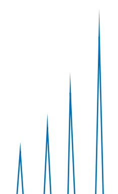
APPLICATION NOTE

YMC

Reliable evaluation of mRNA capping efficiency using YMC Accura Triart Bio C18

Messenger RNA (mRNA) has emerged as a major therapeutic modality following the success of mRNA vaccines, driving increased demand for robust analytical methods and stringent quality control. A critical quality attribute of therapeutic mRNA is the 5' cap structure, which enhances translation efficiency, improves transcript stability, and modulates innate immune recognition. The Cap-0 structure consists of the basic 7-methylguanosine cap, whereas the Cap-1 structure contains an additional methyl group on the first transcribed nucleotide, reducing unwanted immune activation and making it the preferred structure for modern mRNA therapeutics (Figure 1). In manufacturing, the cap can be introduced enzymatically; however, uncapped and partially capped impurities may be generated during this process and can negatively affect product quality and efficacy (Figure 1).

Because intact mRNA molecules are too large for direct chromatographic analysis, capping efficiency is commonly assessed after RNase H-mediated fragmentation, generating short 5'-terminal oligonucleotides that can be analysed by ion-pair reversed-phase liquid chromatography (IP-RP LC). However, the chromatographic separation of structurally similar capped, partially capped, and uncapped N- and (N+1)mer species remains challenging and requires careful optimisation of chromatographic conditions. In this Application Note, an optimised IP-RP LC method using a widepore YMC Accura Triart Bio C18 column with bioinert hardware is presented for the efficient separation of capped and uncapped *in vitro* transcribed (IVT) mRNA species. The method was subsequently applied to the LC-MS analysis of Cap-1-modified mRNA species.



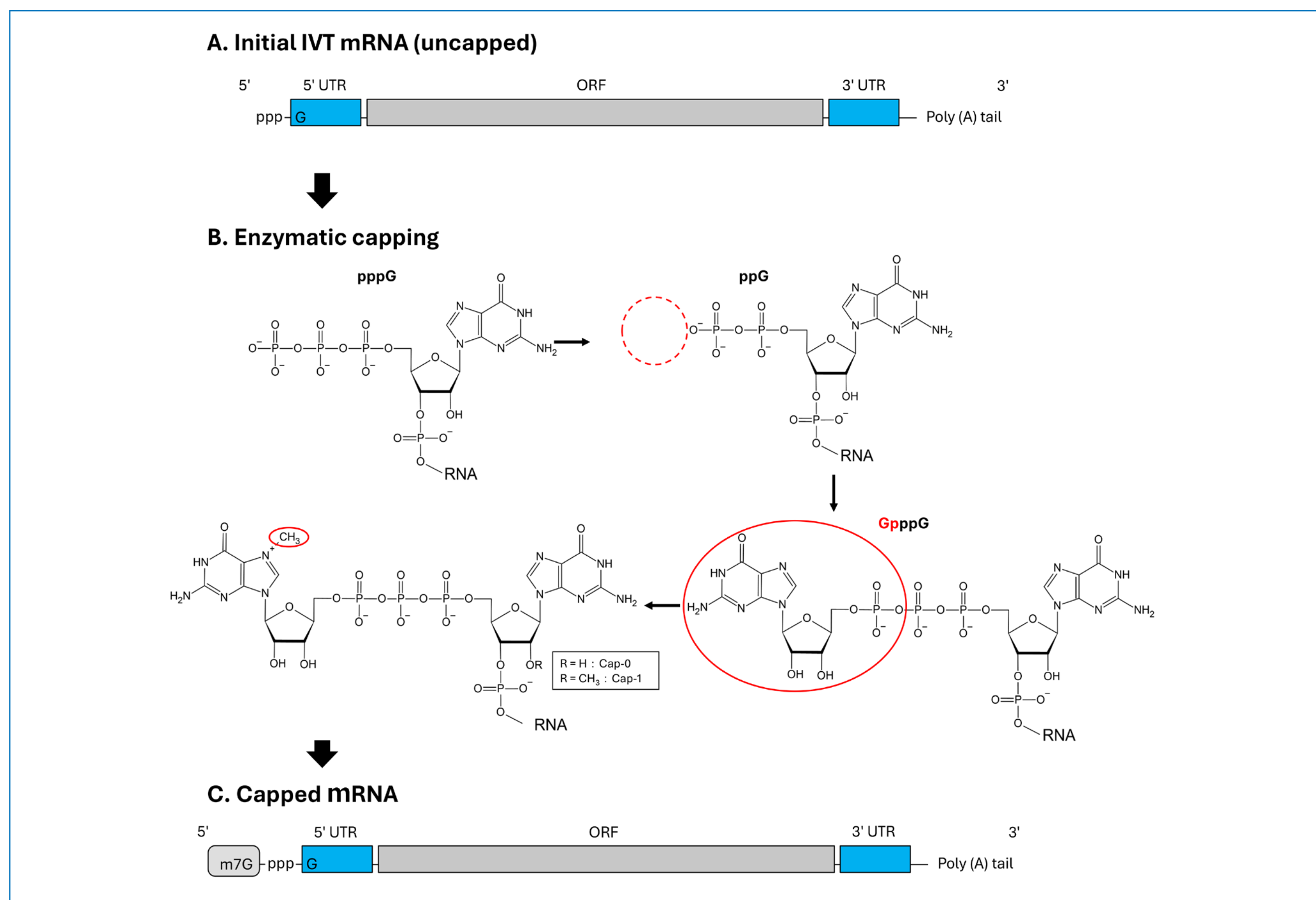


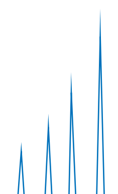
Figure 1: Schematic overview of enzymatic 5' capping of IVT mRNA. (A) Uncapped IVT mRNA; (B) Enzymatic capping reaction; (C) Final capped mRNA containing the 5' cap structure.

The chromatographic conditions were systematically optimised to improve the separation of structurally similar capped and uncapped IVT mRNA species. Key parameters including the organic solvent composition, column temperature, gradient slope, and eluent composition were investigated to enhance selectivity and resolution. In combination with the bioinert-coated YMC Accura Triart Bio C18 column, the optimised conditions enabled improved separation of capped and uncapped species differing by one nucleotide shown in Figure 2.

Table 1: Method conditions for LC-UV analysis.

Column:	YMC Accura Triart Bio C18 (1.9 µm, 30 nm) 100 x 2.1 mm ID
Part No.:	TA30SP9-10Q1PTC
Eluents*:	A) 5 mM HA-100 mM HFIP B) 5 mM HA-100 mM HFIP in water/ACN/IPA (50/12.5/37.5)
Gradient:	0.26%B/min (0.13% organic solvent/min)
Initial %B:	30.5%B
Flow rate:	0.2 mL/min
Temperature:	80 °C
Injection:	1 µL (100 ng/µL)
Detection:	UV at 260 nm
Sample:	Cap-0-modified IVT mRNA

** ACN: acetonitrile; HA: hexylamine; HFIP: 1,1,1,3,3,3-hexafluoro-2-propanol; IPA: 2-propanol*



YMC Europe GmbH · Schöttmannshof 19 · 46539 Dinslaken · Phone +49 2064 427-0 · Fax +49 2064 427-222 · Email info@ymc.eu · **www.ymc.eu**

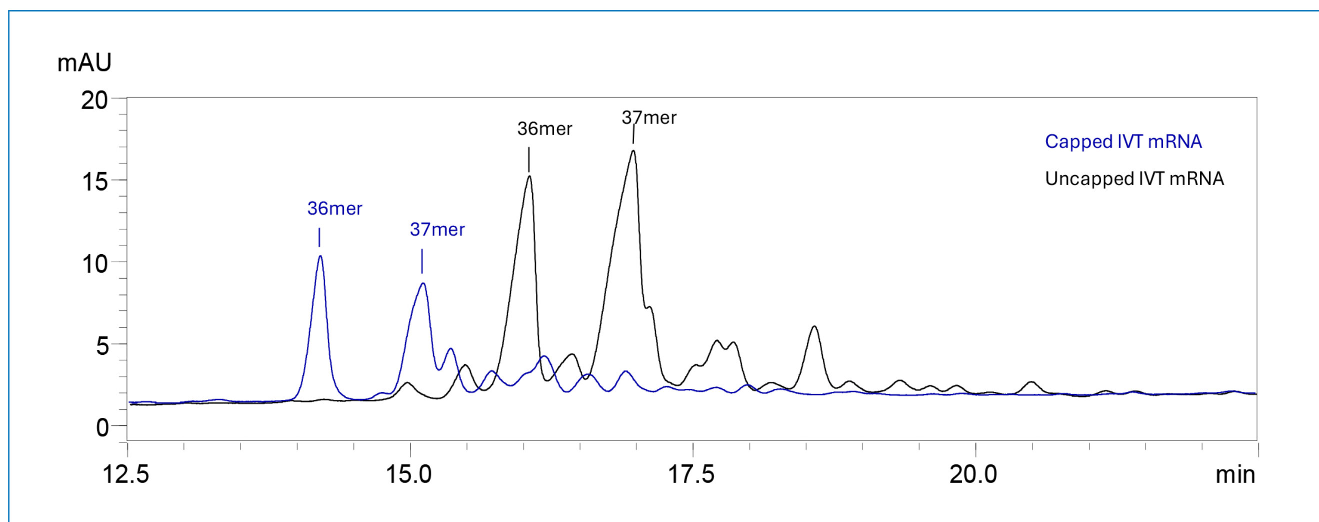


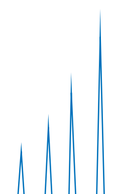
Figure 2: Optimised method for analysis of capped and uncapped IVT mRNA species using a YMC Accura Triart Bio C18 column.

The optimised IP-RP LC conditions were successfully applied to LC-MS analysis of short Cap-1-modified IVT mRNA species. Species differing by only one nucleotide were efficiently separated, while mass-extracted ion chromatograms (MICs) enabled the identification of capped, partially capped, and uncapped mRNA species, including differentiation between Cap-0 and Cap-1 structures. Minor impurity peaks corresponding to uncapped initial and 5'-diphosphate species were also detected. These results demonstrate that the YMC Accura Triart Bio C18 column with bioinert-coated hardware provides a robust platform for LC-MS-based assessment of mRNA capping efficiency and impurity profiling.

Table 2: Method conditions for LC-MS analysis.

Column:	YMC Accura Triart Bio C18 (1.9 µm, 30 nm), 100 x 2.1 mm ID
Part No.:	TA30SP9-10Q1PTC
Eluents*:	A) 5 mM HA-100 mM HFIP B) 5 mM HA-100 mM HFIP in water/ACN/IPA (50/12.5/37.5)
Gradient:	0.26%B/min (0.13% organic solvent/min)
Initial %B:	35.5%B
Flow rate:	0.2 mL/min
Temperature:	80 °C
Injection:	10 µL (100 ng/µL)
Detection:	Q-Tof-MS, ESI negative mode
Sample:	Cap-1-modified IVT mRNA

** ACN: acetonitrile; HA: hexylamine; HFIP: 1,1,1,3,3,3-hexafluoro-2-propanol; IPA: 2-propanol*



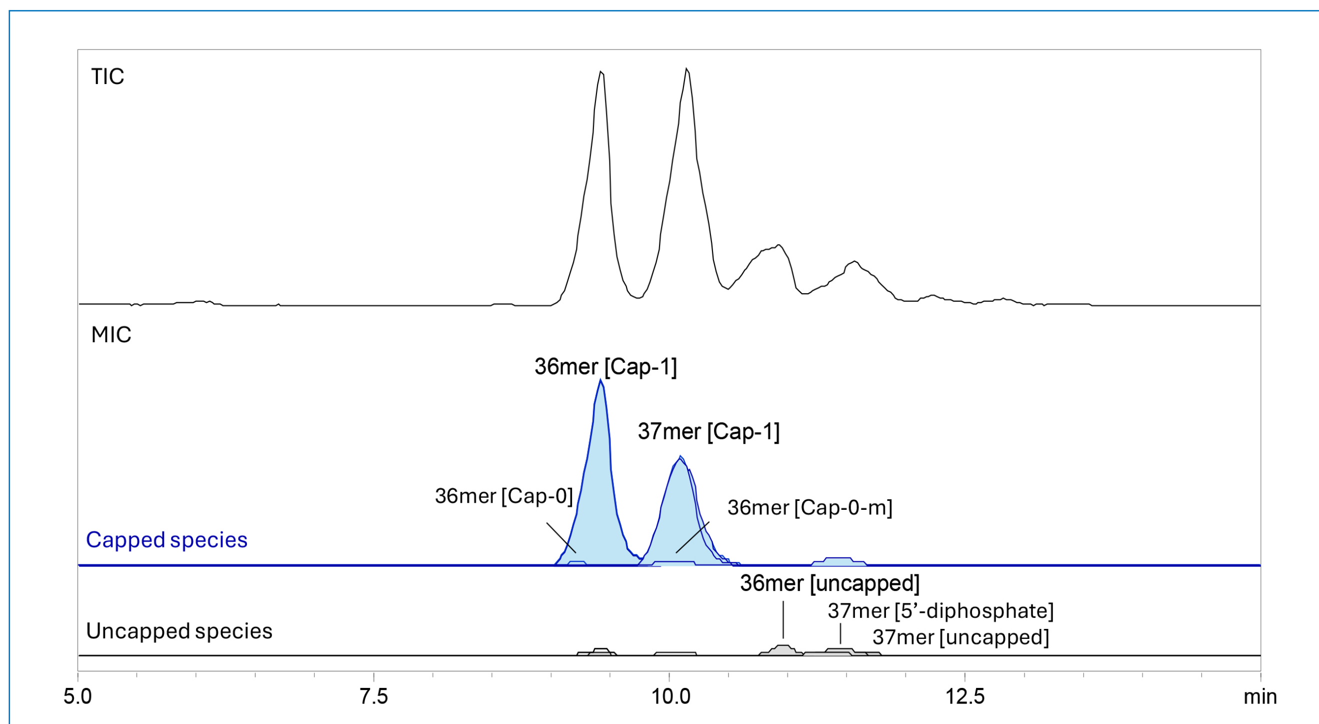


Figure 3: LC-MS analysis of short Cap-1-modified IVT mRNA species with mass-extracted ion chromatograms (MICs) enabling identification of capped and uncapped 36- and 37mer species, including Cap-1, Cap-0, Cap-0 methylated intermediates, 5'-diphosphate species, and uncapped mRNA impurities.

Conclusion

A robust IP-RP LC and LC-MS workflow was established for the analysis of capped and uncapped IVT mRNA species using the YMC Accura Triart Bio C18 column with bioinert hardware. Optimisation of chromatographic conditions enabled improved separation of structurally similar species differing by one nucleotide, while LC-MS provided reliable identification of capped, partially capped, and uncapped impurities. These results demonstrate both the suitability of the developed method for assessing mRNA capping efficiency and impurity profiling, as well as the excellent performance of the YMC Accura Triart Bio C18 column for quality control of mRNA therapeutics.

Application data by YMC CO., LTD.