeted analysis of global modification levels in eukaryotic mRNA

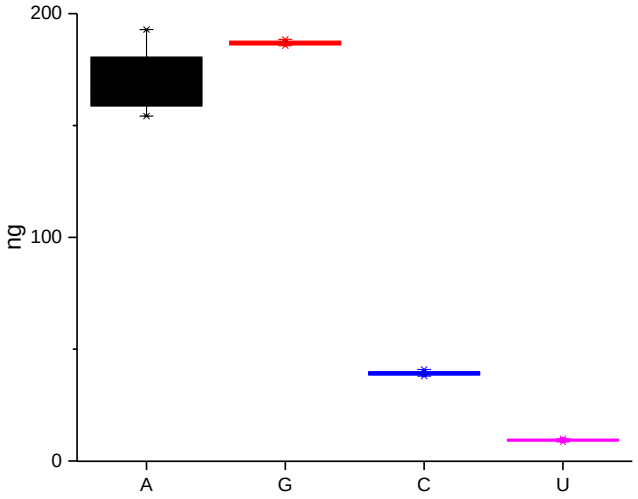
Replicate (ng)	A	G	C	U	Am	Gm	Cm	m7G	I	m1A/m6A	m2G	m5C	m5U
tRNA_1	154.2176	185.6838	38.9601	8.568	-0.2262	2.0748	0.4716	4.4535	-0.0482	0.5035	3.2849	0.0269	0.6433
tRNA_2	168.3678	186.8875	40.8943	9.981	-0.2241	1.8757	0.5286	4.5976	-0.0676	0.4919	3.1179	0.0295	0.7199
tRNA_3	163.1332	186.371	38.8409	9.2623	-0.2224	2.0292	0.526	4.6448	-0.0072	0.4964	3.2466	0.0326	0.7642
tRNA_4	192.8613	188.5319	37.9696	9.6352	-0.2201	2.2802	0.579	4.7487	0.0633	0.5869	3.4571	0.034	0.9042
WT_1	31.2655	26.4209	14.3024	6.0406	-0.0991	-2.3721	-0.123	0.8947	0.1234	0.0818	-0.4495	0.0208	-0.8219
WT_2	34.6262	29.057	15.3255	5.4026	-0.0926	-2.3575	-0.1098	0.8989	0.1715	0.0738	-0.4372	0.0082	-0.8129
WT_3	33.9048	29.1903	14.9435	5.4159	-0.1011	-2.3433	#N/A	0.8965	0.0878	0.0502	-0.4252	0.0077	-0.8216
WT_4	36.0858	31.3199	15.4197	5.4381	-0.0918	-2.3397	-0.1473	0.8978	0.1125	0.0354	-0.4222	0.034	-0.822
KD_1	38.4673	36.336	16.9219	5.5723	-0.0368	-2.3185	-0.1454	0.89	0.4385	-0.0042	-0.4044	0.0083	-0.8224
KD_2	43.7527	39.9296	14.7369	5.1281	-0.05	-2.3073	-0.1489	0.8932	0.4696	-0.001	-0.395	0.0079	-0.8221
KD_3	43.1213	40.3504	16.6305	4.7533	-0.0363	-2.3057	-0.1543	0.8929	0.3667	-0.0164	-0.3937	0.01	-0.8222
KD_4	44.8967	37.9349	17.266	5.1784	-0.0426	-2.3088	#N/A	0.8937	0.2985	-0.0397	-0.3963	0.0085	-0.8221

Targeted analysis of global modification levels in eukaryotic mRNA

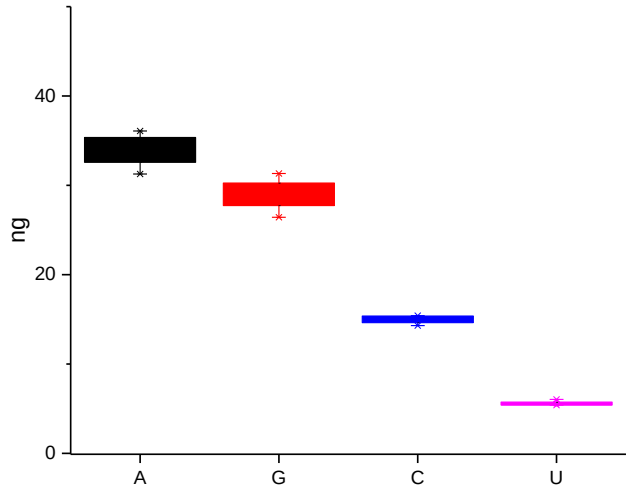
Canonical RNs

E. coli tRNA

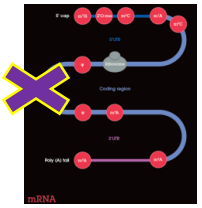


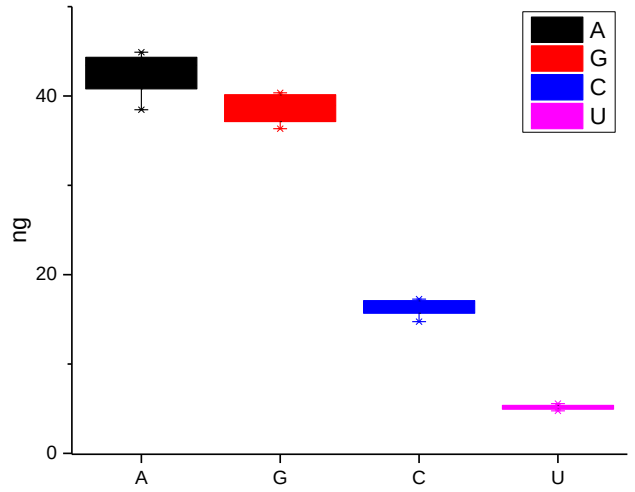


WT mRNA



KD mRNA

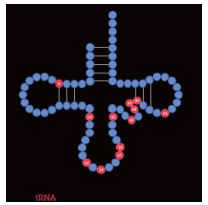




Targeted analysis of global modification levels in eukaryotic mRNA

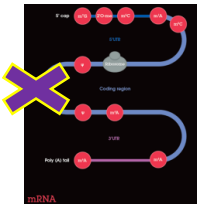
Modified RNs

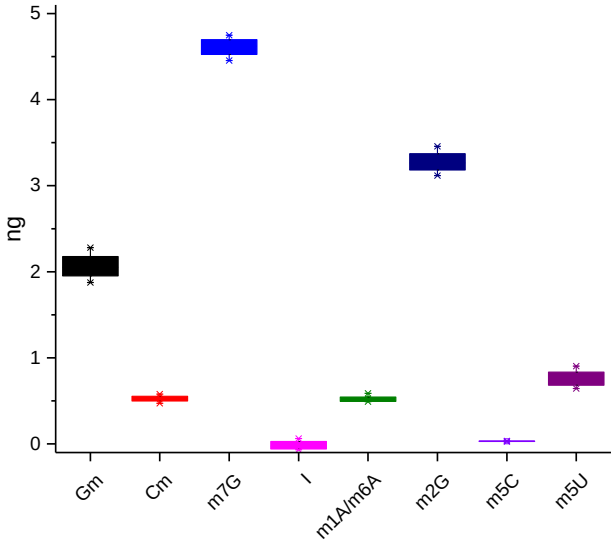
E. coli tRNA

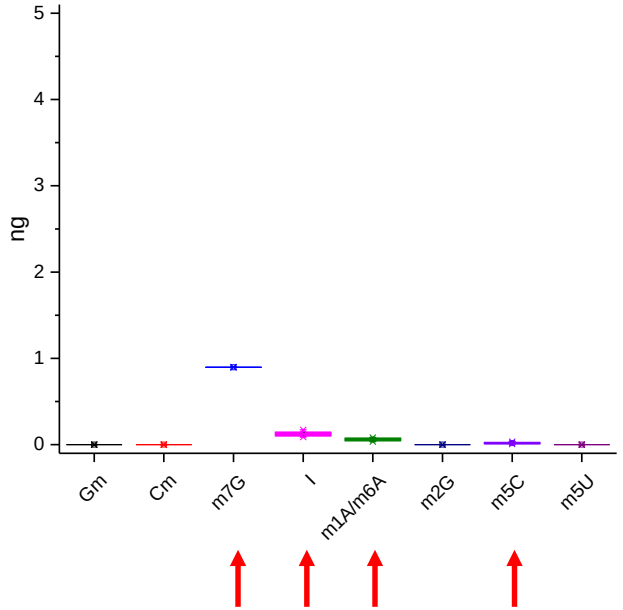


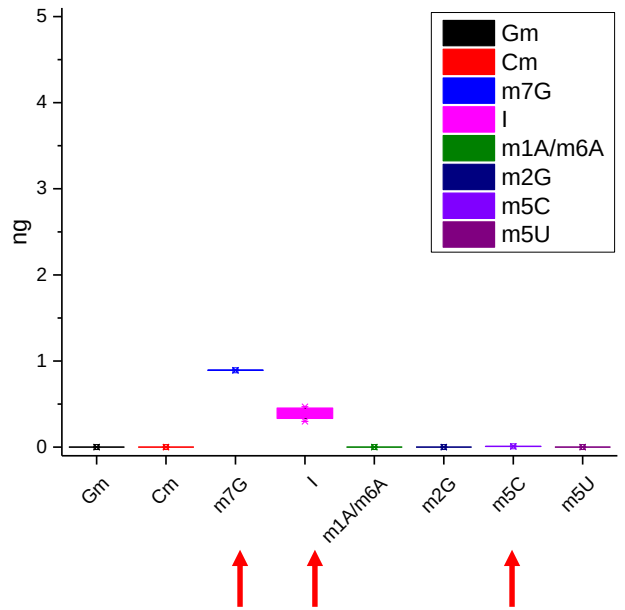
WT mRNA

KD mRNA









Targeted analysis of global modification levels in eukaryotic mRNA

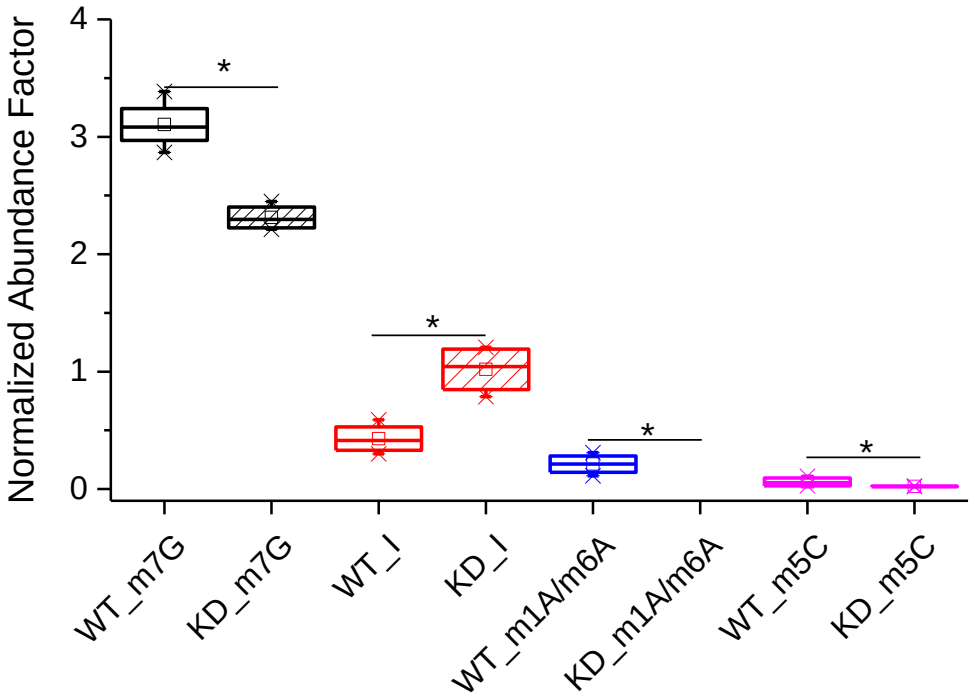
Normalized Abundance Factor

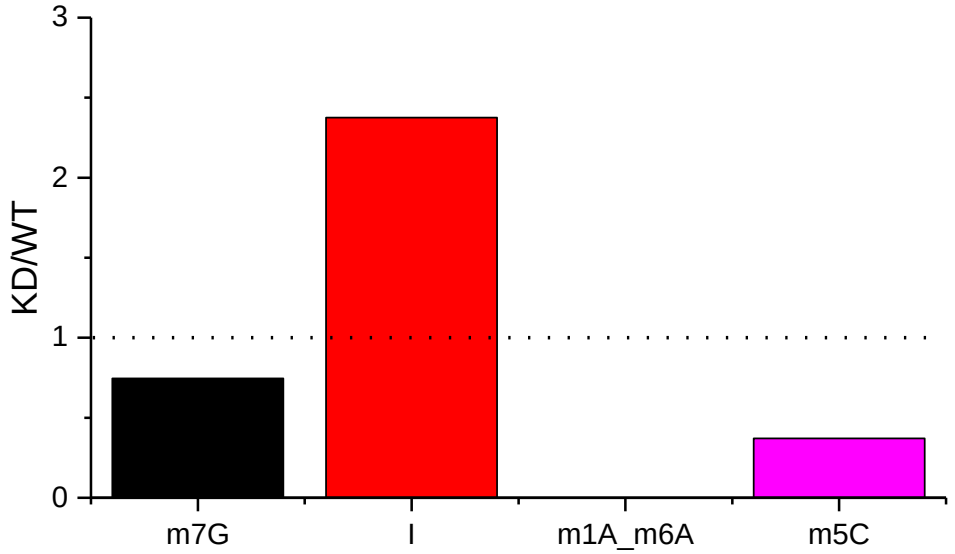
$$NAF_X = X/(X+Y)*100$$

X = Absolute quantity of modified RN_X
Y = Absolute quantity of canonical RNs

Relative abundance level of RNs in Knock-down vs. Wide-type cells

- Decrease in level of m7G and m5C
- Increase in the level of inosine
- m1A/m6A was not detected in the KD cells





Summary

- Using two degrees of separation (retention time of [MH^+] and m/z of MS/MS product ions), natural and modified ribonucleosides were detected and quantified at the sub-nanogram level.
- Natural and modified ribonucleosides were observed from ~500ng of E. coli tRNA and mammalian mRNA.
- Predicted fragmentation parameters from nucleosides standards were imported into Skyline 4.2.0 for interpretation and quantification in quadruplicates.
- Positional isomers with same product ion mass and similar retention time could be distinguished using RawConverter (Anal. Chem., 2015, 87) and an in-house R-script.
- Further method development would be required to expand on the number of detectable and quantifiable modified ribonucleosides in hope of providing a comprehensive epitranscriptomic profile of RNA modifications in different cellular states.
- A G U Cs are not entirely boring, especially in their modified forms!

Ideas and questions welcome!