



### Introduction

Structural characterization of therapeutic mRNA, including confirmation of the oligonucleotide sequence and assessment of 5' and 3' capping efficiency, is crucial for ensuring safety and efficacy. LC-MS/MS digested oligonucleotide sequence mapping is a powerful tool for evaluating these three critical quality attributes within a single assay. In this study, we demonstrate the application of a novel oligonucleotide mapping workflow to fully map the eGFP mRNA sequence, detect and quantify the m7Gppp 5' cap, and measure the PolyA tail length distribution. The eGFP mRNA samples were digested with hRNase 4 endoribonuclease and analyzed using ion pairing reverse phase liquid chromatography-high resolution electrospray tandem mass spectrometry (IP-RPLC-MS/MS).



Oligo

Identification, annotation of Oligonucleotide molecules



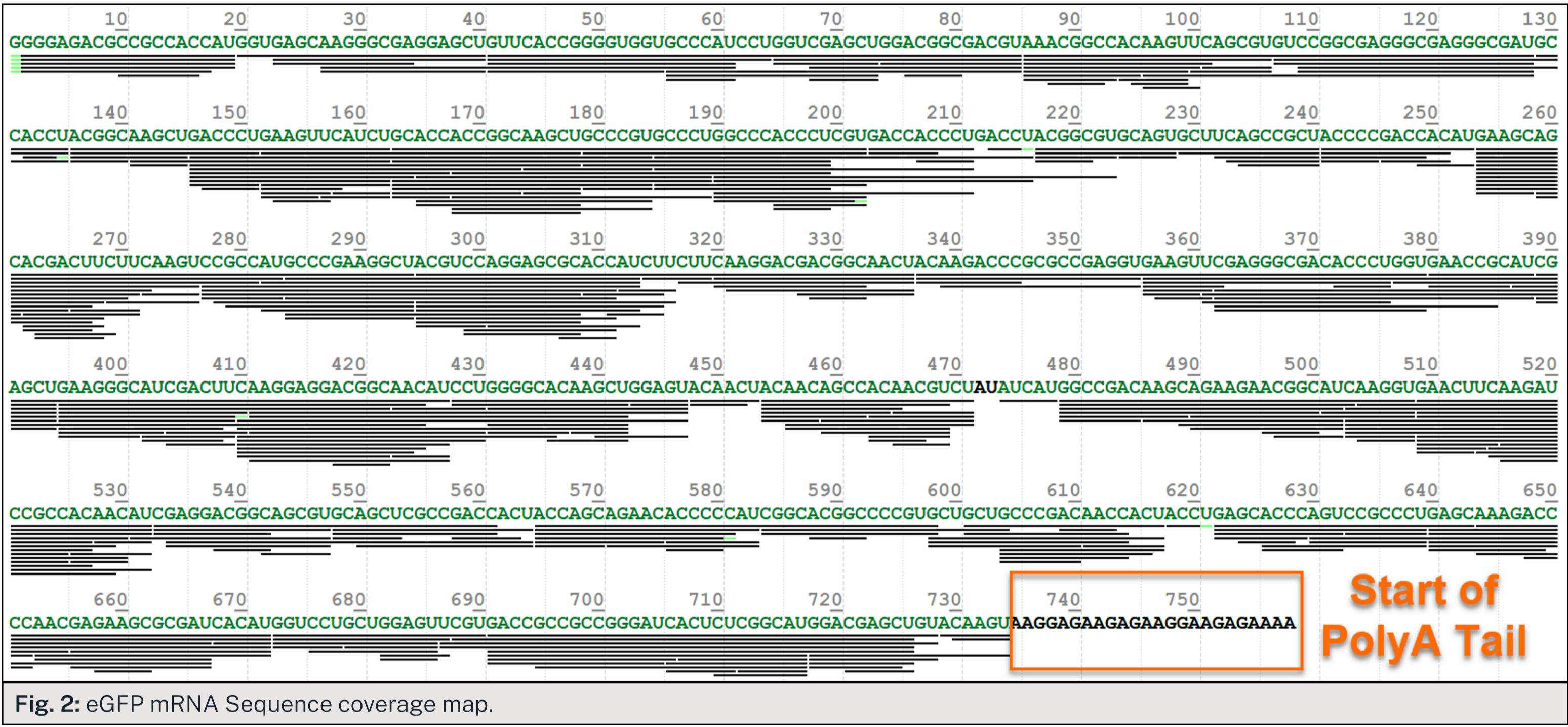
Digested Oligonucleotides

Enzymatically digested oligonucleotides

eGFP mRNA was digested with **hRNase4** and analyzed by IP-RPLC-MS

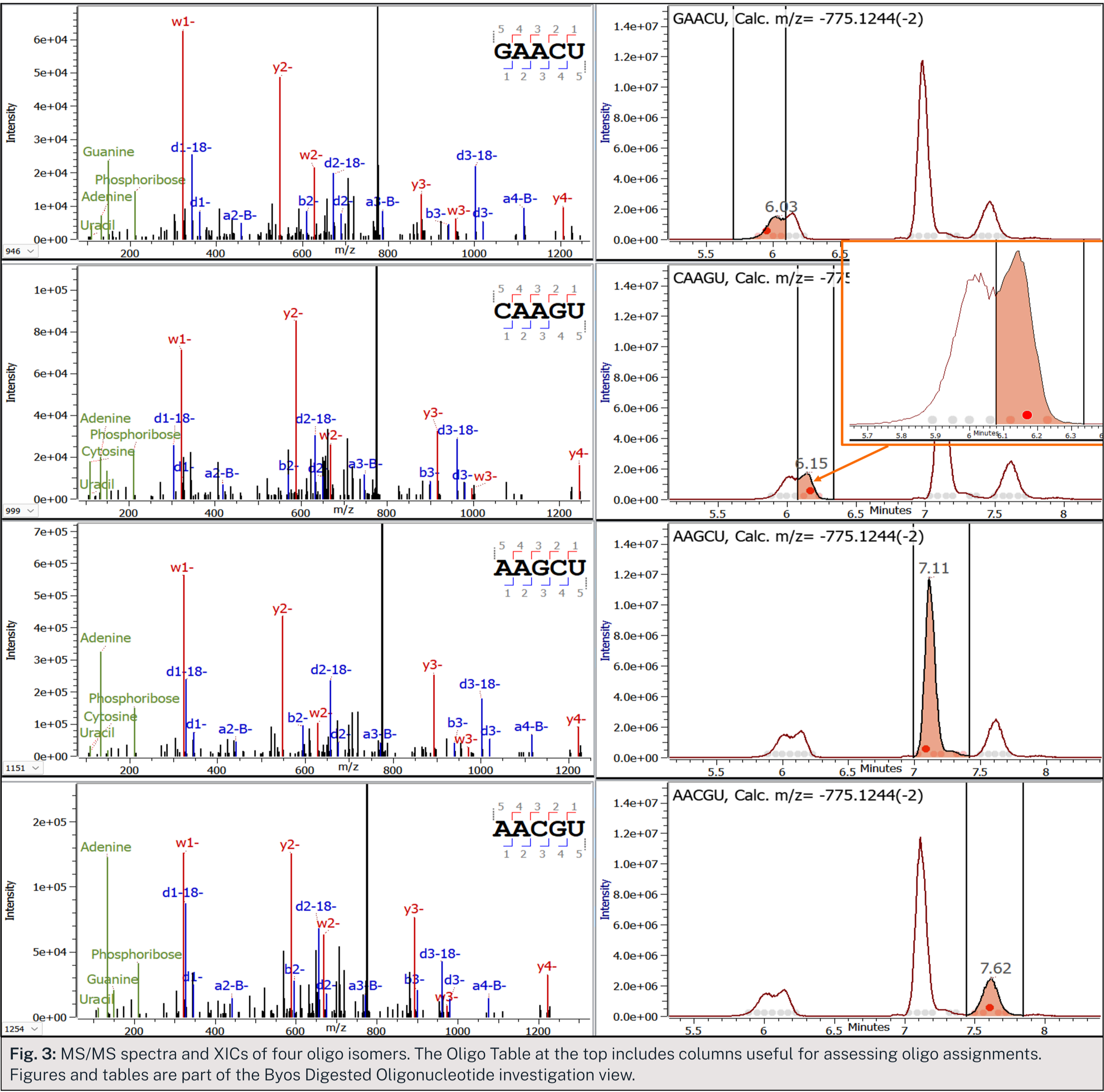
70-minute IP-RPLC gradient using the **Phenomenex bioZen2.6 µm Oligo 150 x 2.1 mm column (PN: 00F-4790-AN)** and DDA Neg mode using the Thermo Q-Exactive Plus

Data processing with **Byos Digested Oligonucleotide** and **Byos Oligo (v5.6)**



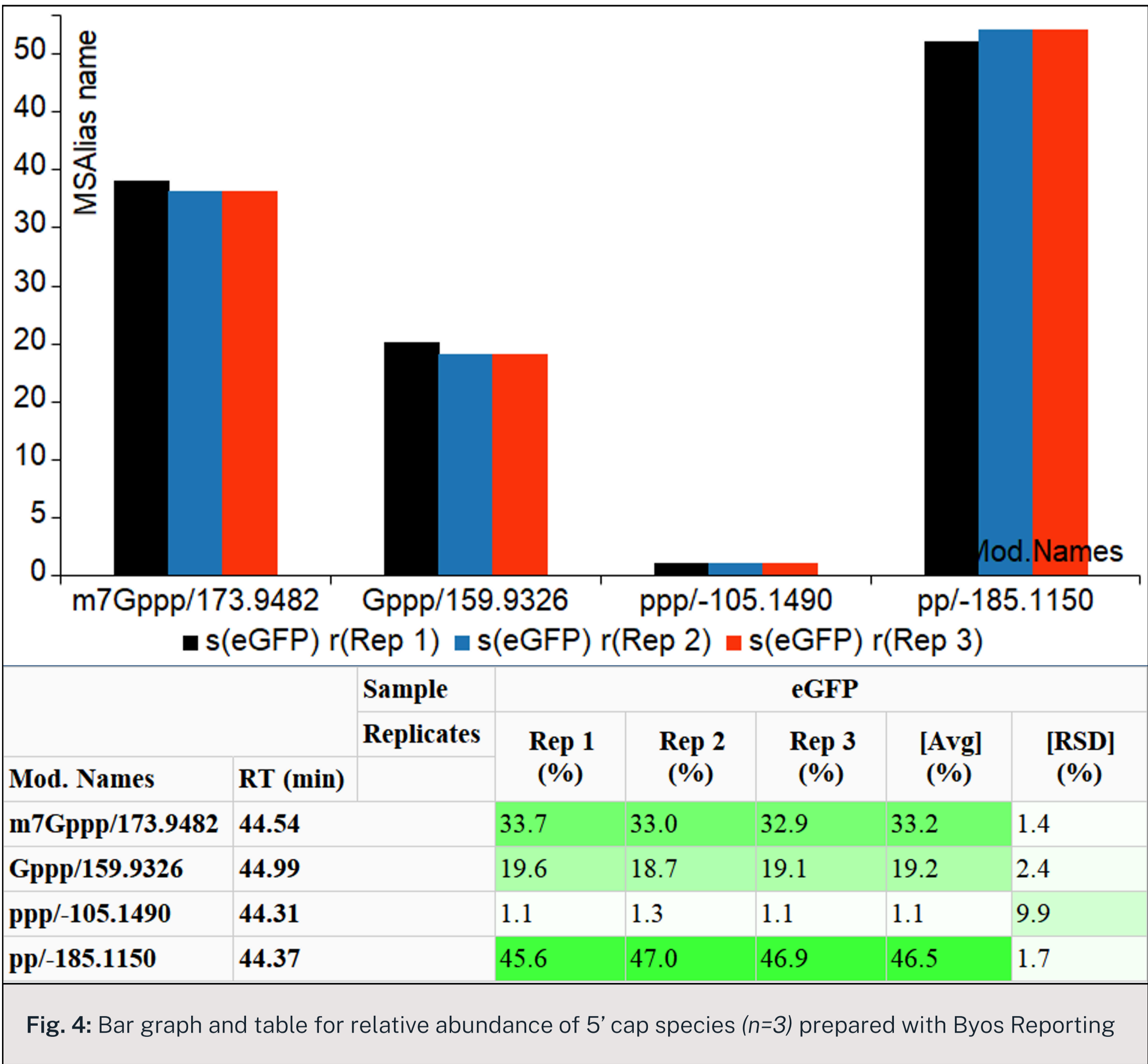
Key isomer distinguishing fragment ions observed in annotated MS/MS spectra.

Row#	Sequence	Calc.M	Apex Time (Posit)	Score	Delta Score	XIC area summed
> 1	U.GAACU.U	1552.26	6.03	416	40 - 143	1.37e+07
> 2	U.CAAGU.C	1552.26	6.15	372	89 - 112	1.13e+07
> 3	C.AAGCU.G	1552.26	7.11	362	33 - 43	6.63e+07
> 4	C.AACGU.C	1552.26	7.62	359	51 - 68	2.04e+07

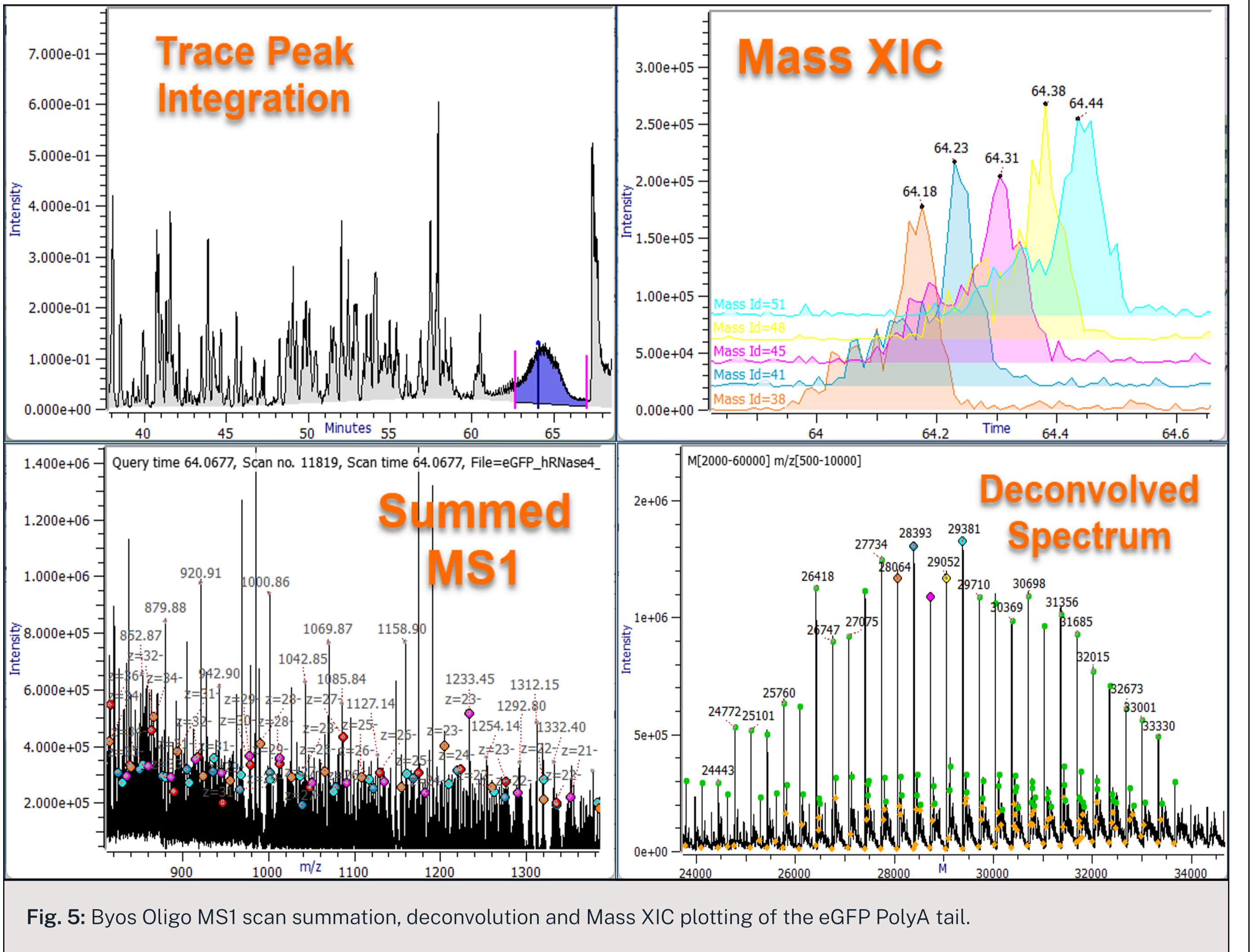


## Results - Oligo Sequence Mapping, 5' cap and 3' PolyA Tail

- >99% eGFP seq. coverage (Fig. 2)
- Byos automatically assigns oligo isomers by MS/MS (Fig 3). IPRP separation is critical for reliable isomer assignment.
- Grey/Red dots in XIC Plot show isomeric OSMs. Grey dots represent assigned OSMs isomers. (see Fig 3 zoom in to the left).
- Delta Score:** MSMS Score difference between the assigned oligo and the next best scoring oligo/isomer (isomer "uniqueness" score).
- Key isomer distinguishing fragment ions observed in annotated MS/MS spectra.



- m7Gppp 5' cap relative abundance of 33.2% %RSD=1.4% (n=3). (Fig. 4)
- Observed 5' cap intermediates Gppp (19%), ppp (1%), and pp (47%). (Fig. 4)



- PolyA tail lengths of 51-84 are mass matched in deconvoluted spectrum. (Fig. 5)
- Mass XIC plot shows LC resolution of PolyA tails. (Fig. 5)

### CONCLUSION

A novel oligonucleotide mapping workflow that enables automated assignment of MS/MS spectra, oligonucleotide sequence mapping, relative quantitation for capping efficiency calculations, deconvolution of the PolyA tail spectra, and report generation.