Analysis of mRNA poly(A) tails with single-nucleotide resolution by capillary gel electrophoresis with UV detection (CGE-UV)

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The poly(A) tail is an essential part of messenger RNA (mRNA). For mRNA-based therapeutics and vaccines, the length of the poly(A) tail is a critical quality attribute (CQA) since it directly affects the stability and translation efficiency of the mRNA. In this technical note, we describe a workflow for mRNA poly(A) tail analysis by CGE-UV using the ssDNA 100-R kit on the PA 800 Plus Pharmaceutical Analysis system. Single-nucleotide resolution was obtained over an extended size range of 9 to 156 nucleotides (nt), enabling accurate poly(A) tail length determination and peak quantitation with excellent assay repeatability. The simple sample preparation and ready-to-use ssDNA 100-R kit on a 21 CFR Part 11 compliance-ready platform make this a potential workflow for development of a QC release test.

Key features of mRNA poly(A) tail analysis by CGE-UV on the PA 800 Plus system

- **Exceptional resolving power:** Achieve single-nucleotide resolution critical for accurate poly(A) length determination and peak quantitation of each poly(A) tail species
- **Extended size coverage:** Sustain single-nucleotide resolution in the size range of 9 to 156 nt
- **Excellent assay repeatability:** Obtain consistent poly(A) tail profiles in multiple injections
- **Streamlined workflow:** Achieve direct measurement of poly(A) tail length and distribution with streamlined sample preparation, using the ready-to-use ssDNA 100-R kit on a 21 CFR Part 11 compliance-ready platform. This method has the potential to be developed for QC testing

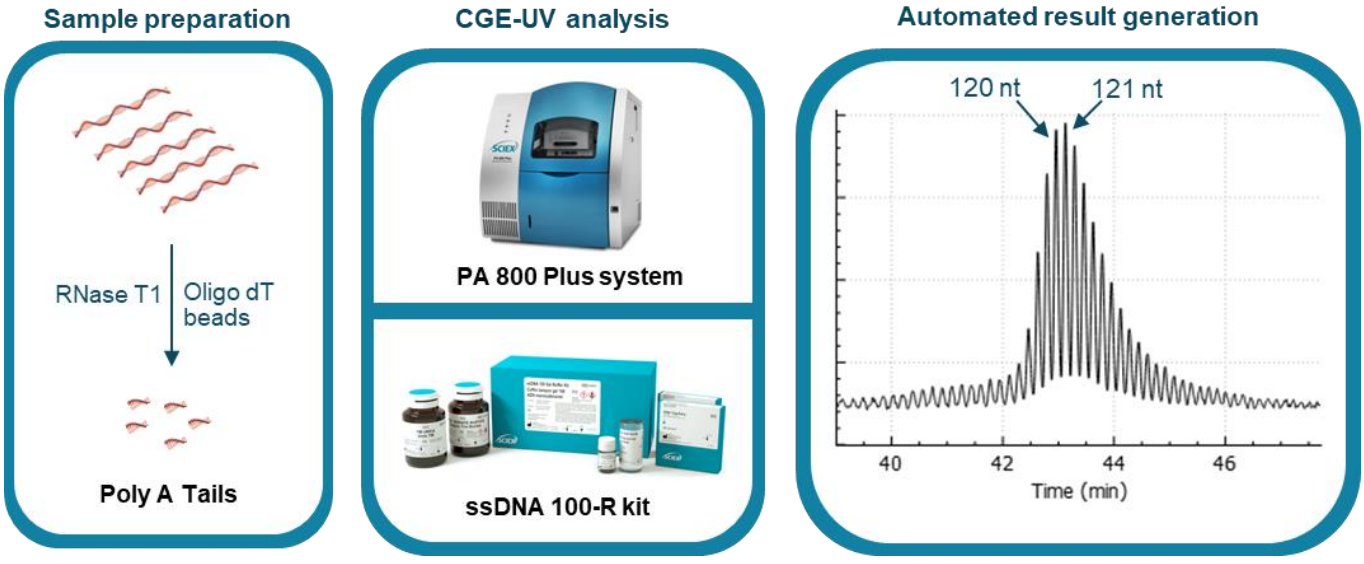

Figure 1. Workflow for mRNA poly(A) tail analysis by CGE-UV. The mRNA sample is digested with RNase T1 first. The poly(A) tails released are then purified with oligo dT conjugated magnetic beads and separated on the PA 800 Plus using the ssDNA 100-R kit. The electropherogram in the right panel demonstrates single-nucleotide resolution in a typical poly(A) tail profile obtained with an mRNA sample.

Introduction

Since the approval of mRNA vaccines against SARS-CoV-2virus, in vitro transcribed (IVT) mRNA has gained popularity in vaccine development, protein replacement therapies, and cancer immunotherapies. The poly(A) tail length is a CQA for mRNA-based therapeutics. One challenge in studying poly(A) tails is that they are difficult to sequence and accurately measure. Various analytical methods have been developed to characterize this CQA. Ion-pair reversed-phase liquid chromatography (IPRP-LC) allows quantification of the percentage of mRNA containing a poly(A) tail but lacks the ability to determine the specific length of the poly(A) tail. Next-generation sequencing (NGS) can achieve single-nucleotide resolution in separating poly(A) tail length. However, its implementation for release testing of manufactured mRNA poses complexity and requires powerful bioinformatics software tools. In this technical note, single-nucleotide resolution was achieved across an extended size range from 9 to 156 nt using a CGE-UV workflow, allowing for precise determination of poly(A) tail length and accurate quantitation of peaks with outstanding assay repeatability.

Methods

Materials: The ssDNA 100-R kit (P/N 477480) containing DNA Capillary, ssDNA 100-R Gel (lyophilized), Tris-Borate buffer, 7M Urea, and pd(A) 40-60 Test Mix; Universal Vials (P/N A62251), Universal Vial Caps (P/N A62250), PCR Microvials (P/N 144709), CE Grade Water (P/N C48034), and NanoVial (P/N 5043467) were from SCIEX (Framingham, MA). Nuclease-free water (NFW, P/N AM9932), RNase T1 (1000 U/µL, P/N EN0541), Dynabeads Oligo $(dT)_{25}$ (P/N 61002), and Zeba Spin Desalting columns (7K MWCO, 75 µL, P/N 89877) were from Thermo Fisher Scientific (Waltham, MA). The 10x RNase H Reaction Buffer (Component #B0297SVIAL) of the RNase H kit (P/N M0297L) was from New England Biolabs (Ipswich, MA). The firefly luciferase (FLuc) mRNA (P/N: L-7602) was from TriLink BioTechnologies (San Diego, CA). The FLuc mRNA contains a 5' Cap and a 3' poly A tail, mimicking an mRNA drug substance. The 120 nucleotide (nt) polyA size marker was custom-synthesized by Integrated DNA Technologies (Coralville, IA).[1] The Agencourt SPRIPlate 96S Super Magnet (P/N A32782) was from Beckman Coulter Life Sciences (Indianapolis, IN). The 0.2 µm (P/N: 4612) and the 0.45 µm (P/N 4654) syringe filters were from PALL (Port Washington, NY). The LABQUAKE rotator (model 400100) was from Barnstead International (Dubuque, IA).

Instruments and software: A PA 800 Plus Pharmaceutical Analysis System (P/N A66528) equipped with an ultraviolet (UV) detector was from SCIEX. The UV wavelength used was 254 nm. Data acquisition and analysis were performed using 32 Karat software version 10.

Tris-Borate-Urea (TBU) buffer preparation: This step must be done one or a few days before running samples. To rehydrate the buffer, 135 ml of 0.2 µm filtered deionized water was added to the bottle containing the dry Tris-Borate from the ssDNA 100-R kit. The buffer solution was mixed with a clean magnetic stirring bar for about 20–30 minutes until boric acid was completely dissolved. The dry 7M Urea was then slowly added to the Tris-Borate buffer while stirring the solution. The urea was completely dissolved after about 2 hours and the solution became clear. The reconstituted Tris-Borate-Urea buffer should be good for 30 days if stored at 2°C to 8°C after preparation. The entire bottle of buffer should be brought to ambient temperature before use. The required volume to be used for the day was removed and filtered through a 0.2 µm disposable syringe filter into a clean container.

ssDNA 100-R Gel buffer reconstitution: Five milliliters of freshly filtered TBU buffer was added to the bottle with lyophilized gel. The gel bottle was then placed onto a rotator in a cold room (2°C to 8°C) for 72 hours with gentle rotation or stirred at room temperature for 5 to 6 hours. The prepared gel buffer can be used up to 30 days after preparation if stored at 2°C to 8°C. The gel buffer was brought to ambient temperature and filtered through a 0.45 µm disposable syringe filter before use.

Cartridge assembly: A DNA capillary (PN 477477) from ssDNA 100-R kit was installed according to the instructions in the ssDNA 100-R kit application guide.[2] The total capillary length was 30.2 cm, with 20 cm as the length to the detection window. A 100 x 200 µm aperture was used for better resolution when using a UV detector. Since the inner wall of the DNA capillary is coated, the cartridge assembly should be carried out promptly. Excessive exposure to air may damage the inner coating and cause clogging. The capillary ends must be immersed in liquid (water or buffer) when the cartridge assembly is complete to prevent the coating from drying out.

Preparation of buffer trays and sample trays: Vial positions for buffer trays are indicated in Figure 2A. Each CE Water vial was

filled with 1.5 mL CE Grade Water. The waste vial was filled with 1.0 mL CE Grade Water. The 100-R Gel vial was filled with 1.5 mL ssDNA 100-R Gel buffer, and the TBU Buffer vials were filled with 1.5 mL TBU buffer.

Instrument setup: The Initial conditions and UV detector initial conditions were set up as indicated in the ssDNA 100-R kit application guide.[2] The same setup was used for all methods: the new capillary-conditioning method, the gel-filling method, the separation method and the shutdown method. The time program settings for the capillary-conditioning method and for the gel-filling method are provided in the ssDNA 100-R kit application guide.[2] Time program settings for the separation method are shown in Figure 2B.

Sample preparation: First, the FLuc mRNA was digested with RNase T1 to release the polyA tails. The RNase T1 reaction with a total volume of 100 µL was assembled by mixing 63 µL of 1 mg/mL (100 pmoles) FLuc mRNA that was preheated at 70°C for 5 minutes and chilled on ice for 5 minutes, 10 µL of the 10 x RNase H reaction buffer, 24.5 µL of NFW, and 2.5 µL of the RNase T1. [1] The mixture was incubated at 37°C at for 1 hour in a thermal cycler. Secondly, the released polyA tails were purified using the Dynabeads Oligo $(dT)_{25}$. For 100 pmoles of mRNA samples, 1 mg (or 200 µL) of the Dynabeads Oligo $(dT)_{25}$ was washed and resuspended in 100 µL of the binding buffer following the manufacturer's instructions. The RNase T1 digested Fluc mRNA sample (100 µL) was added to 100 µL of the binding buffer and heated at 65°C for 2 minutes in a thermal cycler to unfold possible secondary structures. Immediately after the heat treatment, the sample was placed on ice. In a 500 µL microcentrifuge tube, the 200 µl of mRNA and binding buffer mixture were then mixed with 100 µL of Dynabeads Oligo $(dT)_{25}$ prepared as described above and mixed thoroughly by vortex. The released polyA tails in the RNase T1 treated FLuc mRNA sample were annealed to the oligo $(dT)_{25}$ on the Dynabeads by rotating continuously on a rotator for 30 to 60 minutes at room temperature. The tube was then placed on the magnet for 1-2 minutes. The supernatant was carefully removed while the tube was still on the magnet. Next, the tube was removed from the magnet, and beads were resuspended in 200 µl of washing buffer B. The beads were washed 3 times with the washing buffer B. To elute the polyA tails, 50 µL of ice-cold NFW was added to the beads. The mixture was transferred to a PCR tube, heated at 80°C for 2 minutes in a thermal cycler, and then immediately placed on the magnet. After 1-2 minutes on the magnet, the eluted polyA tails were transferred to a new RNase-free tube. Finally, the sample was desalted with the Zeba column following the instructions from the manufacturer. About 45 µl of purified polyA tail was collected at the end, and 10 ul of this sample was transferred to a Nanovial for CE analysis.

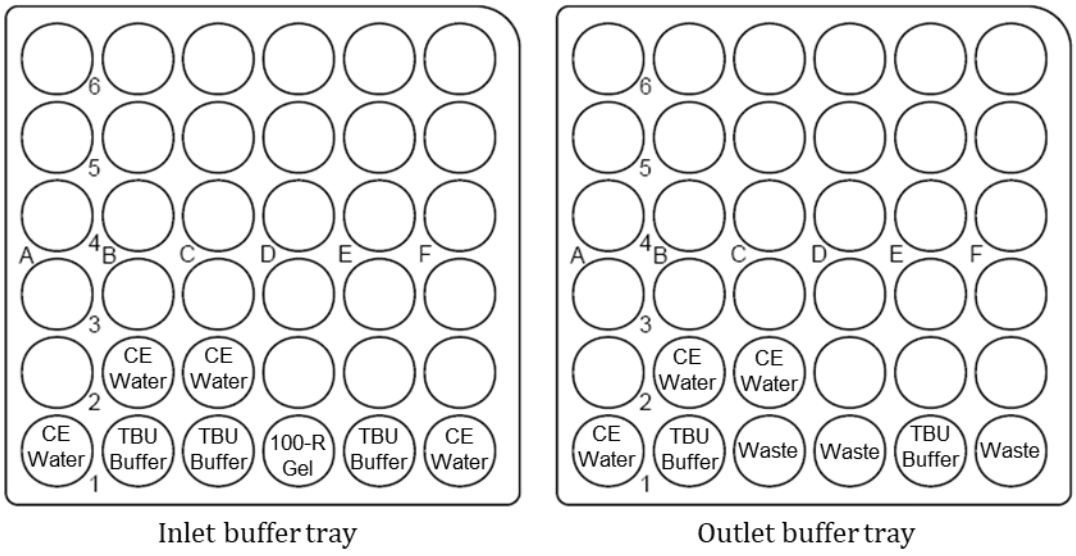

Figure 2. Schematic setup for inlet (left) and outlet (right) buffer trays.

Initial Conditions | UV Detector Initial Conditions | Time Program

	Time (min)	Event	Value	Duration	Inlet vial	Outlet vial	Summary	Comments
1		Wait		0.00 min	BI:A1	BO:A1		Water dip to clean the capillary tip
2		Inject - Voltage	5.0 KV	10.0 sec	SI:A1	BO:B1	Override, reverse polarity	Sample injection
3		Wait		0.00 min	BI:B2	BO:B2		Water dip to prevent sample carry-over
4		Wait		0.00 min	BI:C2	BO:C2		Water dip to prevent sample carry-over
5	0.00	Separate - Voltage	11.0 KV	55.00 min	BI:E1	BO:E1	0.17 Min ramp, reverse polarity, both	Separation in ssDNA 100-R gel
6	1.00	Autozero						
7	55.00	End						
8								

Figure 3. Time Program settings for the separation method.

Poly(A) tail analysis workflow by CGE-UV with single nucleotide resolution

The workflow of Poly(A) tail analysis by CGE-UV is illustrated in Figure 1. The mRNA sample is digested with RNase T1 first. The poly(A) tails released are then purified with oligo dT conjugated magnetic beads and separated on the PA 800 Plus system using the ssDNA 100-R kit that contains a replaceable gel buffer and a coated capillary for enhanced reproducibility. The electropherogram in the right panel demonstrates single-nucleotide resolution in a typical poly(A) tail profile obtained with an mRNA sample.

Poly(A) tail length determination

To determine the poly(A) tail length, the poly(A) tails released from the TriLink FLuc mRNA sample were mixed with a 120 nt size marker before CE separation on the PA 800 Plus. As a control, the poly(A) tail samples were also analyzed alone. Results obtained with the poly(A) tail sample and the spiked-in 120 nt size marker are shown in the upper panel of Figure 4, while the results obtained with the poly(A) tail sample alone are shown in the lower panel. Single-nucleotide resolution was achieved in both separation results and maintained throughout the entire poly(A) tail profile. The arrow in the upper panel indicates the 120 nt full-length size marker comigrating with one of the poly(A) tail peaks in the sample, indicating the length of this poly(A) tail peak is 120 nt. This 120 nt peak and the 121 nt peak are the most abundant poly(A) tail species in this sample, consistent with the theoretical 120 nt poly(A) tail length design by TriLink. This sample's poly(A) tail profile included a length distribution from 97 nt to 156 nt. This heterogeneity in poly(A) length may be caused by transcription slippage of the RNA polymerase used to synthesize the FLuc mRNA, as indicated previously.[3]

Single-nucleotide resolution maintained in the size range of 9 nt to 156 nt

Figure 5 shows results obtained with a 120 nt size marker (in the pink trace) and with poly(A) tails from the TriLink FLuc mRNA (in the blue trace). The 120 nt full-length product for this size marker and n-1 species down to the size of 9 nt were well separated with single nucleotide resolution. The single-nucleotide resolution was also achieved in analyzing the poly(A) tails from the TriLink FLuc mRNA with a length distribution of 97 to 156 nt. These results demonstrate a sustained single-nucleotide resolution in the size range of 9 nt to 156 nt. For mRNA-based therapeutics, it was shown that a poly (A) tail length of approximately 100 nt is optimal to minimize degradation.[4] However, it has been reported that short poly(A) tails are associated with highly expressed, well-translated genes.[5] Some very stable natural transcripts, such as beta-actin, were shown to have a short poly(A) tail of less than 30 nucleotides. Therefore, the poly(A) tail analysis workflow by CGE-UV is suitable for analyzing mRNA-based therapeutics and natural transcripts.

Excellent assay repeatability

To assess the assay repeatability, poly(A) tails from the TriLink FLuc mRNA sample were injected 4 times, and the results are shown in Figure 6. Each panel displays the electropherogram from 1 injection. The poly(A) tail profiles in all 4 injections were consistent, with 120 nt and 121 nt peaks as the most abundant species, and single nucleotide resolution was maintained throughout the profile for each

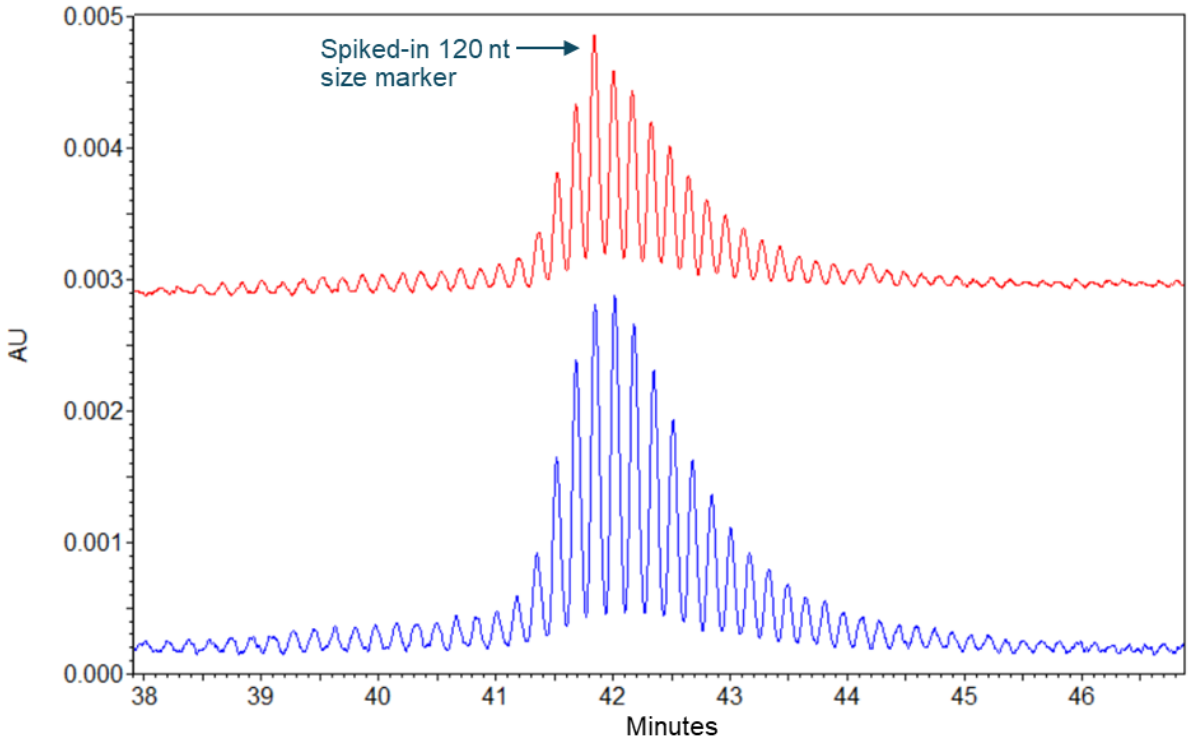

Figure 4: Analysis of polyA tails from TriLink FLuc mRNA. Upper panel: Electropherogram of poly(A) tails from TriLink FLuc mRNA with spiked-in 120 nt size marker. Lower panel: Electropherogram of poly(A) tails from TriLink FLuc mRNA without the spiked-in size marker.

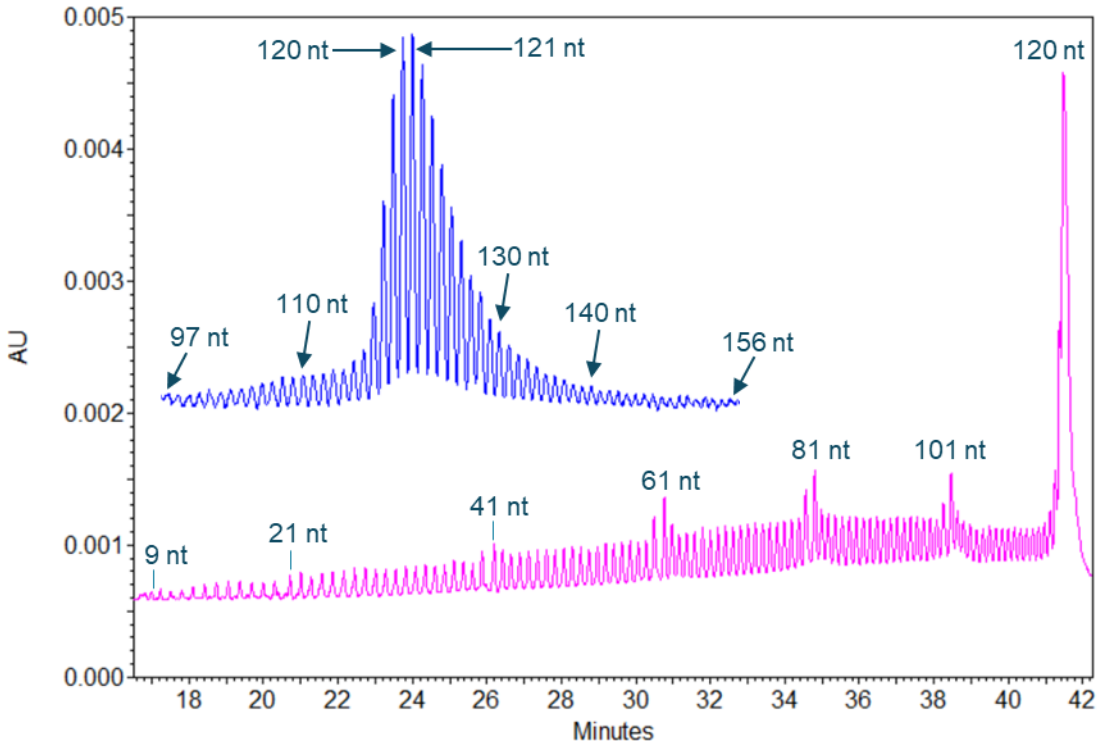

Figure 5: Single-nucleotide resolution over a size range from 9 nt to 156 nt. The Blue trace: poly(A) tails from TriLink FLuc mRNA. Pink trace: 120 nt poly(A) size marker and its n-1 species.

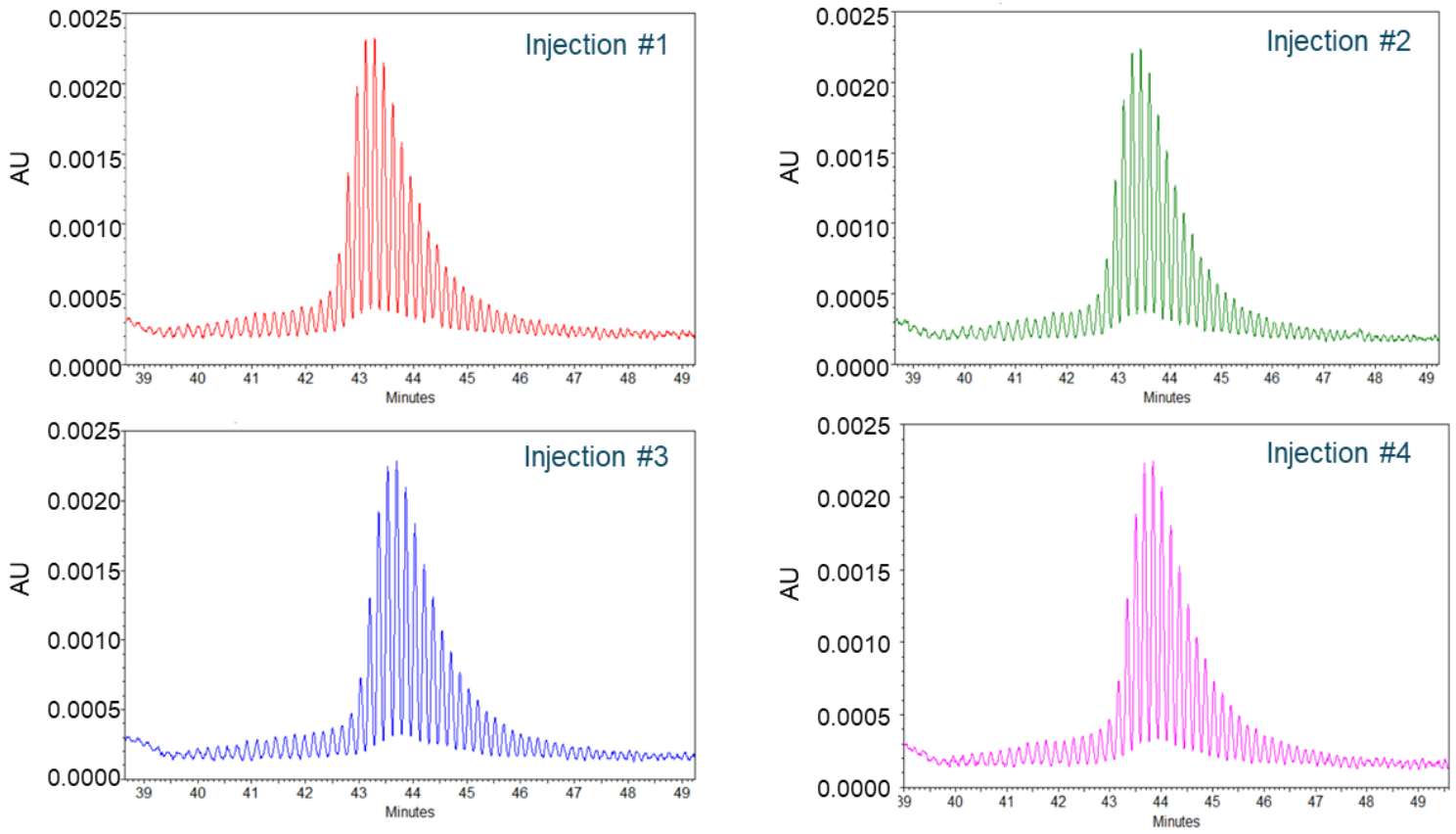

Figure 6: Injection repeatability. The same sample was injected 4 times. Each panel shows the electropherogram from 1 injection.

injection. The CVs for the migration times and the corrected peak area percentage (CPA%) of the 120 and 121 nt species across 4 injections were better than 0.60% and 2.50%, respectively, demonstrating excellent assay repeatability.

Peak quantitation of poly(A) tail species

Maintaining single-nucleotide resolution throughout the entire poly(A) tail profile enabled the execution of peak integration. The CPA% of each poly(A) tail species of the TriLink FLuc mRNA sample was determined using the 32 karat software, and results were summarized in Figure 7. The poly(A) tail distribution of the TriLink FLuc mRNA covers 60 fragments in a size range of 97 nt to 156 nt. Each fragment peak had a signal-to-noise ratio of 5 or higher. The most abundant 2 species (dark green) are the 120 nt and the 121 nt fragments, each representing about 9%. These 2 species, together with 17 additional species with lengths ranging from 116 nt to 132 nt, cover about 80% of all poly(A) tails. This quantitative information adds value to the characterization and quality monitoring of mRNA-based therapeutics.

Size (nt)	CPA%	Size (nt)	CPA%	Size (nt)	CPA%
97	0.15	117	2.65	137	0.70
98	0.23	118	4.99	138	0.61
99	0.25	119	7.60	139	0.49
100	0.28	120	9.09	140	0.47
101	0.35	121	9.42	141	0.40
102	0.32	122	8.63	142	0.36
103	0.38	123	7.45	143	0.31
104	0.45	124	6.10	144	0.27
105	0.48	125	5.06	145	0.23
106	0.50	126	4.13	146	0.29
107	0.57	127	3.31	147	0.19
108	0.66	128	3.04	148	0.20
109	0.68	129	2.19	149	0.15
110	0.76	130	1.82	150	0.17
111	0.75	131	1.55	151	0.23
112	0.84	132	1.26	152	0.17
113	0.96	133	1.10	153	0.16
114	1.03	134	0.99	154	0.15
115	1.15	135	0.82	155	0.11
116	1.48	136	0.66	156	0.10

Figure 7. CPA% of poly(A) tails of the TriLink FLuc mRNA.

Conclusions

- Single-nucleotide resolution was achieved with the CGE-UV workflow, enabling accurate and direct measurement of poly(A) tail length
- The single-nucleotide resolution was sustained across the entire poly(A) tail profile in the size range of 9 to 156 nt, facilitating peak integration of each individual poly(A) tail species
- Excellent assay repeatability was demonstrated by multiple injections of the same poly(A) tail sample with CVs for the migration times and the CPA% of the 120 and 121 nt species across 4 injections better than 0.60% and 2.50%, respectively
- Potential of CGE-UV workflow for development of QC assay for poly(A) tail analysis with simple and fast sample preparation and ready-to-use ssDNA 100-R kit on a 21 CFR Part 11 compliance-ready platform

References

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   https://doi.org/10.1038/nsmb.3499

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