Analytical Challenges, Solutions and Perspective on the Future Analysis and Characterization of mRNA

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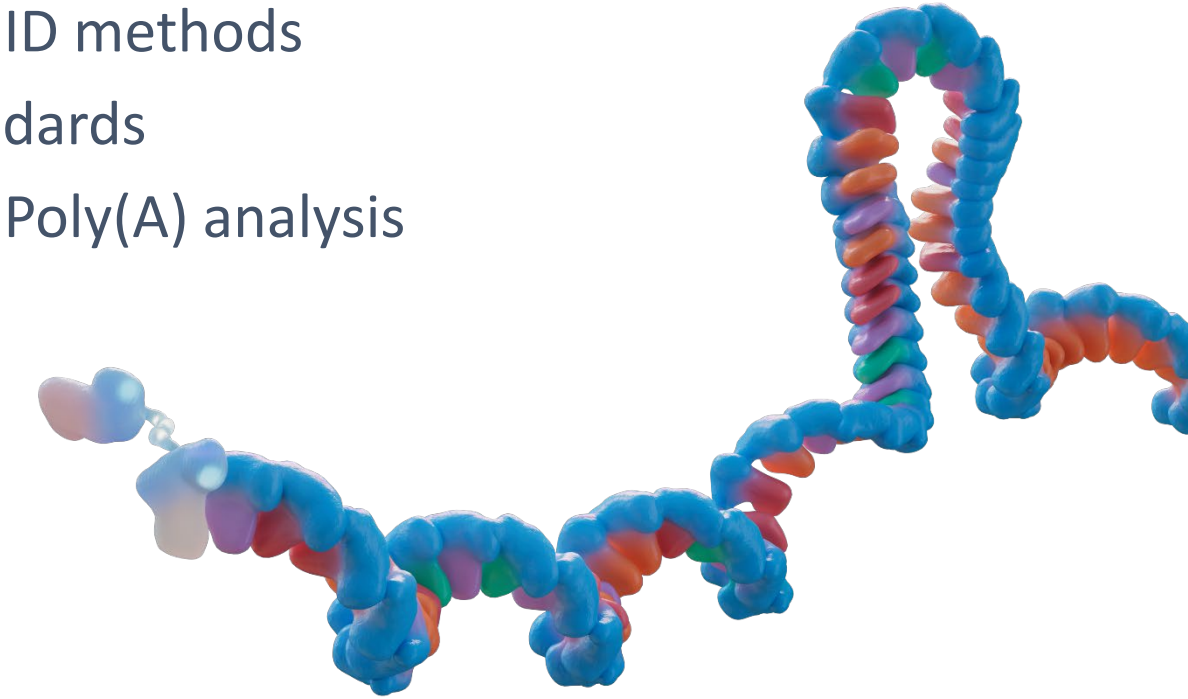
Agenda

This presentation will discuss the criticality of establishing appropriate orthogonal analytical methods for a well characterized mRNA.

1. mRNA background and anatomy
2. Analytical consideration for analysis and characterization of mRNA
   - Importance of orthogonal analytical methods
   - Orthogonal ID methods
   - Poly(A) standards
   - Orthogonal Poly(A) analysis



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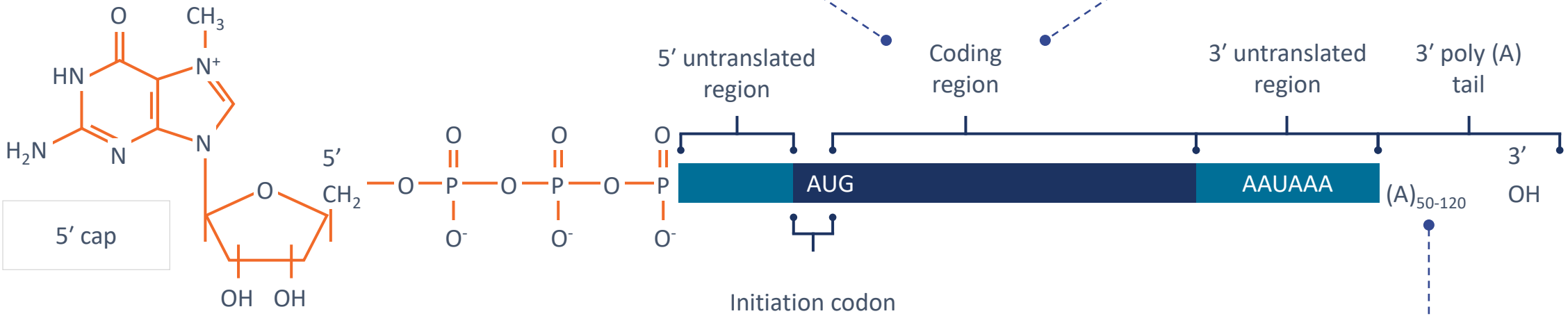
mRNA structure

Nucleoside-modified mRNA

e.g., Ψ, m1Ψ, 5meC

Sequence-engineered mRNA

e.g., GC motif, structural UTR elements



Cap analogs

e.g., CleanCap® capping analogs

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Analytical consideration for analysis and characterization of mRNA

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mRNA critical quality attributes and analytical methods

Integrity and purity

- Gel electrophoresis
- Capillary Electrophoresis
- IP-RP-HPLC
- SEC-HPLC
- AX-HPLC

Identity

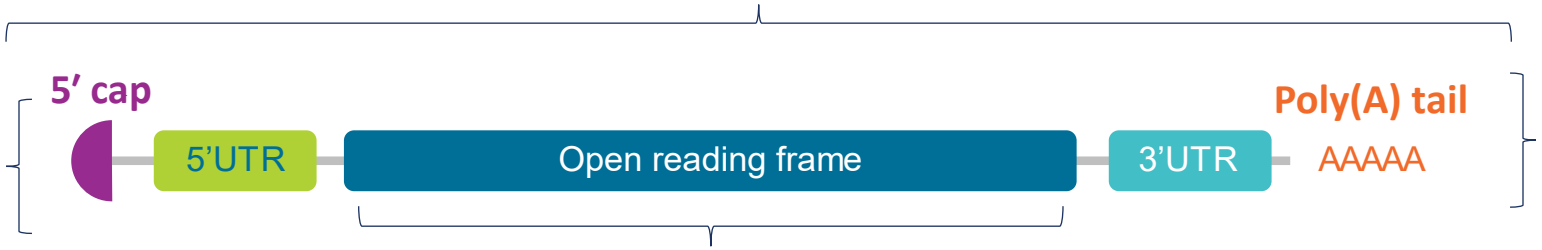
- Sequencing (Sanger, NGS)
- RT-PCRA
- "Fingerprinting" by LC-MS/MS
- Base composition Assay

Content

- UV-Vis Spectroscopy
- RT-qPCR
- RT-dPCR
- IP-RP-HPLC

Capping efficiency

LC-MS
LC-UV



Tail length

LC-MS
LC-UV

Functionality

- In-vitro translation/Western blot (Cell Free assay)
- Cell-based assays (contract out)

Impurities

- **DNA:** qPCR
- **Protein:** Nano Orange; BCA
- **NTPs**: AX-HPLC
- **Solvents:** GC
- **dsRNA:** Immunoblot, ELISA

Others

- **Appearance:** USP <1>, <790>
- **pH:** USP <791>

Safety

- **Endotoxin:** USP <85>
- **Bioburden:** USP <61>, <62>, <1115>

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Case study |Orthogonal mRNA ID methods

1. Development of three (3) orthogonal ID methods for mRNA:
    a. Sequencing (Sanger)
    b. Finger Printing LC-MS/MS
    c. Base composition by LC
2. Case study using Fluc mRNA with a mixture of Uridine and N1-Methylpseudouridine as compared to a FLuc WT mRNA

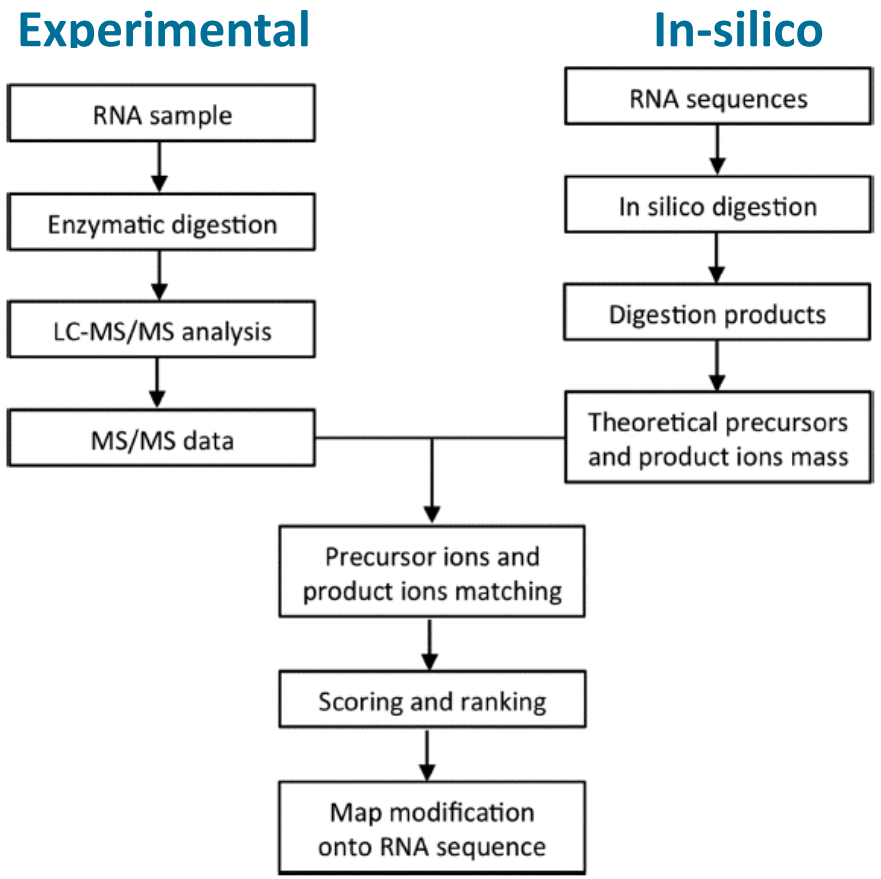
Sanger sequencing

Sample	Result
Control Fluc WT	The sequence was a match
Uridine and N-Me-PseudoU mixture	The sequence was a match

mRNA fingerprinting workflow by LC-MS/MS

Partial mRNA digestion using magnetic beads with immobilized RNase T1

1. Controlled enzymatic Digestion with RNase T1 mag beads.
2. Incubate Reaction for 2-30 min depending on construct.
3. Separate digested mRNA fragments from mag beads.
4. Analyze by LC-MS/MS.
5. Analyze data using Biopharma Finder Software.



Yu, et al 2017

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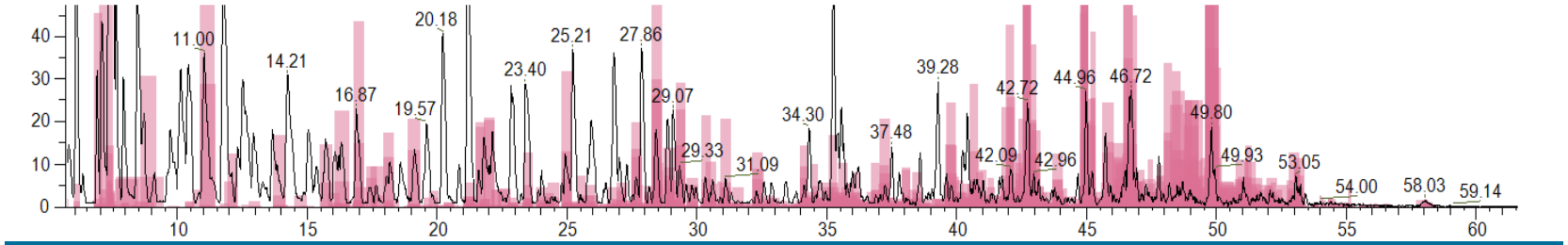
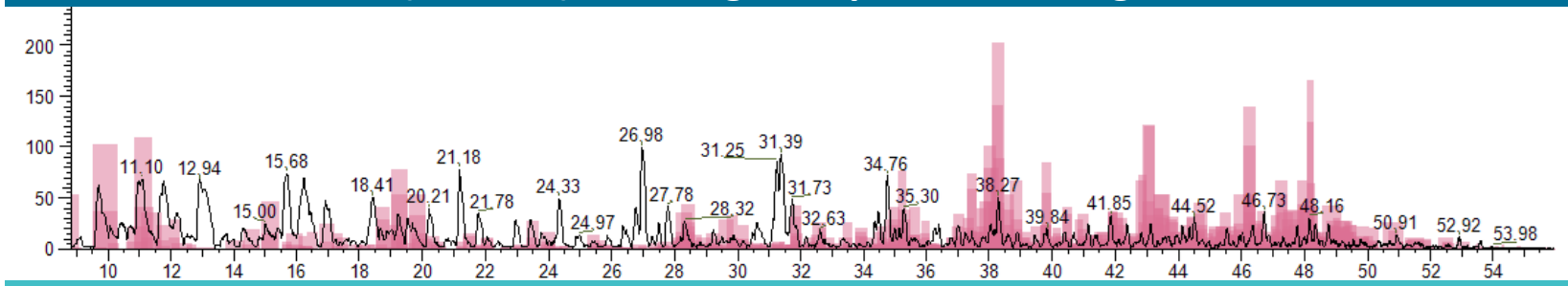
mRNA fingerprinting by LC-MS/MS

Target sequence coverage achieved for different constructs with and without modified NTPs

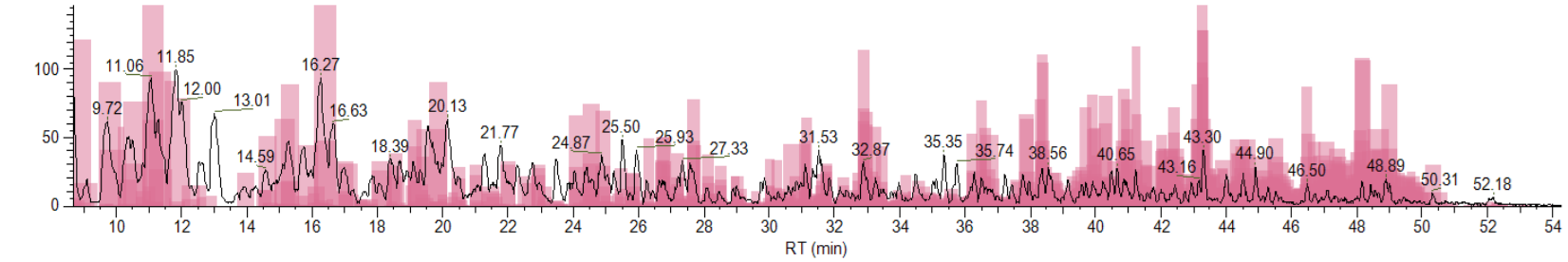
eGFP (996nt) WT: target sequence coverage 87.9%



FLuc (1929nt) WT: target sequence coverage 93.7%



Cas9 (4521nt) WT: target sequence coverage 97.3%



- TIC chromatogram highlighting the peaks used to calculate sequence coverage.
- Unhighlighted peaks were not used for identification.

	Sequence Coverage (%)	RSD (n = 3)
eGFP	87.9	0.0
FLuc	93.7	0.1
Cas9	93.3	1.1

~ 100% coverage of ORF plus UTRs

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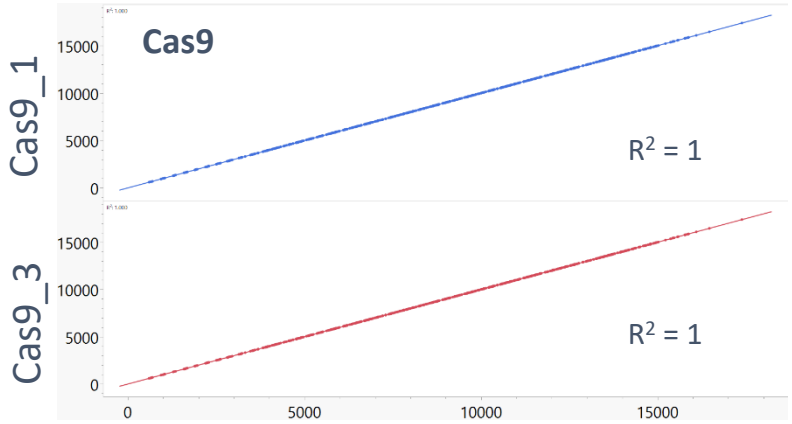
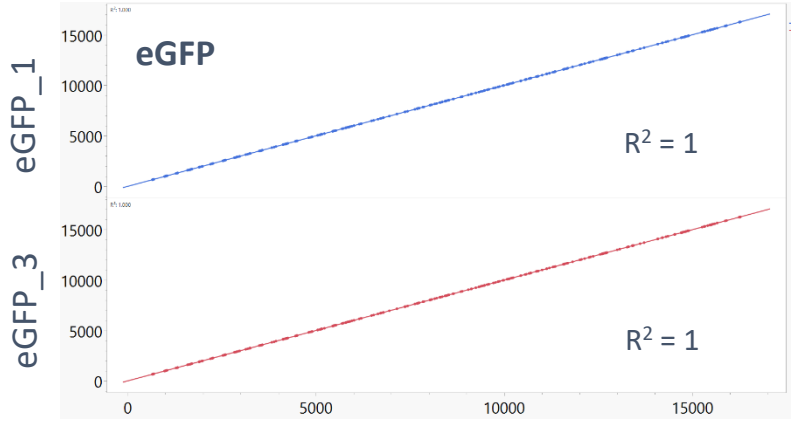
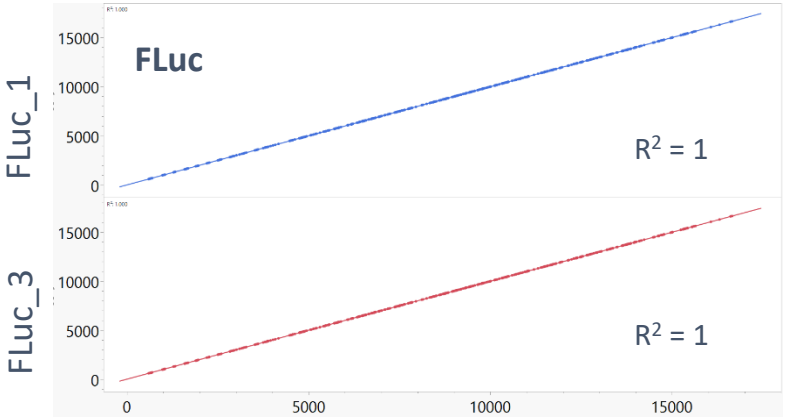
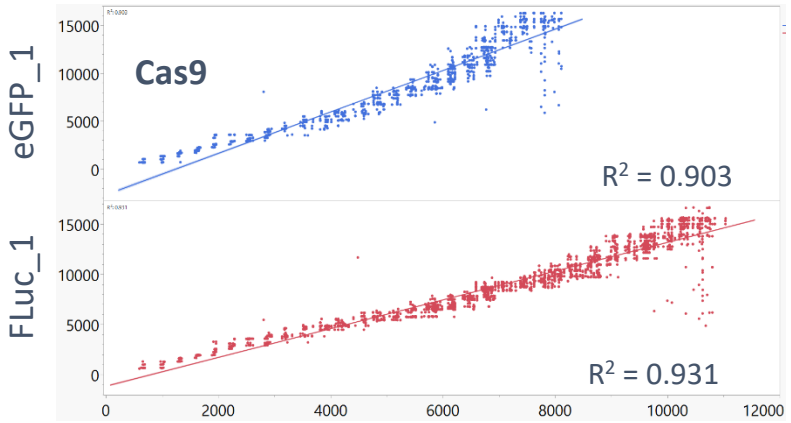
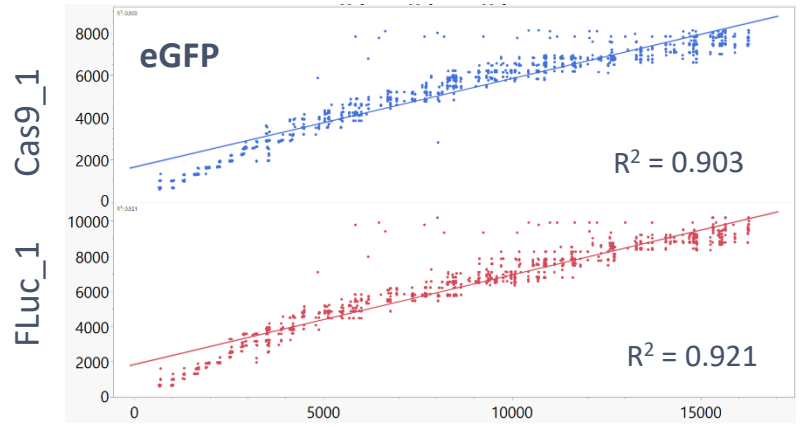
Accuracy and reproducibility of the method

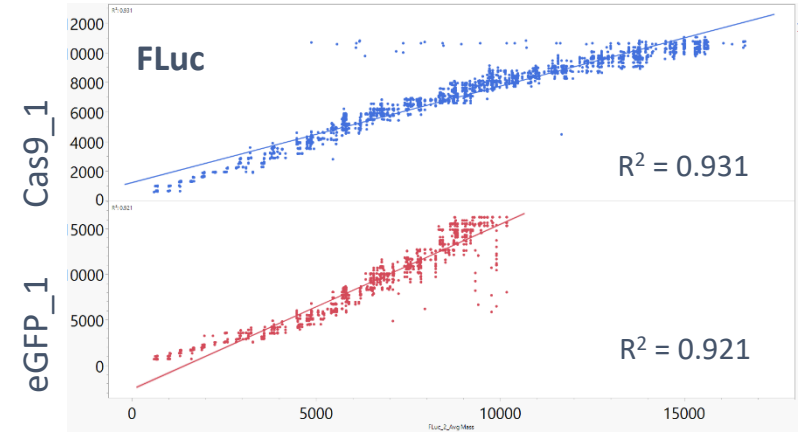












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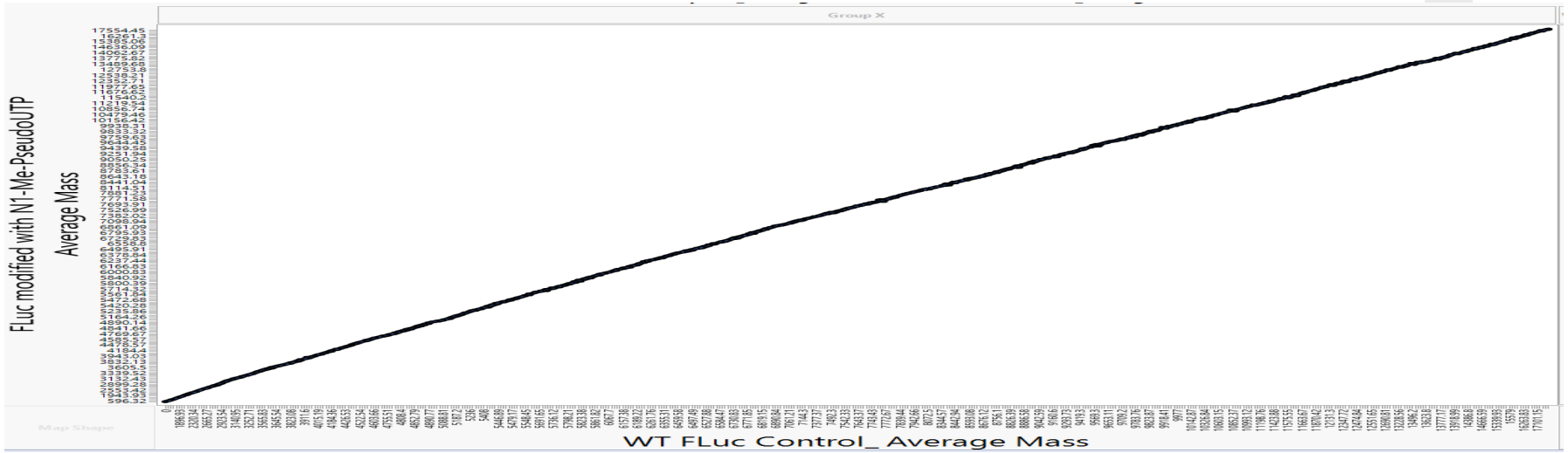
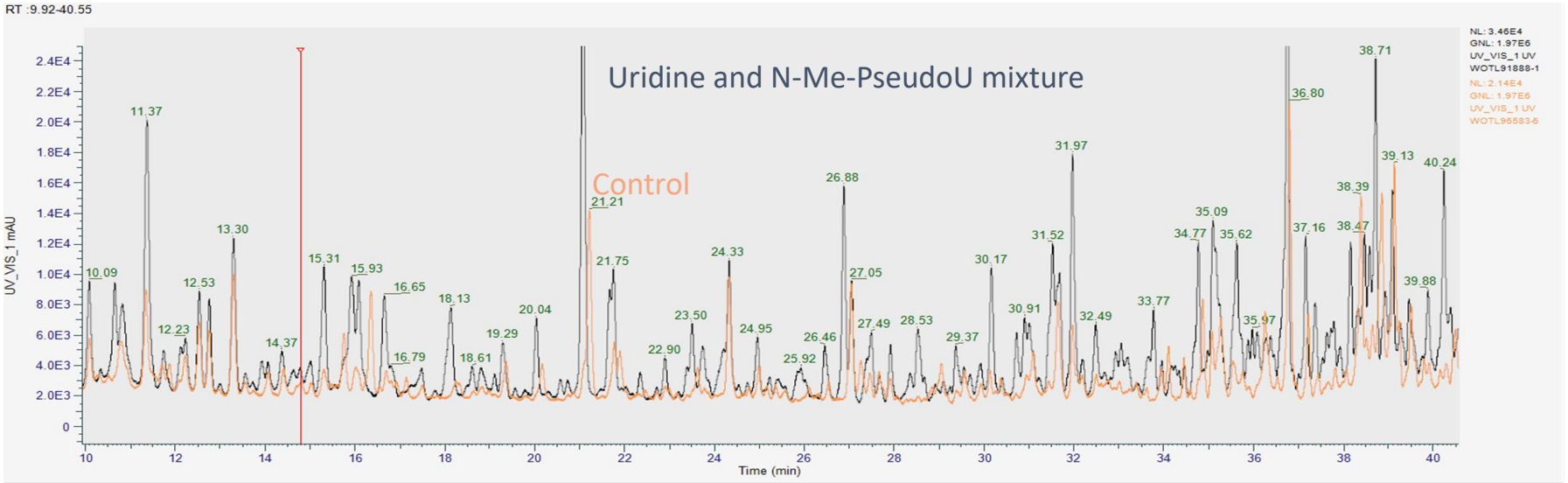
Fingerprinting analysis of uridine and modified uridine sample





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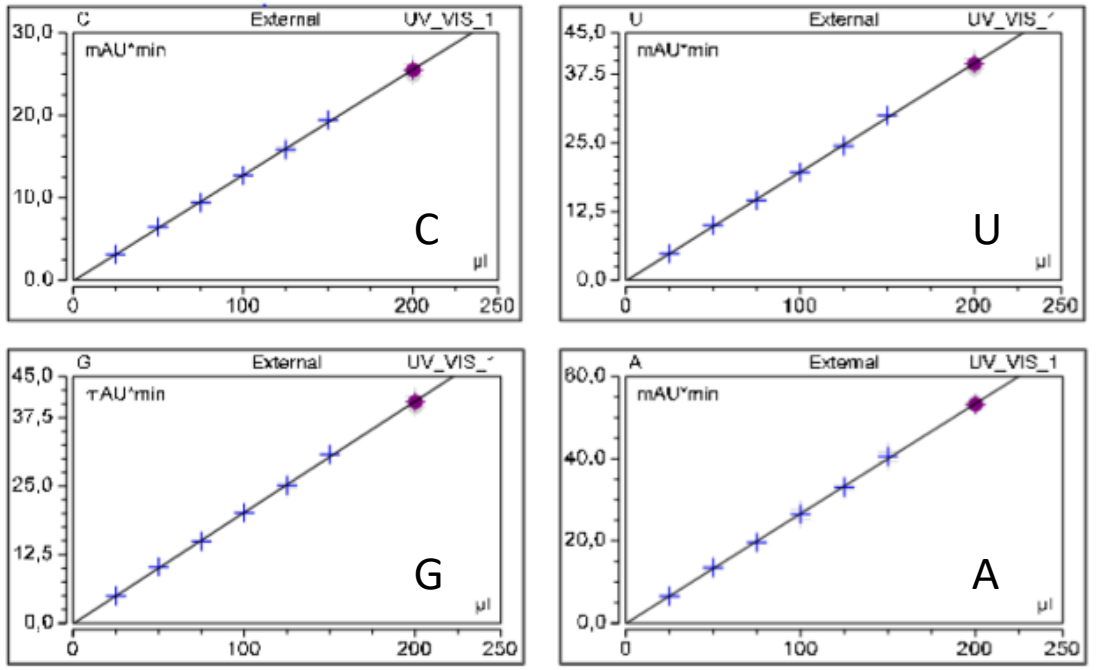
Base composition assay

Digest mRNA sample using snake venom phosphodiesterase

Converting to nucleosides using Calf Intestinal Phosphatase (CIP)

Analysis and quantitation of nucleosides sample by RP-HPLC against a nucleosides bases standard curve



Representative Calibration Curves
Linear range 0.4 – 200 µM

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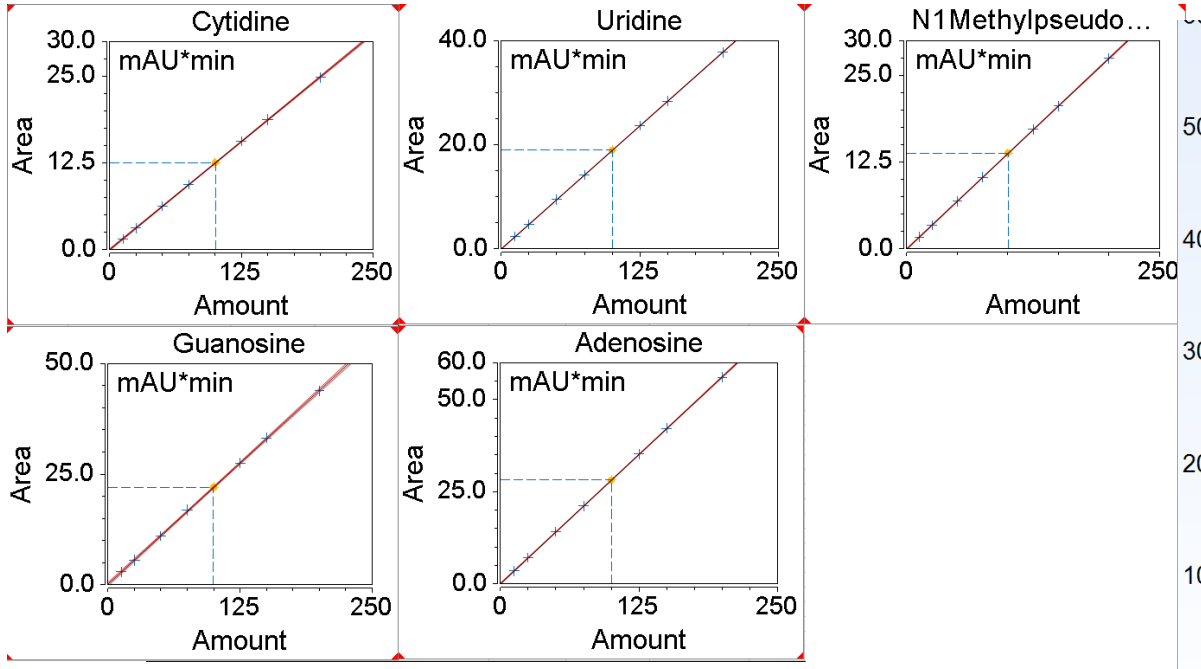
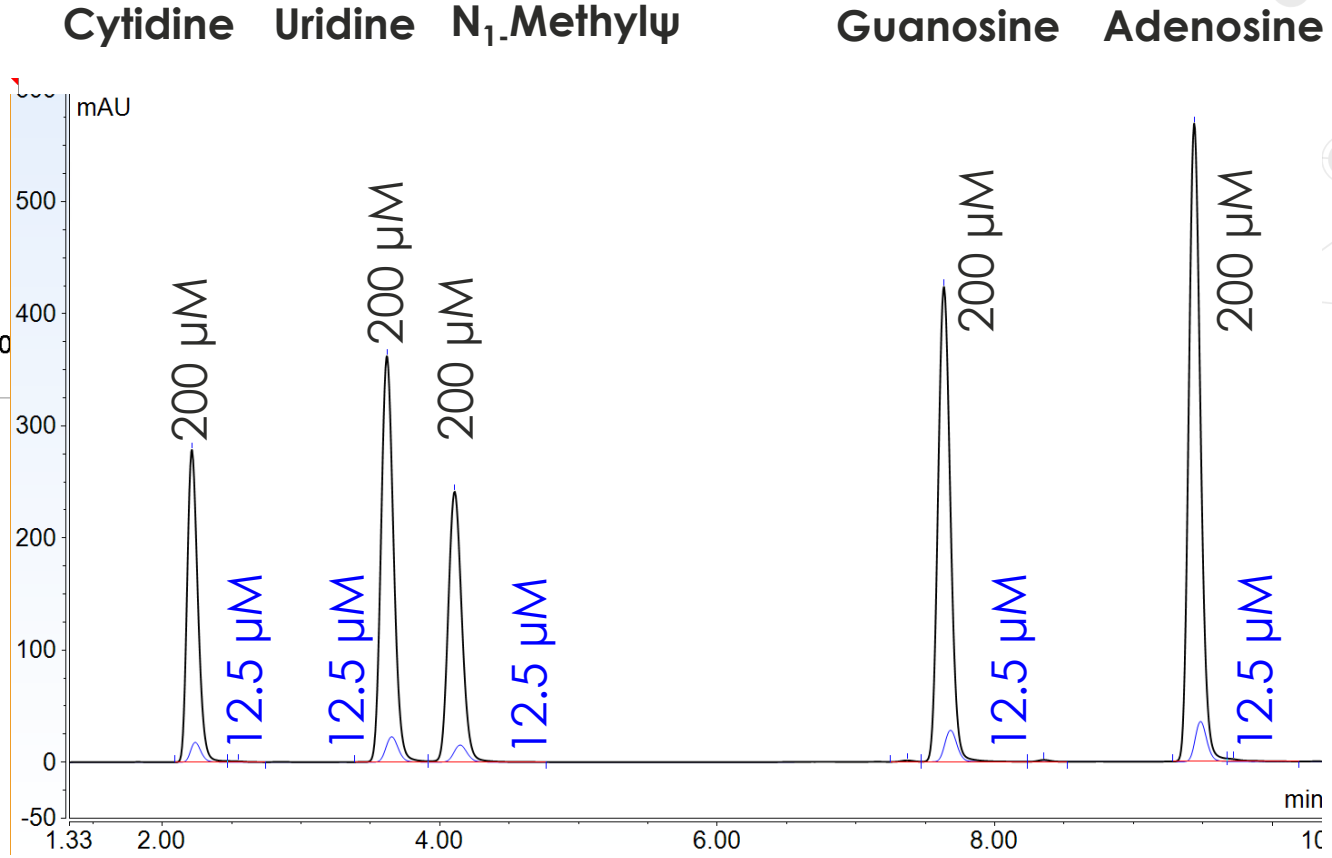
Base composition assay



R^2: 1.000



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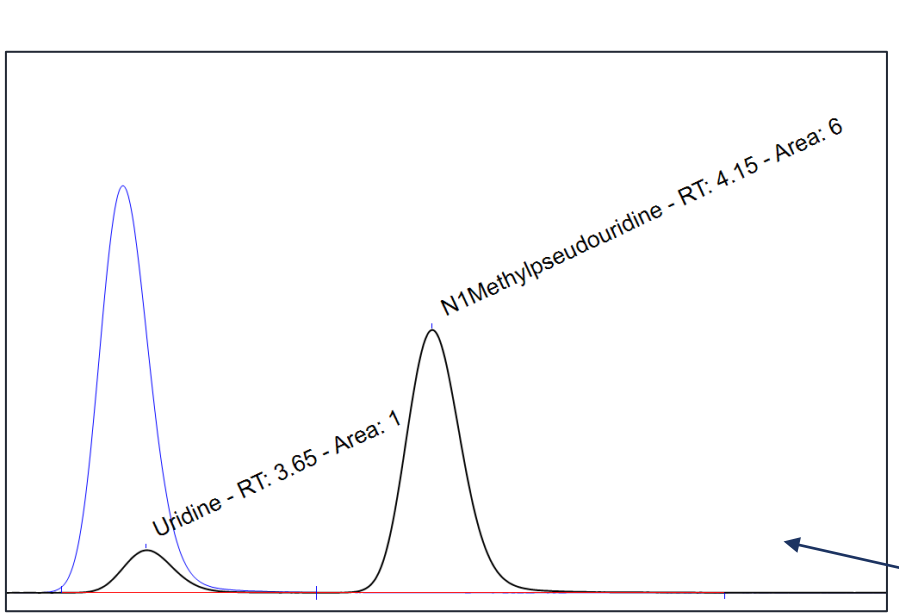
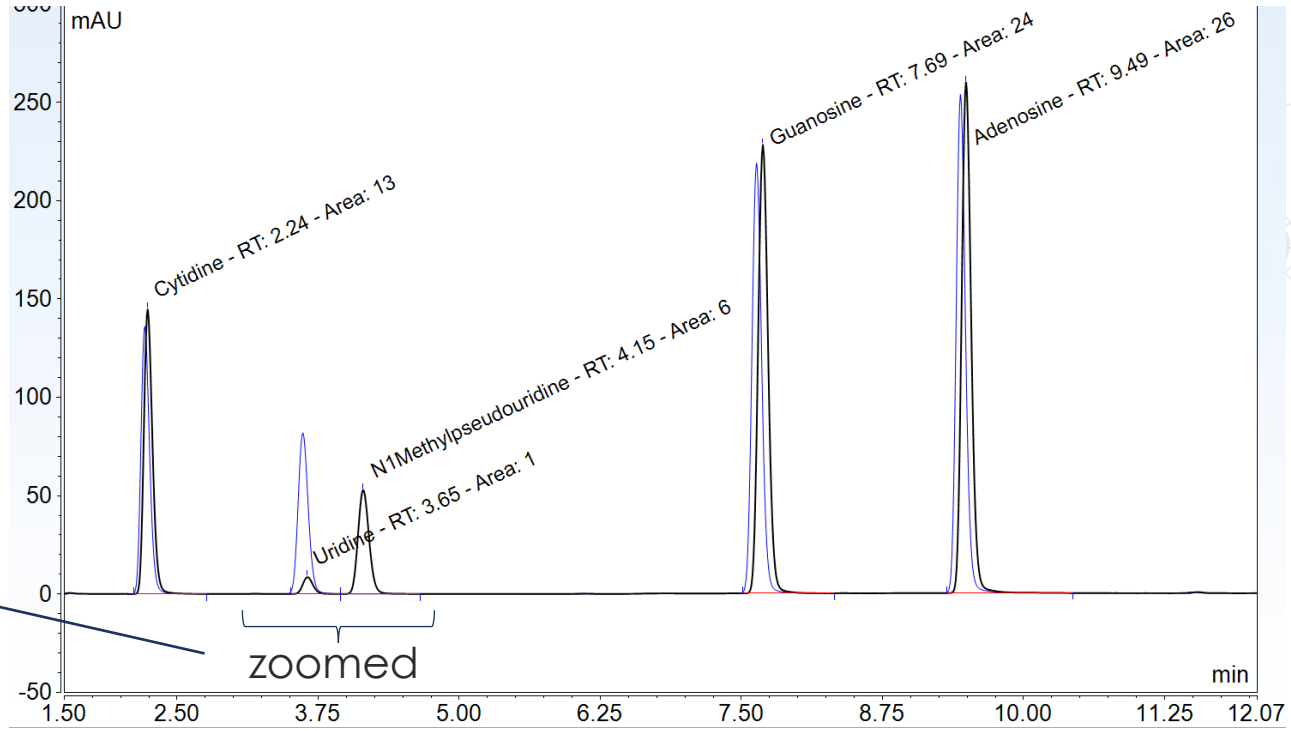
Base composition assay

Fluc WT mRNA control (**blue**) overlayed with N1081 modified + UPT spiked sample (**black**)





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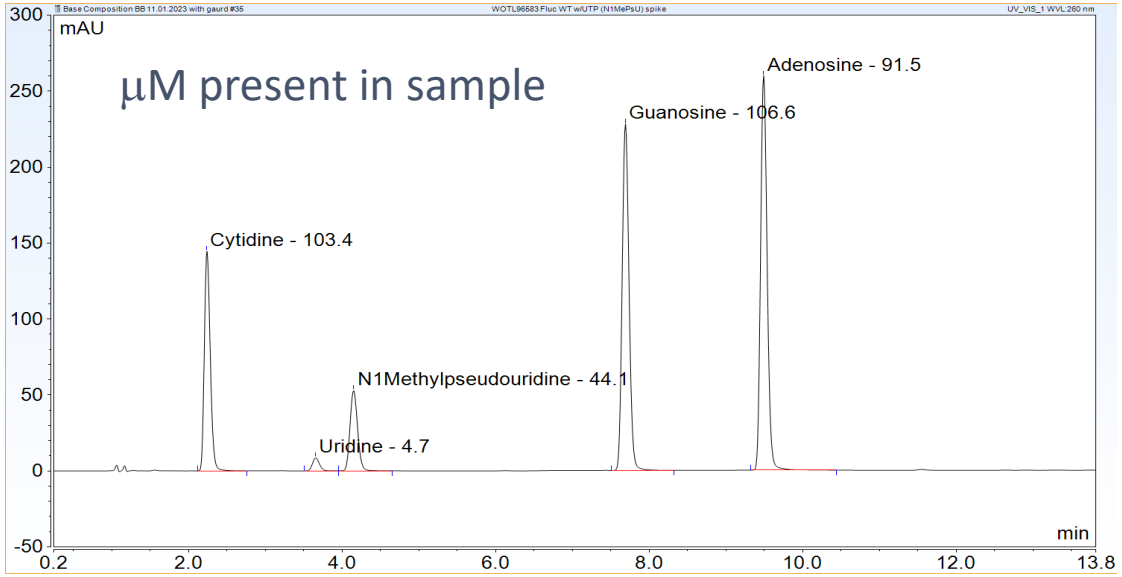
Base composition assay



Sample	# C	# U	# G	# A	# N1-MethylΨ	Total
Fluc WT theoretical	594	273	555	500	0	1922
Fluc with N1-MethylΨ spiked with UTP	567	26	585	502	242	1922
Fluc WT control	569	262	587	504	0	1922

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Summary | Orthogonal mRNA ID methods

1. Each ID methods provide unique information regarding the identity of mRNA
    a. Sequencing (Sanger) provides a sequence coverage of the ORF but would not directly differentiate between WT and modified nucleotides.
    b. Finger Printing LC-MS/MS provides sequence coverage of the ORF and UTR's and indication of any possible nucleotides mismatching.
    c. Base composition by LC provides specific details regarding the base composition of the sequences and how does it match to the theoretical sequence base composition.
2. When combined, the three orthogonal ID methods can provide a tool for investigational and some detailed levels of ID testing.

Methodologies for characterization of Poly(A) tail

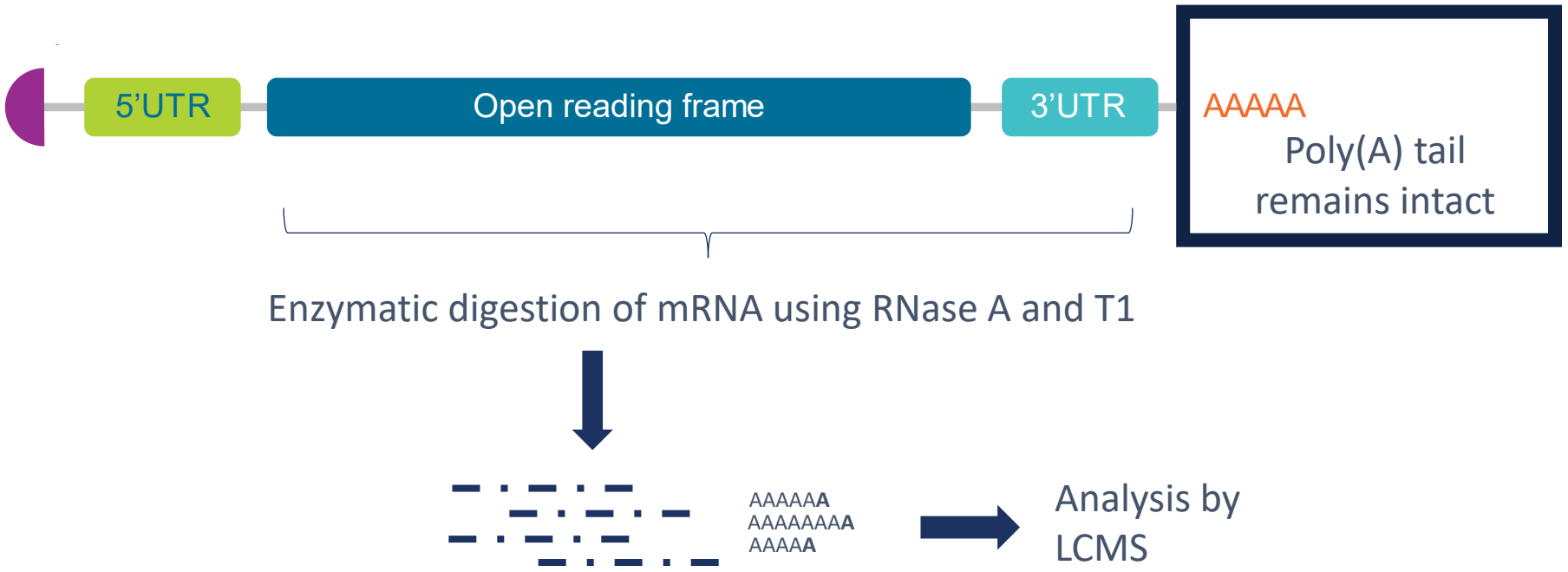
Due to the size of the mRNA, direct analysis of mRNA by Liquid Chromatography - Mass Spectrometry (LC-MS) can be challenging at time. As such, this requires to digest the mRNA prior to analysis. Main methods of digesting mRNA:

- Annealing at the 3’ UTR using DNAzyme. This yield the poly (A) + x Bases and untailed mRNA- x Bases
- Enzymatic digestion such as RNase which yield a complete digestion except for the Poly (A) portion.
- Base composition

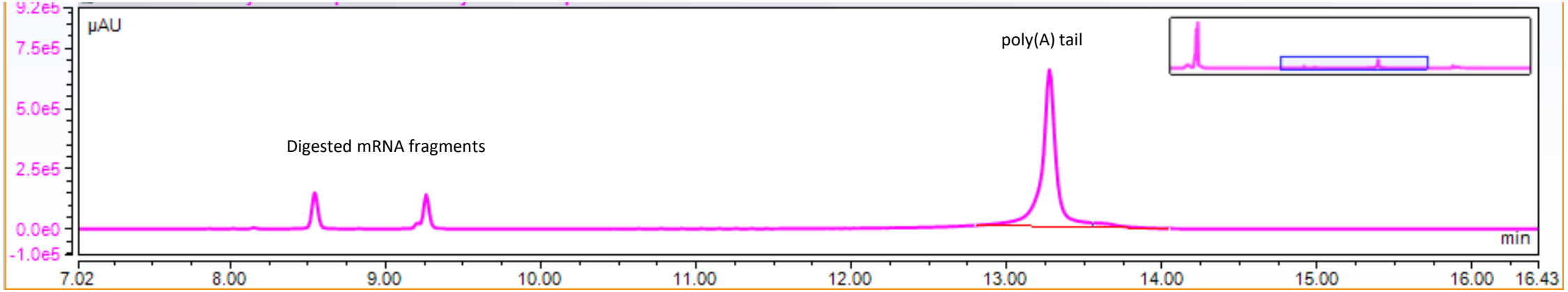
Once the sample is prep, then it can be analyzed by a wide variety of analytical methods

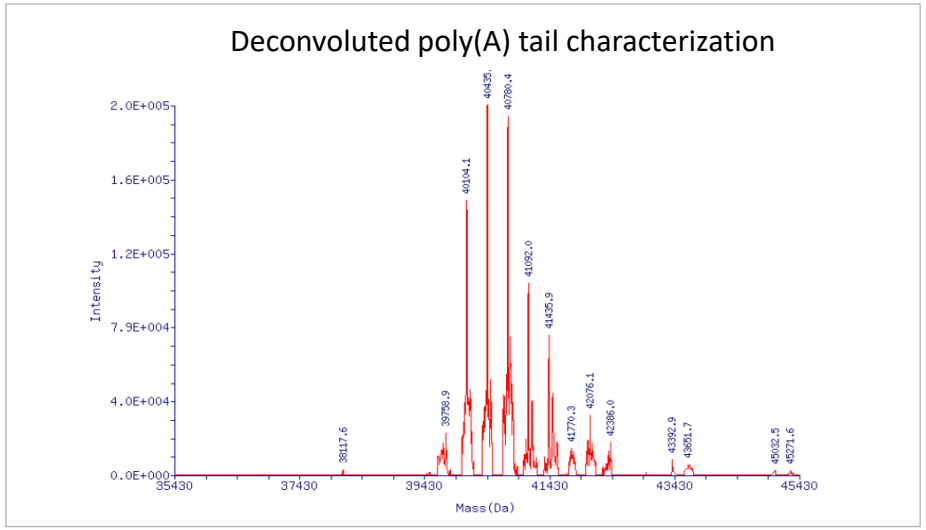
Recently, various publication on this subject and quite a few approaches are still being vetted and evaluated for fit for use in a QC environment.

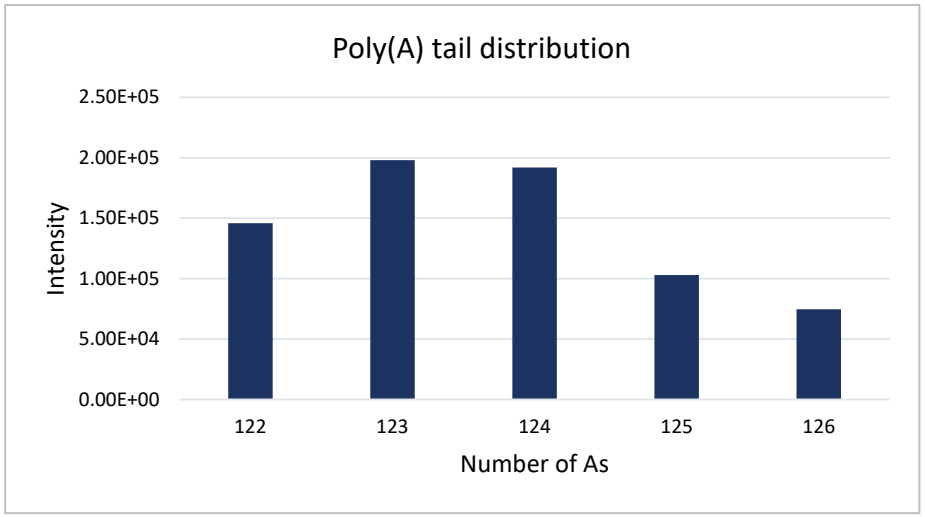
Poly(A) tail enzymatic digestion method by LC-MS



Fluc mRNA poly(A) tail by LC-MS







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Determination of poly(A) tail length by base composition method

1. Use of developed ID composition assay to determine poly (A) length

    a. Tested 6 lots of Fluc mRNA made by the same IVT process and backbone were analyzed against an mRNA without a Poly (A) tail.

    b. Samples were prepared and analyzed for the amount of A present.

    c. Poly (A) length was determine based on the concentration of A and that was compared to values of Poly (A) by LC-MS method.

FLuc mRNA	Lot 1	Lot 2	Lot 3	Lot 4	Lot 5	Lot 6
Observed # A	498	496	498	500	502	504
theoretical # A without tail			**380**			
Average Tail #	118	116	118	120	122	124
Tail Length by LCMS	**124**	**123**	**123**	**123**	**124**	**124**
% difference	95%	94%	96%	98%	98%	100%

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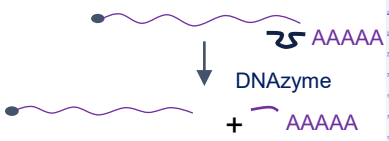
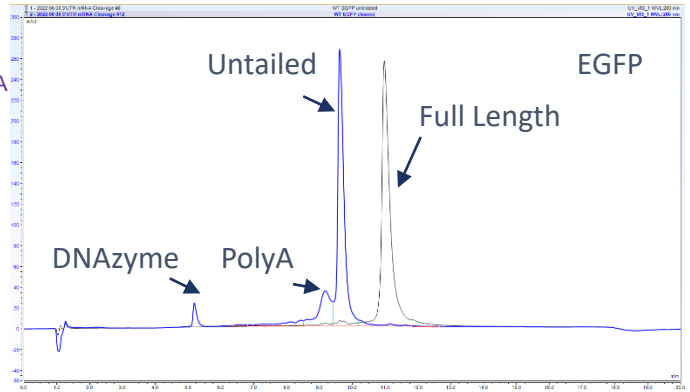
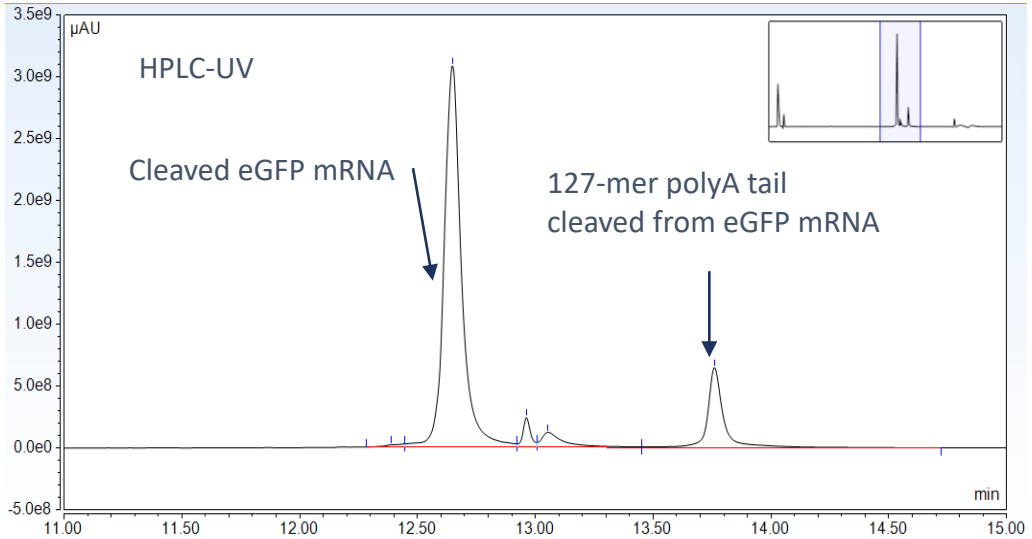
Direct analysis of poly(A) tail DNAzyme cleavage method

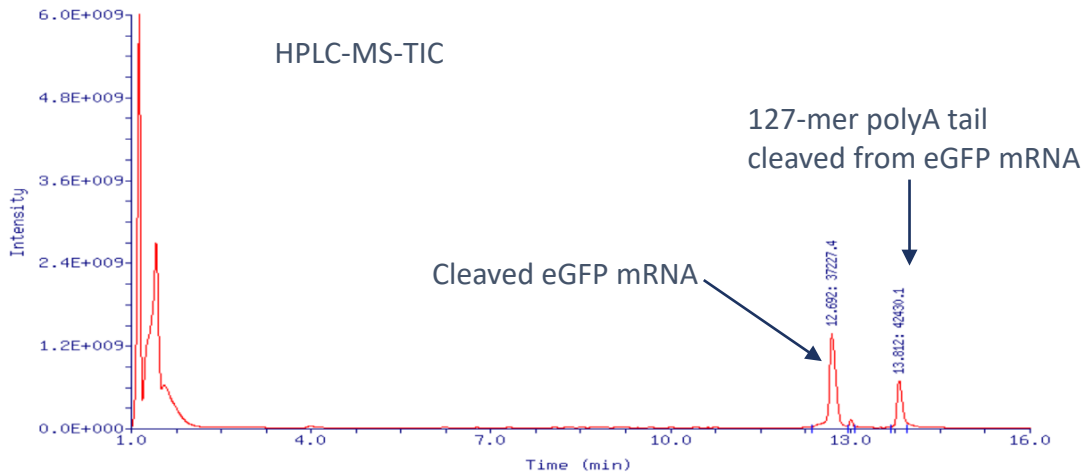


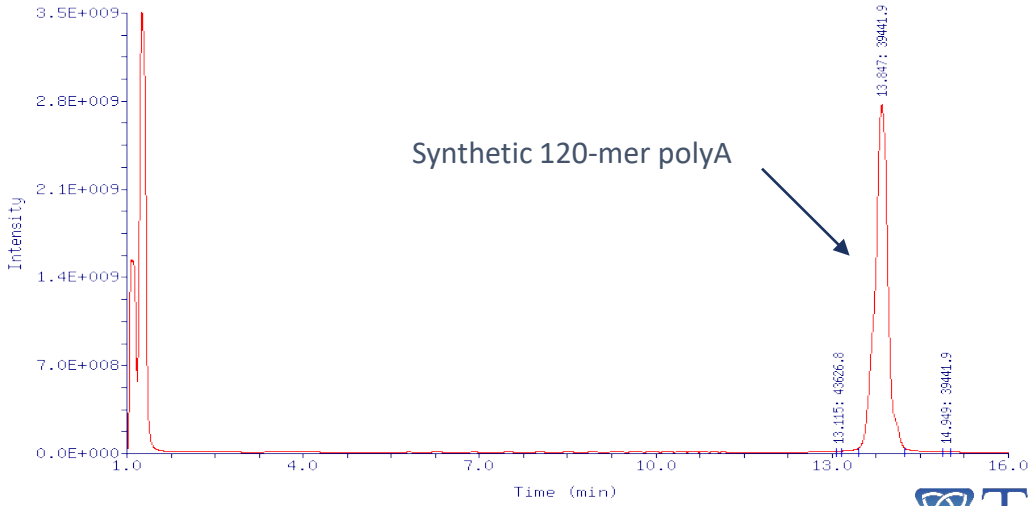


Reaction steps

- DNAzyme reaction
- Annealing
- Cleavage







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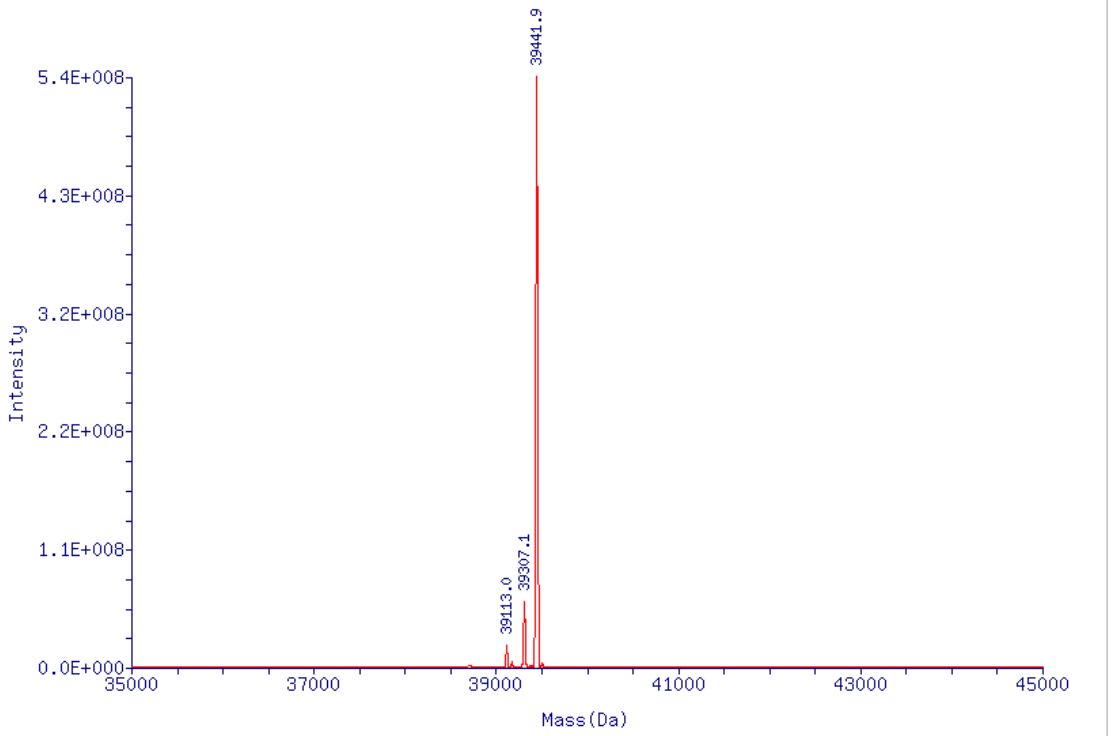
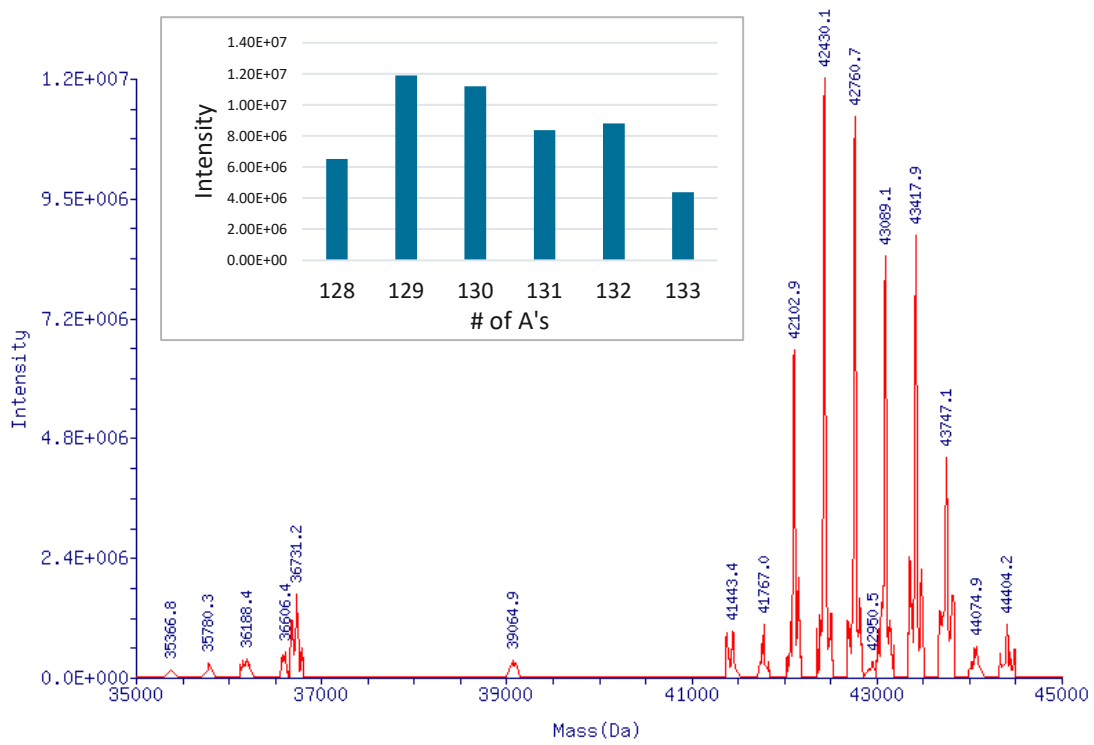
Another approach for poly(A) tail analysis

Deconvoluted spectra ofsSynthetic 120-mer poly(A) tail



Deconvoluted spectra of poly(A) tail cleaved by DNAzyme from eGFP mRNA



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Synthetic poly(A) standards

Synthetic poly(A) standards

- This has been a cross functional collaborative effort to provide better solution to enhance customer's journey
  - AS/QC team
  - Oligo team
  - Core mRNA

- Five synthetic Poly (A)s 39, 59, 79, 99, 120
- Analysis of synthetic Poly (A)s by various methods
  - Size exclusion
  - Modified IPRP-HPLC
  - IPRP-HPLC
  - Octylamine HPLC
  - AX-HPLC
  - Fragment analyzer

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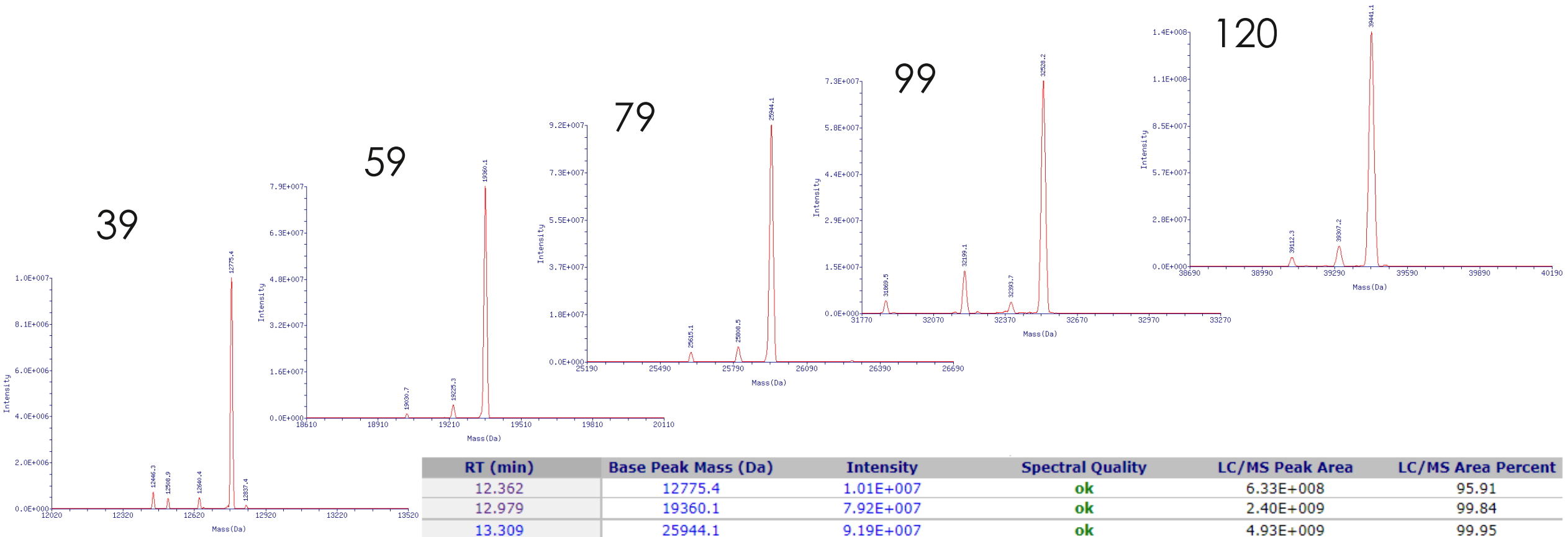
MS deconvoluted data for size determination



RT (min)	Base Peak Mass (Da)	Intensity	Spectral Quality	LC/MS Peak Area	LC/MS Area Percent
12.362	12775.4	1.01E+007	ok	6.33E+008	95.91
12.979	19360.1	7.92E+007	ok	2.40E+009	99.84
13.309	25944.1	9.19E+007	ok	4.93E+009	99.95
13.463	32528.2	7.27E+007	ok	7.05E+009	99.98
13.528	39441.1	1.42E+008	ok	9.68E+009	99.98

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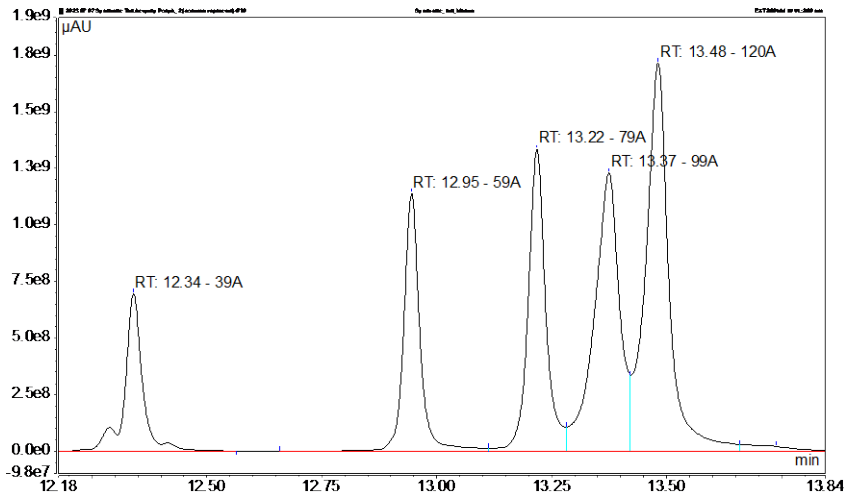
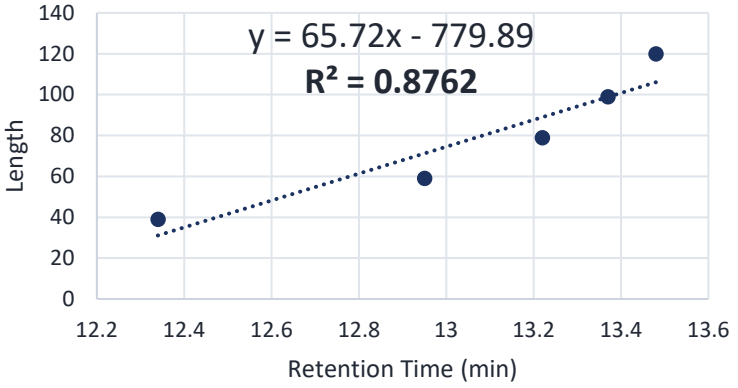
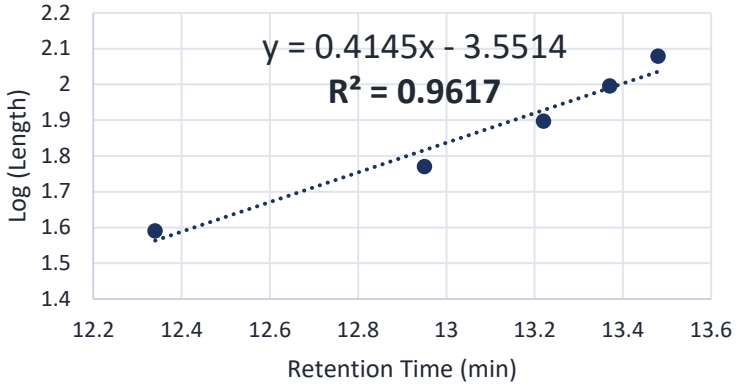
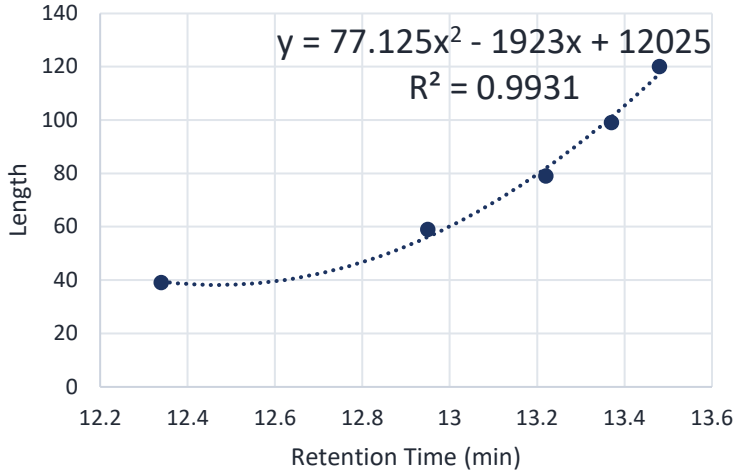
Length vs relative retention time standard curves

Poly(A) tail mixture









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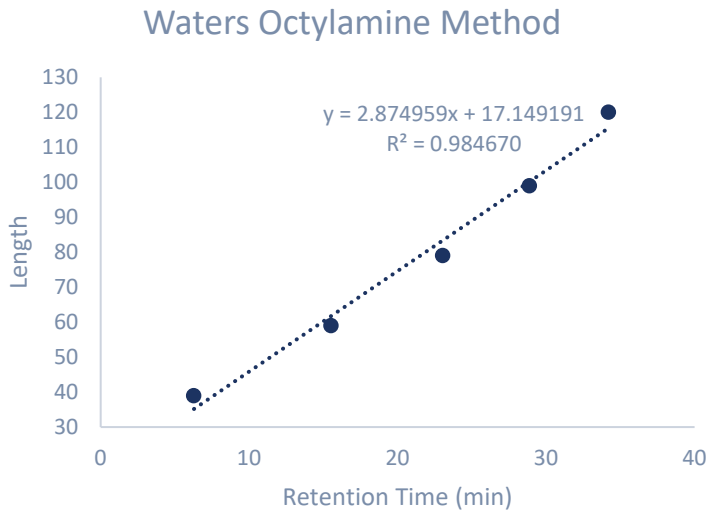
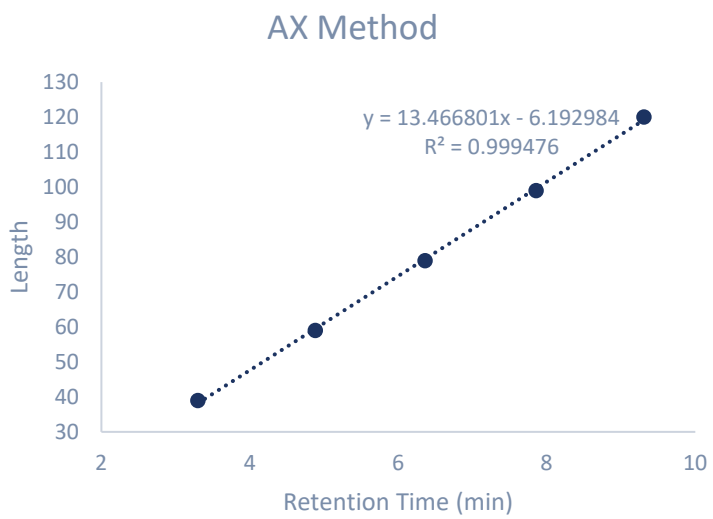
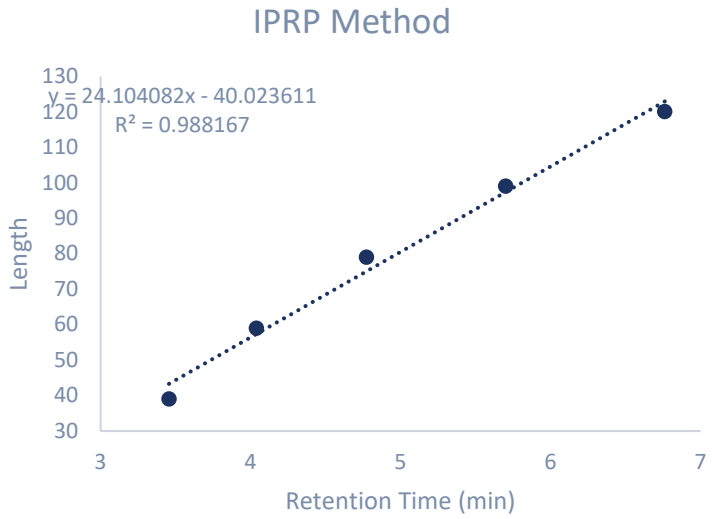
Poly(A) standards results

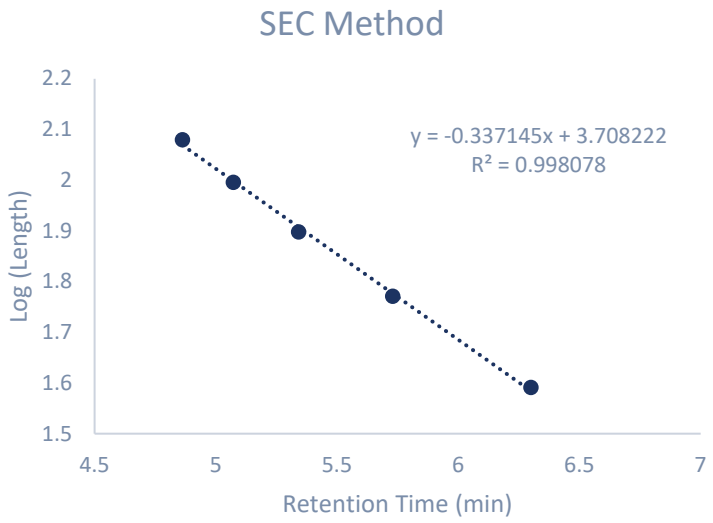
Length	Target Mass (Da)	Observed (Da) PolyA method	LCMS Area Percent PolyA method
120 A's	39443.2	39442.7	99.99
99 A's	32529.8	32529.1	99.97
79 A's	25945.6	25945.1	99.94
59 A's	19361.4	19360.4	99.96
39 A's	12777.2	12776.2	99.88

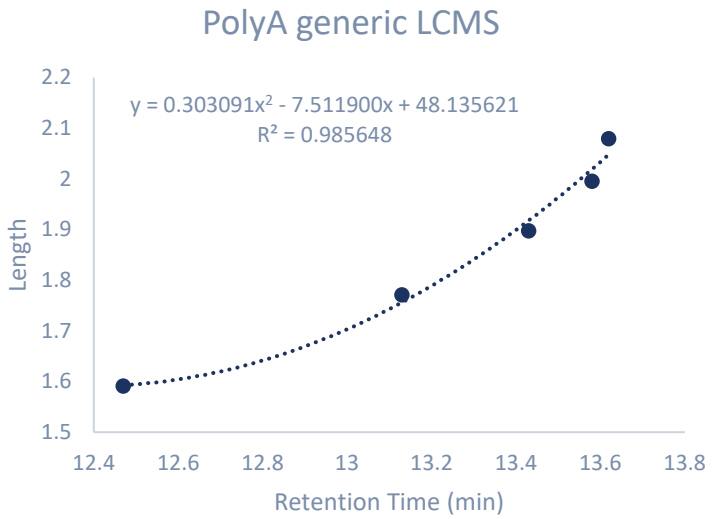
Length versus RT graphs constructed with synthetic poly(A) tail standards











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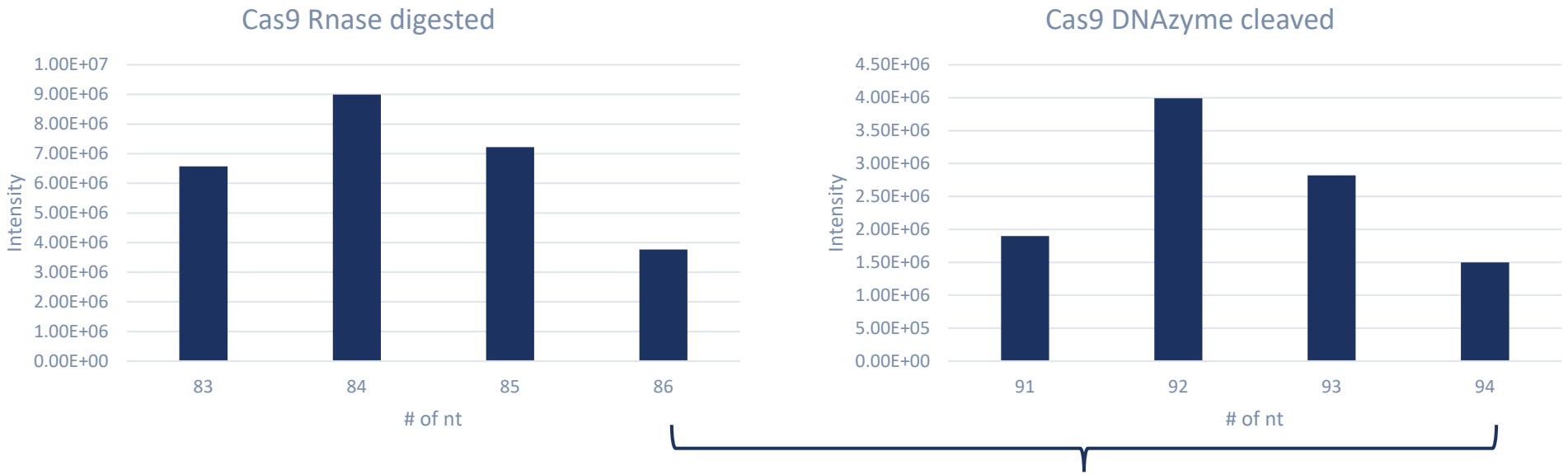
MS results for Cas9 80A mRNA– generic Poly(A) tail LC-MS



	Theoretical
Cas9 digested	80
Cas9 cleaved	85

8 nt difference between the cleavage and RNase treatment

Result does not match

5-mer of 3’UTR region with tail

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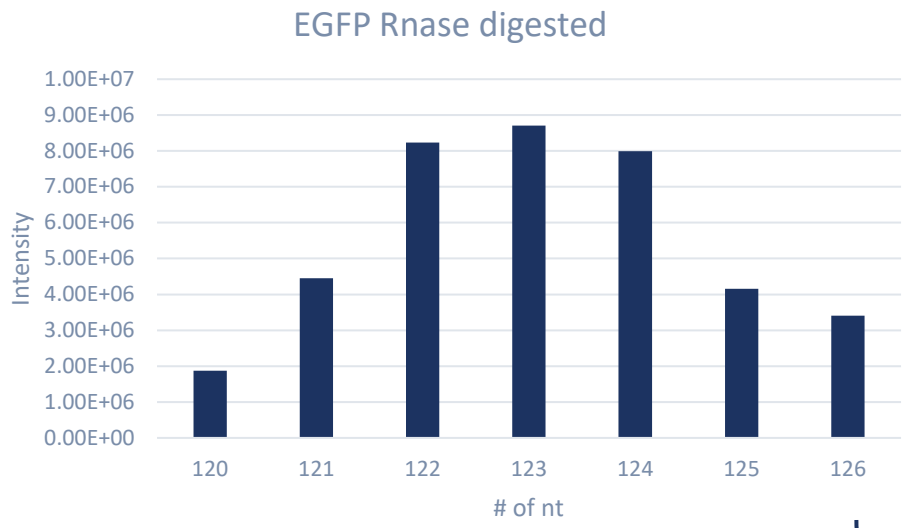
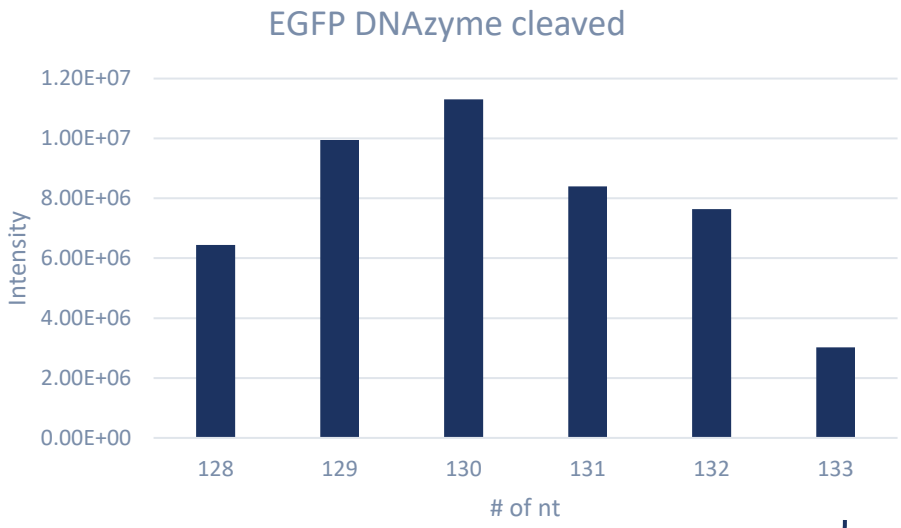
MS results for eGFP 120A mRNA – generic Poly(A) tail LC-MS





	Theoretical
eGFP digested	120
eGFP cleaved	127

7 nt difference between the cleavage and RNase treatment

Result matches expectations

7-mer of 3’UTR region with tail

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Calculated Poly(A) tail length summary of orthogonal methods

Calculated Length	Octylamine	AX	SEC	mRNA IPRP	Generic PolyA LCMS	Theoretical Length
eGFP digested	117	122	120	130	120	120
Cas9 digested	89	84	86	79	82	80
eGFP cleaved	126	125	141	130	116	127
Cas9 cleaved		86		79	74	85

- Poly(A) tail lengths were calculated with length vs RT graphs shown on the previous slide.

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Summary

1. Robust analytical methods for mRNA characterization
2. Orthogonal ID Methods
3. Multiple approaches for poly(A) analysis
4. Poly(A) standards

Identity, purity and characterization testing	Safety and general testing
• Identification • Quantitation • Product characterization • Functionality • Impurity profiling	• Safety (microbial and endotoxin) • Stability • Qualitative assessment

Custom services

Construct-specific method development, qualification/validation, analytical testing and stability studies

Achieve your analytical objectives from process development to scale-up and cGMP manufacturing

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Analytical methods lifecycle by phase

	Discovery	Preclinical/Toxicology	Phase I	Late Phase
Type of material	RUO material	Engineering material	Phase I GMP material	Late Phase GMP material
Level of analytical method evaluation	Feasibility recommended	Feasibility	Method* qualification/ verification	Method* validation
Parameters	Generic method based on structure	Generic method based on structure	Specific method • Specificity** • Linearity • Dynamic range • Accuracy • Precision**/IP • LOD**/LOQ	Specific method • Specificity** • Linearity • Dynamic range • Accuracy • Precision**/IP • LOD**/LOQ • Robustness • Sample stability

Evaluation of stability indicating methods

Cross-labs qualification, validation, and training

*For intended use e.g. to support safety, integrity, strength, purity, and quality
** Qualitative assessment

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Analytical Services and Quality Control laboratory expansion

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TriLink's Analytical Sciences Center of Excellence (ASCE)

Centralized hub to drive innovation in nucleic acid analytical methodologies

- Develops additional methodologies for characterization of nucleic acids
- Provides method development, qualification, validation, stability testing, and product characterization
- Offers standalone support for RUO and non-clinical mRNA

Extensive advanced
in-house instrumentation (4,000 ft^2 lab space)

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ACKNOWLEDGEMENTS

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Analytical Services team

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Thank you

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