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

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Introduction

An essential step in manufacturing high-quality mRNA is to perform comprehensive quality control prior to its usage 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 requires 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. An evaluation has been performed to compare the results obtained from EZ-QC™ assay kits with results from various LC-MS methods.

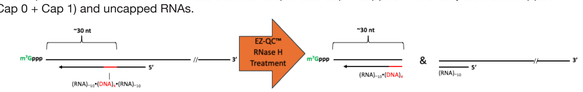
[1]USP-NF (2024). Analytical Procedures for mRNA Vaccine Quality. Draft Guidelines: 3rd Edition.

Importance of mRNA Capping

Capped mRNA is essential for stability and cellular expression. Enzymatic capping is catalyzed by mRNA Capping Enzyme and produces a Cap 0 cap structure. Cap 0 caps can be converted into Cap 1 caps with the additional treatment of an mRNA 2′-O-methyltransferase which catalyzes the 2′-O-methylation of the ribose sugar of the first base of the RNA transcript. Most Cap 1 mRNAs are expressed at higher levels in cells relative to their Cap 0 counterparts since the Cap 1 helps to ensure low immunogenicity by helping cells identify the mRNA as “self” RNA.

EZ-QC™ XBG mRNA Capping Efficiency Assay Kit (Catalog Number XBG250310)

The EZ-QC™ XBG mRNA Capping Efficiency Assay Kit utilizes a chimeric RNA-DNA-RNA Targeting Oligonucleotide which hybridizes near the 5′ end of an RNA mixture. This hybridization complex serves as a substrate for 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 allows for the calculation of the percent capped RNA content of the sample. This kit does not differentiate between Cap 0 and Cap 1 capped RNAs, only between capped (Cap 0 + Cap 1) and uncapped RNAs.



Hybridize the mRNA of interest to a chimeric RNA-DNA-RNA targeting oligo, then digest hybrid with EZ-QC™ RNase H.

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

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

In contrast to LC-MS:

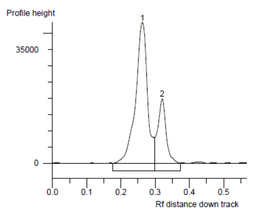
- EZ-QC™ assays do not have the strict (13-23 nt) 5′-end size limitation.
- EZ-QC™ assays do not require clean-up to remove targeting oligo, protein, and mRNA 3′ ends prior to analysis.
- EZ-QC™ assays do not require knowledge of the expected mRNA fragment lengths and sequences for identification.

EZ-QC™ XBG mRNA Capping Efficiency Assay Kit Results

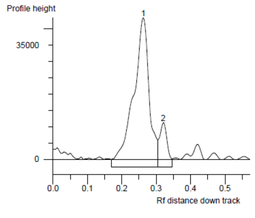
Lane	Sample
1	10/60 ssDNA ladder
2	80% Capped/20% Uncapped XBG RNA Control (included with the kit)
3	90% Capped/10% Uncapped 55 nt mRNA Sample

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

Lane 2 – Kit Control



Lane 3 – 55 nt mRNA



Electropherogram of results from PAGE after using the **EZ-QC™ XBG mRNA Capping Efficiency Assay Kit**.

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 of 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.

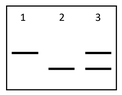
Compiled EZ-QC™ Assay and LC-MS Results

Lane	Sample	EZ-QC™ Assay % Capped	LC-MS % Capped
1	80% Capped/20% Uncapped XBG RNA Control	75.1%	Not performed
2	90% Capped/10% Uncapped	**89.1%**	**93.0%**

Comparable results were obtained between **EZ-QC™ XBG mRNA Capping Efficiency Assay Kit and LC-MS.**

EZ-QC™ mRNA Cap 1 Efficiency Assay Kit (Catalog Number ONE240910)

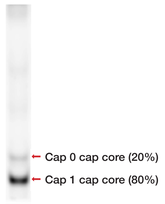
The EZ-QC™ mRNA Cap 1 Efficiency Assay Kit labels mRNA ends with the fluorescent dye Cyanine5 (Cy5). The labeled mRNA is then digested with the EZ-QC™ RNase mixture releasing Cy5-labeled 5′ Cap 1 and Cap 0 cap cores. These are resolved using PAGE and analyzed using a fluorescent gel imager to measure 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.



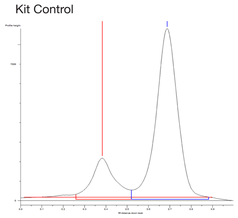
1) Released Cap 0 cap core: **Cy5**-m^7G(5′)ppp(5′)N1p Dinucleotide molecule
2) Released Cap 1 cap core: **Cy5**-m^7G(5′)ppp(5′)$\mathbf{m^{2'\text{-}O}}$$N_1$pNp Trinucleotide molecule
3) Mixed sample of Cap 0 & Cap 1 cap cores

Example Data from EZ-QC™ mRNA Cap 1 Efficiency Assay Kit:

Each EZ-QC™ mRNA Cap 1 Efficiency Assay Kit includes an 80% Cap 1/20% Cap 0 control that can be run alongside samples to aid in analysis of Cap 1 and Cap 0 content.



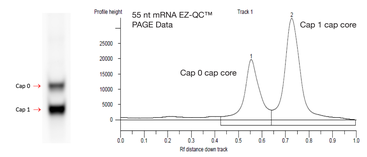
PAGE separation of the 80% Cap 1/20% Cap 0 control included with every EZ-QC™ mRNA Cap 1 Efficiency Assay Kit.



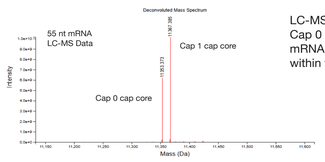
Electropherogram of PAGE results after using the EZ-QC™ mRNA Cap 1 Efficiency Assay Kit.

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

Due to the ease and flexibility of the the EZ-QC™ mRNA Cap 1 Efficiency Assay Kit, full length mRNA can be processed directly using this kit to quantitate Cap 0 and Cap 1 content. The 90% Capped/10% Uncapped 55 nt mRNA 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, testing of this 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.



PAGE results and electropherogram showing quantitation of the relative Cap 0 and Cap 1 cap cores present in the sample using the **EZ-QC™ mRNA Cap 1 Efficiency Assay Kit.**



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 found within the sample are highlighted here for clarity.

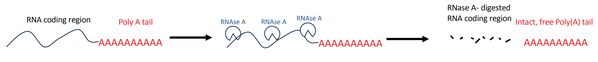
Compiled EZ-QC™ Assay and LC-MS Results

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**

Comparable results were obtained between **EZ-QC™ mRNA Cap 1 Efficiency Assay Kit** and LC-MS for determination of Cap 1 and Cap 0 content.

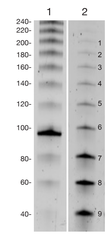
EZ-QC™ mRNA Poly(A) Tail Length Assay Kit (Catalog Number PAT240910)

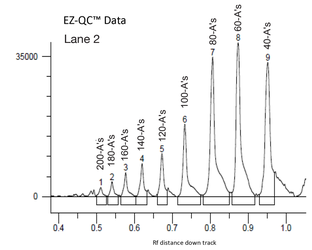
3′-end polyadenylation is a critical modification that influences mRNA stability and expression levels in eukaryotic cells with longer tails generally providing increased stability and translation efficiency. 3′-poly(A) tails can be added co-transcriptionally when encoded by template or post-transcriptionally 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.



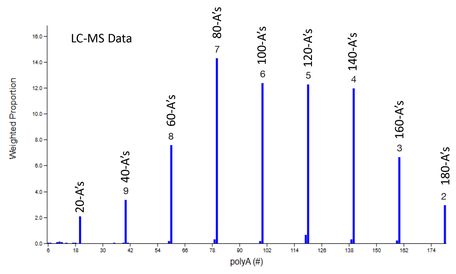
Side-by-Side Evaluation of the EZ-QC™ mRNA Poly(A) Tail Length Assay Kit Compared to LC-MS

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.

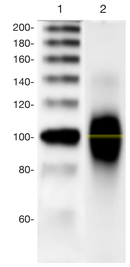


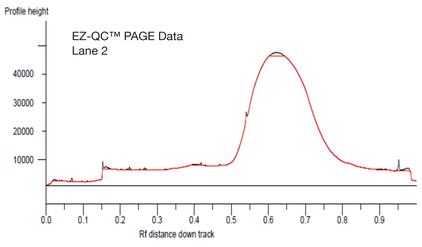


PAGE results and corresponding electropherogram showing lengths of the Poly(A) 20-mer Ladder included with the **EZ-QC™ mRNA Poly(A) Tail Length Assay Kit**. Lane 1: EZ-QC™ Poly(A) 20-mer Ladder.
Lane 2: a variation of the Poly(A) 20-mer Ladder submitted for LC-MS. The 20-A peak is also present but was not captured here. The peaks are labeled 1-9 and their quantitation is denoted in the electropherogram.

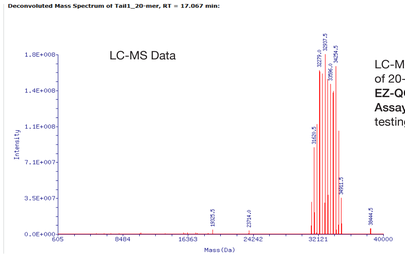


LC-MS Analysis Summary of a Poly(A) 20-mer Ladder submitted for LC-MS. The 200-A peak and lengths greater than 200 were not detectable via LC-MS due to limitations of the platform.





PAGE results and electropherogram showing lengths of a sample processed with the **EZ-QC™ mRNA Poly(A) Tail Length Assay Kit**. Lane 1: EZ-QC™ Poly(A) 20-mer Ladder. Lane 2: a 20-mer RNA substrate with tail.



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.

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**

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

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	<35 nt is the max fragment length to achieve optimal analysis
Length of mRNA for Cap 1 analysis	No max length restrictions	<35 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 pico-moles 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, scal-able 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 facilitate easy, affordable, and accurate methods to determine the 5′-cap and 3′-polyadenylation status of mRNA using standard laboratory equipment for running polyacrylamide gels. The same samples were tested 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)**

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RNA for Translation in Cells

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