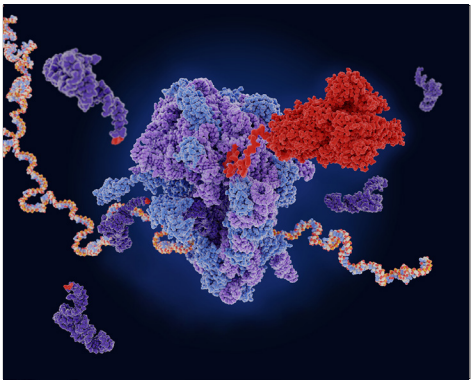


EZ-QC™ mRNA PAGE-based Assay Kits Provide a Viable Alternative to LC-MS

Chateen Krueger, Daniel Gerhardt, Ryan Lahr, Ron Meis, Johnathan Parker, Kari Weber, and Jerry Jendrisak

CELLSCRIPT™, 726 Post Road, Madison, WI 53713 USA

Introduction

An essential step in manufacturing high-quality mRNA is to perform comprehensive quality control (QC) prior to use in downstream applications. Evaluating 5′-capping efficiency and 3′-poly(A) tail length is vital for producing high-quality mRNA. The USP has issued draft guidelines[1] for mRNA QC analysis and recommends reverse-phase liquid chromatography and mass spectrometry (LC-MS), which require special expertise with complex and expensive equipment. The EZ-QC™ mRNA assay kits allow for concurrent analysis of multiple samples without the added complexity, expense, stringent sample requirements and long turnaround times associated with LC-MS. Here we present an evaluation to compare the results obtained from EZ-QC™ assay kits with results from various LC-MS methods.

Side-by-Side Evaluation of the EZ-QC™ XBG mRNA Capping Efficiency Assay Kit Compared to LC-MS

Fully-capped mRNA is essential for the mRNA's stability and cellular expression. Enzymatic capping is catalyzed, *in vitro*, by two cap-building enzymes, mRNA Capping Enzyme and mRNA 2′-O-Methyltransferase. Capping Enzyme produces a Cap 0 structure on the RNA, which is then converted into Cap 1 structures by the 2′-O-Methyltransferase. Most Cap 1 mRNAs are expressed at higher levels in cells relative to their Cap 0 counterparts since the Cap 1 modification helps to identify the mRNA as "self" RNA, thereby reducing the innate immune response to the mRNA.

The EZ-QC™ XBG mRNA Capping Efficiency Assay Kit provides an efficient way to assess the quality of your mRNA by allowing quantitation of the percentage of capped mRNA in an mRNA sample. This kit utilizes a chimeric RNA-DNA-RNA Targeting Oligonucleotide which hybridizes near the 5′-end of the mRNAs in the mixture. This hybridization complex serves as a substrate for EZ-QC™ RNase H cleavage, releasing the capped and uncapped 5′-end fragments which can subsequently be resolved via polyacrylamide gel electrophoresis (PAGE). Staining with SYBR™ Gold and quantification by gel image band analysis enables calculation of the percentage of capped mRNA in the sample. This kit is designed to work with XBG 5′-UTR-containing mRNA. For users requiring an alternative custom targeting oligo, the EZ-QC™ mRNA Capping Efficiency Assay Kit is available.

Due to the large size and complexity of full-length mRNA, enzymatic processing to cleave the 5′-capped end is required prior to LC-MS analysis. The EZ-QC™ XBG mRNA Capping Efficiency Assay Kit was used to process the mRNA for compatibility with LC-MS analysis. The products from the EZ-QC™ assay were submitted to a third-party laboratory specializing in LC-MS sample analysis. A 55 nt mRNA test sample, formulated to 90% capped/10% uncapped RNA, was first processed using the EZ-QC™ XBG mRNA Capping Efficiency Assay Kit and then analyzed on an Orbitrap Liquid Chromatography High Resolution Mass Spectrometry platform. Subsequent analysis of the results was performed with deisotoping and charge deconvolution software, ProMass.

Compared to LC-MS:

- EZ-QC™ assays do not have the strict (13-23 nt) 5′-end size limitation.
- EZ-QC™ assays do not require clean-up to remove targeting oligo, protein, and mRNA 3′ ends prior to analysis.

Figure 1. EZ-QC™ XBG mRNA Capping Efficiency Assay Kit and LC-MS Results

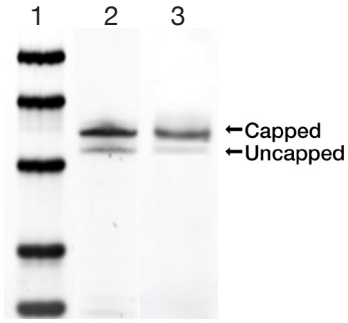


Figure 1A. PAGE separation of samples processed with the EZ-QC™ XBG mRNA Capping Efficiency Assay Kit.

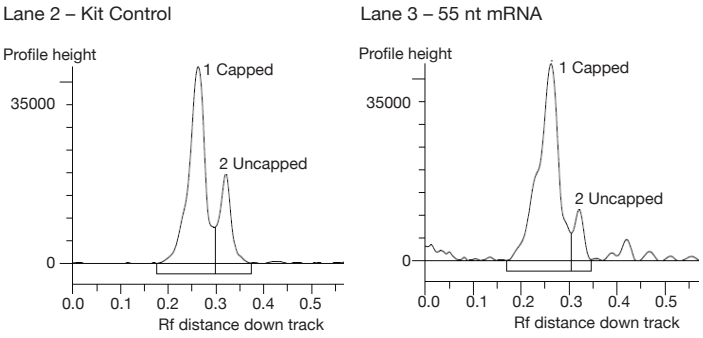


Figure 1B. Electropherogram of results from PAGE for lanes 2 and 3 (Figure 1A) after using the EZ-QC™ XBG mRNA Capping Efficiency Assay Kit showing the relative quantities of capped to uncapped RNA.

Table 1. A snapshot of the different moieties found within the sample when evaluated using LC-MS. Relevant mRNA components found within the sample via LC-MS testing were identified based on mass-to-charge ratio (m/z) due to several molecules having similar retention times. Numerous species were detected that were irrelevant to the capping identity of the sample and only a subset is shown here.

LC-MS Results

RT (min)	Observed Mass (Da)	Identity
17.077	11367.385	10834.3792 (m^7g_cap1)
17.077	11353.373	10834.3792 (m^7g_cap0)
17.283	11074.271	10834.3792 (uncapped triphosphate)
17.077	10994.303	10834.3792 (uncapped diphosphate)
16.913	10914.33	10834.3792 (uncapped phosphate)

Table 2. Comparable results were obtained between EZ-QC™ XBG mRNA Capping Efficiency Assay Kit and LC-MS. The EZ-QC™ XBG mRNA Capping Efficiency Assay Kit provides a streamlined PAGE-based method for quantifying mRNA capping efficiency, yielding results that agree closely (±5%) with those obtained with LC-MS.

Lane	Sample	EZ-QC™ Assay % Capped	LC-MS % Capped
2	80% Capped/20% Uncapped XBG RNA Control	75.1%	Not performed
3	90% Capped/10% Uncapped	**89.1%**	**93.0%**

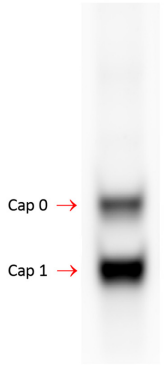
Side-by-Side Evaluation of the EZ-QC™ mRNA Cap 1 Efficiency Assay Kit Compared to LC-MS

The EZ-QC™ mRNA Cap 1 Efficiency Assay Kit labels mRNA ends with the fluorescent dye Cyanine 5 (Cy5). The labeled mRNA is then digested with the EZ-QC™ RNase mixture, releasing Cy5-labeled 5′-Cap 1 and Cap 0 cap cores. These are resolved using PAGE and analyzed using a fluorescent gel imager to measure the relative amounts of Cap 1 to Cap 0 present in the sample. Since RNA 5′-uncapped ends are not labeled, the EZ-QC™ mRNA Cap 1 Efficiency Assay does not differentiate between uncapped and capped RNAs, but only between Cap 0 and Cap 1 capped RNAs.

The EZ-QC™ mRNA Cap 1 Efficiency Assay Kit allows full-length mRNA to be processed directly for quantitation of Cap 0 and Cap 1 content. The 90% Capped/10% Uncapped 55 nt RNA, previously tested using the EZ-QC™ XBG mRNA Capping Efficiency Assay Kit, was also assessed for Cap 1 and Cap 0 content using the EZ-QC™ mRNA Cap 1 Efficiency Assay Kit. This sample was preformulated to contain 65% Cap 1 and 35% Cap 0 RNA. For LC-MS testing, the sample first required processing using the EZ-QC™ XBG mRNA Capping Efficiency Assay Kit to cleave the 5′-capped end to allow for more efficient detection via LC-MS due to size limitations.

Figure 2. EZ-QC™ mRNA Cap 1 Efficiency Assay Kit and LC-MS Results

Figure 2A. PAGE separation of samples processed with the EZ-QC™ mRNA Cap 1 Efficiency Assay Kit.



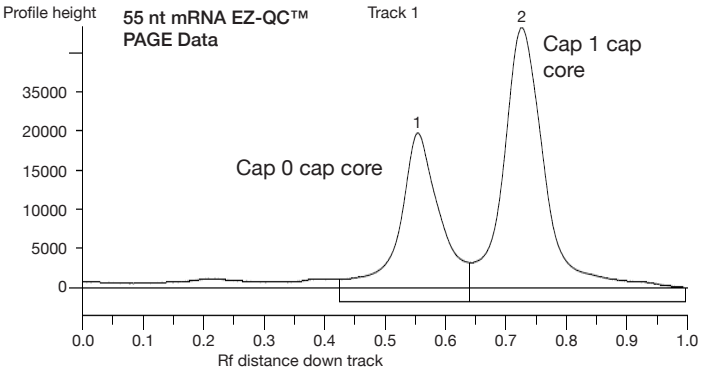


Figure 2B. Electropherogram showing the relative abundance of Cap 0 and Cap 1 cap cores from quantitating the PAGE bands depicted in Figure 2A.

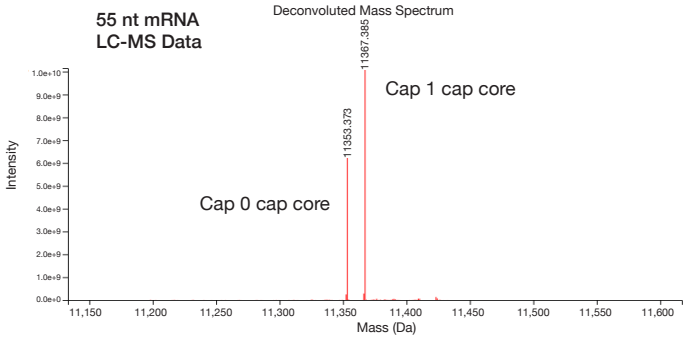


Figure 2C. LC-MS deconvoluted mass spectrum displaying Cap 0 and Cap 1 cap cores for the same 55 nt mRNA. The relevant cap core mRNA structures (Cap 0 and Cap 1) found within the sample are shown here.

Table 3. EZ-QC™ mRNA Cap 1 results compared to LC-MS. The EZ-QC™ mRNA Cap 1 Efficiency Assay Kit provides a convenient PAGE-based method for quantifying mRNA Cap 1 efficiency, yielding measurements that closely mirror those obtained by LC-MS. Cap 1 and Cap 0 quantitation was within 2% when comparing results from EZ-QC™ mRNA Cap 1 Efficiency Assay Kit to LC-MS.

Sample	Method	Cap 1 Relative %	Cap 0 Relative %
Capped 55 nt mRNA	EZ-QC™ mRNA Cap 1 Efficiency Assay Kit	**63.4**	**36.6**
Capped 55 nt mRNA	LC-MS	**62.1**	**37.9**

Side-by-Side Evaluation of the EZ-QC™ mRNA Poly(A) Tail Length Assay Kit Compared to LC-MS

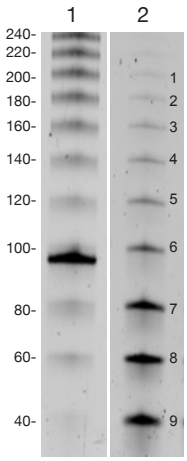
The EZ-QC™ mRNA Poly(A) Tail Length Assay provides a convenient way to assess tail lengths of mRNA. 3′-end polyadenylation is a critical modification that influences mRNA stability and expression levels in eukaryotic cells, with longer tails generally providing increased stability and translation efficiency. 3′-poly(A) tails can be added co-transcriptionally when encoded by the template or post-transcriptionally via enzymatic addition with poly(A) polymerase.

The EZ-QC™ mRNA Poly(A) Tail Length Assay employs EZ-QC™ RNase A to digest the mRNA molecule but leave the poly(A) tail intact. In conjunction with the EZ-QC™ Poly(A) 20-mer Ladder, the poly(A) tail length can be determined using PAGE followed by staining with SYBR™ Gold plus Poly(A) Fluorescence Enhancer.

While various LC-MS methods are constrained to analyzing maximum poly(A) tail lengths of just 120-150 bases, the EZ-QC™ mRNA Poly(A) Tail Length Assay exceeds those capabilities. The EZ-QC™ mRNA Poly(A) Tail Length Assay enables assessment of full-length mRNA with tails up to 300 adenosines, and with minor adjustments to gel running conditions, tails with up to 400 adenosines.

Figure 3. EZ-QC™ mRNA Poly(A) Tail Length Assay Kit and LC-MS Results.

Figure 3A. PAGE separation of the Poly(A) 20-mer Ladder included with the EZ-QC™ mRNA Poly(A) Tail Length Assay Kit. Lane 1: EZ-QC™ Poly(A) 20-mer Ladder. Lane 2: a variation of the Poly(A) 20-mer Ladder submitted for LC-MS. The 20-A peak is also present but was not captured here.



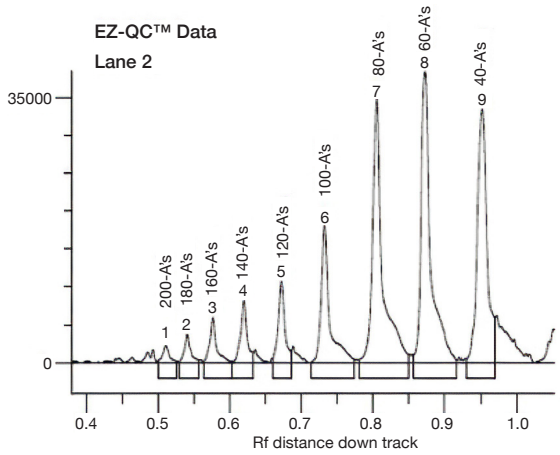


Figure 3B. The electropherogram shows the relative intensities of the peaks depicted in Figure 3A, lane 2. The peaks are labeled 1-9 and their quantitation is denoted in the electropherogram.

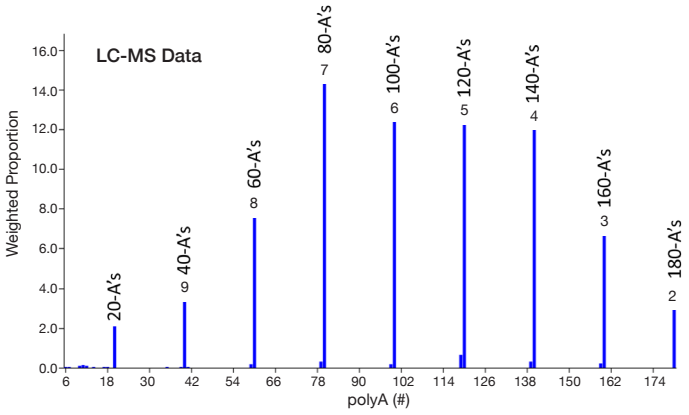


Figure 3C. LC-MS Analysis Summary of a Poly(A) 20-mer Ladder submitted for LC-MS. The 200-A peak and lengths greater than 200 were not detectable via LC-MS due to limitations of the platform.

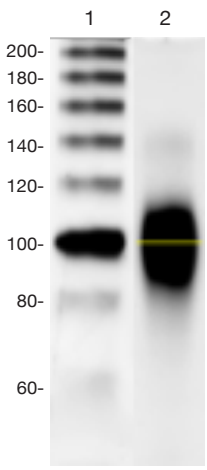


Figure 3D. PAGE separation of samples processed with the EZ-QC™ mRNA Poly(A) Tail Length Assay Kit.

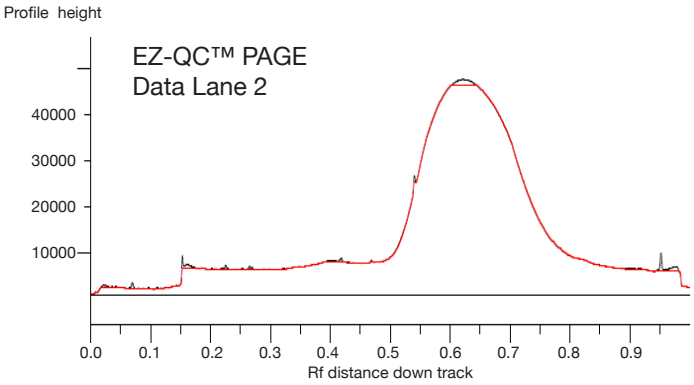


Figure 3E. Electropherogram depiction of the band obtained in Figure 3D, lane 2.

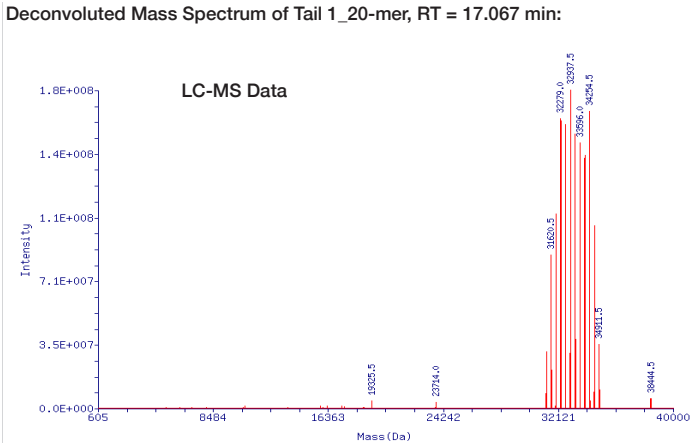


Figure 3F. LC-MS deconvoluted mass spectrum of 20-mer sample processed with the EZ-QC™ mRNA Poly(A) Tail Length Assay Kit and submitted for LC-MS testing.

Table 4. Comparable results were obtained between EZ-QC™ mRNA Poly(A) Tail Length Assay Kit and LC-MS for the same tailed RNA sample.

Compiled EZ-QC™ Assay and LC-MS Results

Sample	EZ-QC™ Assay	LC-MS
20-mer RNA substrate with tail	**~100 A's**	**95-106 A's**

The EZ-QC™ mRNA Poly(A) Tail Length Assay allows for accessible and straightforward determination of poly(A) tail lengths by resolving intact tails using PAGE and permits analysis of longer tails beyond LC-MS capabilities.

Comparison of EZ-QC™ Assay and LC-MS Requirements

Factor	EZ-QC™ Assay Kits	LC-MS
Length of mRNA for capping analysis	No max length restrictions	13-23 nt is the max fragment length to achieve optimal analysis
Length of mRNA for Cap 1 analysis	No max length restrictions	13-23 nt is the max fragment length to achieve optimal analysis
Length of mRNA for Poly(A) tail analysis	No max length restrictions	<200 A's is the max fragment length to achieve optimal analysis
Quantity of mRNA	5-10 picomoles for Capping efficiency kit 12-24 picomoles for Cap 1 efficiency kit 5 picomoles and up for Poly(A) tail length kit	30-50 µl of a 5 µM solution = 150-250 picomoles total required for each QC analysis.
Turnaround time	Same day	>1-5 days, not including shipping time
Cost per sample	4 samples for the cost of 1X LC-MS sample (for evaluation using all 3 EZ-QC™ Assays on a single sample).	1X LC-MS sample cost
Number of samples per run	10-15 samples per gel, scalable based on needs	1 sample per run
Controls required	Included with some EZ-QC™ kits to aid analysis	System suitability controls. Essential to provide additional controls to run alongside unknowns.
Expected molecular weight or length of target needed for analysis	Not required	Yes, so the software can assign expected molecular weights and relative abundance.
Equipment required	Benchtop electrophoresis and gel imager	LC-MS platform
Training required	Basic lab techniques	Yes, specialized training required for using the software, interpreting data and using the equipment. Many molecules have similar profiles, making analysis difficult.
Flexibility of testing	Process and analyze samples when convenient	Requires possible outsourcing of testing, account setup, and scheduling of shipment on dry ice. Requires up-front prep of platform and knowledge of the analysis software and data output.

Conclusion

CELLSCRIPT™'s EZ-QC™ mRNA Assay Kits offer an easy, affordable, and accurate approach to determining the 5′-cap and 3′-polyadenylation status of mRNA using standard laboratory equipment for running polyacrylamide gels. The same samples were analyzed using EZ-QC™ mRNA Assay Kits and on a third-party LC-MS platform and evaluated for a variety of factors. The EZ-QC™ mRNA Assay Kits produce results comparable to LC-MS and offer convenience, flexibility, and a cost-effective approach compared to traditional LC-MS methods.

Materials & Methods

All experiments presented here were performed using CELLSCRIPT™ EZ-QC™ Kit protocols and reagents; detailed steps can be found on respective product webpages.

- EZ-QC™ XBG mRNA Capping Efficiency Assay Kit (Catalog Number XBG250310)
- EZ-QC™ mRNA Capping Efficiency Assay Kit (Catalog Number ACE240910)
- EZ-QC™ mRNA Cap 1 Efficiency Assay Kit (Catalog Number ONE240910)
- EZ-QC™ mRNA Poly(A) Tail Length Assay Kit (Catalog Number PAT240910)

[1]USP-NF (2024). Analytical Procedures for mRNA Vaccine Quality. Draft Guidelines: 3rd Edition.