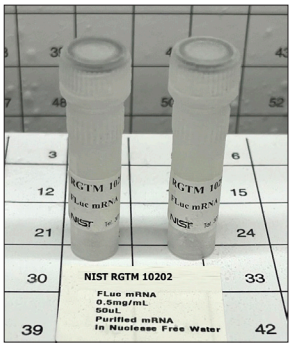


Credit: Mark Lowenthal, NIST

Analytical Characterization of the NIST Firefly Luciferase mRNA Standard:

Integrity, Cap Structure, Poly(A) Tail Length and dsRNA Content

Kari L. Weber PhD and Samara G. Bradtke
CELLSCRIPT™, 726 Post Road, Madison, WI 53713 USA

Abstract

Messenger RNA (mRNA) therapeutics require precise control of structural and biochemical attributes to ensure stability, translation efficiency, and minimal immunogenicity. To facilitate method development and interlaboratory comparisons, the National Institute of Standards and Technology (NIST) has produced and distributed a Research-Grade firefly luciferase mRNA test material for recipients to collaboratively evaluate for potential fitness for purpose as an mRNA Standard Reference Material. We have performed a detailed analytical characterization of this material, including use of CELLSCRIPT™'s EZ-QC™ Assays. The NIST mRNA exhibited high integrity and complete 5′ capping, with 92% of 5′ caps consisting of Cap 1 structure. Poly(A) tail lengths were consistent with template design, and dsRNA content was ~0.05%. These results provide a detailed molecular profile for the NIST mRNA standard, support its use as a standard reference material for mRNA quality control testing, and demonstrate that the CELLSCRIPT™ EZ-QC™ assays provide reliable mRNA quality information consistent with expected results.

Introduction

Messenger RNA (mRNA) therapeutics have emerged as a transformative platform for vaccines, protein replacement therapies and gene editing applications. Their clinical performance depends on a complex set of structural and biochemical attributes, including integrity, 5′-cap structure, poly(A) tail length, and the absence of immunostimulatory byproducts such as double-stranded RNA (dsRNA). Each of these features influences translational efficiency, stability, and immunogenicity, and therefore must be quantitatively characterized during development and manufacturing.

To support analytical method development and interlaboratory comparability, the NIST has produced and distributed an mRNA encoding firefly luciferase (FLuc) for recipients to collaboratively evaluate for potential fitness for purpose as an mRNA Standard Reference Material. Here, we applied a panel of assays, including CELLSCRIPT™'s EZ-QC™ Assays, to characterize key quality attributes of this NIST FLuc mRNA standard. RNA integrity was evaluated by formaldehyde agarose gel electrophoresis. 5′-cap structure was analyzed using EZ-QC™ mRNA Capping Efficiency and mRNA Cap 1 Efficiency Assay Kits. Poly(A) tail length was assessed using the EZ-QC™ mRNA Poly(A) Tail Length Assay Kit. Double-stranded RNA (dsRNA) impurities were quantified by J2 antibody-based immunoblot.

Materials and Methods

Imaging and analysis of all gels and immunoblots were performed using a Syngene™ G:Box gel documentation system and Syngene™ GeneTools software.

mRNA Material

The NIST FLuc mRNA Research Grade Test Material (RGTM 10202) was obtained from the National Institute of Standards and Technology and includes an enzymatically added Cap 1 structure on the 5′ end. Inferring from the sequence provided for the DNA template used by NIST to prepare the FLuc mRNA, the material is expected to be 1938-nt long, including the 5′ cap and a template-encoded, discontinuous 3′-poly(A) tail with poly(A) stretches of 31- and 70-nt interrupted by 10-nt mixed sequence.

mRNA Integrity Assessment

mRNA integrity was assessed on a 1% agarose formaldehyde gel prepared in MOPS buffer. The mRNA sample was prepared in a formaldehyde and formamide containing loading buffer, denatured at 70°C for 10 min, then loaded (100 ng/lane) and electrophoresed at 100 V for 1 hour alongside the Millennium™ RNA Marker (Thermo Fisher Scientific). The gel was stained with SYBR™ Gold (Invitrogen™).

5′-Cap Efficiency Determination

The EZ-QC™ mRNA Capping Efficiency Assay (CELLSCRIPT™, Catalog ACE240910) was performed according to kit instructions. A 55-nt uncapped *in vitro* transcript corresponding to the 5′ end of the NIST FLuc mRNA sequence was prepared to use as a negative control. A 24-nt chimeric RNA-DNA-RNA targeting oligonucleotide (rGrGrGrUrCrUrUrArUrATTTCrUrUrCrUrUrA-rCrUrCrU) was designed per kit instructions to hybridize near the 5′ end of the 55-nt control transcript and/or the NIST FLuc mRNA and, upon digestion with EZ-QC™ RNase H, release an mRNA 5′-end fragment of 34-nt (uncapped) or 35-nt (capped). Digestion products were separated on a 20% polyacrylamide, 8 M Urea gel alongside the DNA oligo 10/60 Ladder (IDT). The relative intensity of the uncapped and capped 5′-end fragment bands were compared to determine what percentage of the mRNA was capped.

5′ Cap Structure Analysis

The EZ-QC™ Cap 1 Efficiency Assay (CELLSCRIPT™, Catalog ONE240910) was performed according to kit instructions. Alongside internal control standards of Cap 0 and Cap 1 mRNA prepared by CELLSCRIPT™, the NIST FLuc mRNA was fluorescently labeled at its ends with Cyanine5, purified, then digested with EZ-QC™ RNase I and EZ-QC™ RNase T1, releasing fluorescently-labeled Cap 1 and Cap 0 cap cores, which resolve on a 15% polyacrylamide gel based on charge, with the trinucleotide Cap 1 cap core migrating faster than the dinucleotide Cap 0 cap core. The relative intensity of the resultant fluorescently labeled Cap 0 and Cap 1 core bands were compared to determine what percentage of the 5′-capped mRNA was Cap 1.

Poly(A) Tail Length Analysis

The EZ-QC™ Poly(A) Tail Length Assay (CELLSCRIPT™, Catalog PAT240910) was performed according to kit instructions. Alongside internal controls including untailed mRNA and enzymatically polyadenylated mRNA with ~180-nt poly(A) tail, NIST Fluc mRNA was digested with EZ-QC™ RNase A, which cleaves only at pyrimidines and leaves stretches of poly(A) intact. Digestion products were separated alongside the EZ-QC™ Poly(A) 20-mer ladder on a 10% polyacrylamide, 8 M Urea gel. Poly(A) tail lengths were interpolated by comparing gel mobility of the RNase A digestion products relative to the Poly(A) 20-mer ladder.

dsRNA Quantification by J2 Dot Blot

Double-stranded RNA (dsRNA) content was determined via immunoblot. A dsRNA standard was prepared by annealing two complementary single-stranded RNAs. The dsRNA standard curve (50–250 pg, with each standard in triplicate) was spotted onto a nitrocellulose membrane and air-dried alongside 1 µg NIST FLuc mRNA and, as a positive control, 1 µg NIST FLuc mRNA plus 100 pg dsRNA standard. The membrane was blocked with 5% (w/v) skim milk in TBS-T for 1 hour, incubated overnight with SCICONS™ anti-dsRNA antibody, clone J2 (Nordic-MUbio) at 1 µg/ml in blocking solution, washed, then incubated with Anti-mouse IgG, HRP-linked antibody (Cell Signaling Technology) diluted 1:1000 in blocking solution for 1 hour, followed by washing. Detection was performed using enhanced chemiluminescence (ECL). The ECL signal of the test sample and positive control exceeded the ECL signal of the highest standard curve standard, but tentative dsRNA content of the test sample and the positive control were estimated by standard curve extrapolation.

Results

RNA Integrity Assessment by Formaldehyde Agarose Gel Electrophoresis

To evaluate integrity of the NIST FLuc mRNA, 100 ng was run on a 1% agarose formaldehyde gel alongside RNA size marker. The NIST FLuc mRNA displayed a sharp, distinct band without smearing, indicating high integrity, and appears to be the expected size of 1938-nt.

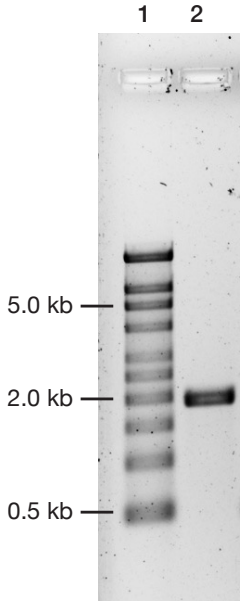


Figure 1. RNA integrity assessment by formaldehyde agarose gel.

5′-Cap Efficiency Determination

The EZ-QC™ mRNA Capping Efficiency Assay produced no RNase H cleavage product at the position of the uncapped 5′-end standard, indicating that uncapped NIST FLuc mRNA was below the limit of detection of the assay.

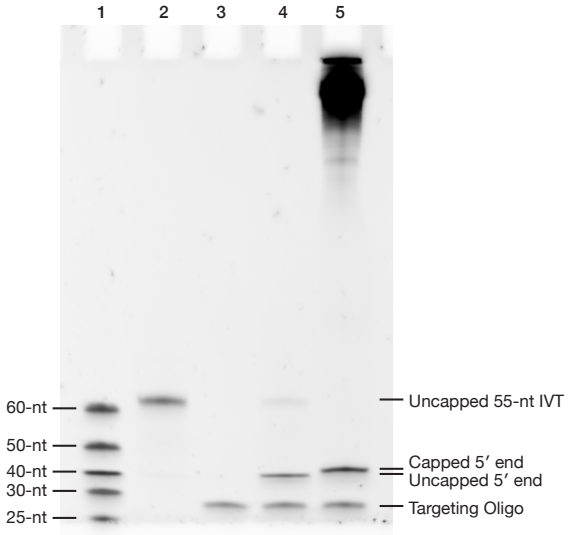


Figure 2A. 5′-end capping determination gel.

Lane assignments: (1) Oligo ladder; (2) 55-nt Uncapped 5′-end standard; (3) 24-nt RNA-DNA-RNA targeting oligonucleotide; (4) 55-nt Uncapped 5′-end standard incubated with 24-nt targeting oligo and digested with EZ-QC™ RNase H; (5) NIST FLuc mRNA incubated with 24-nt targeting oligo and digested with EZ-QC™ RNase H.

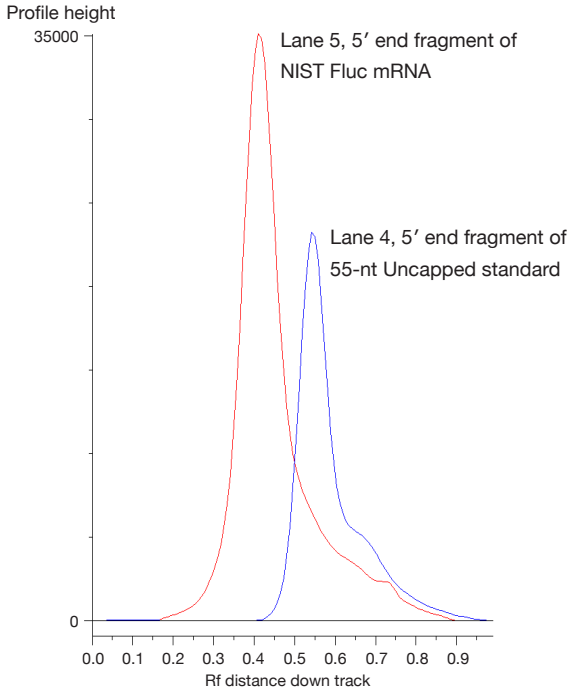
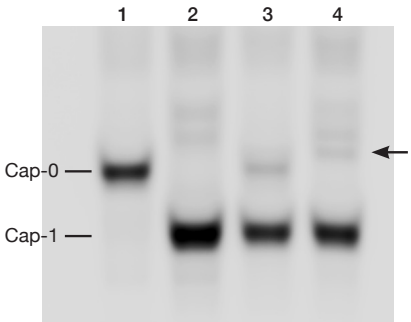


Figure 2B. Overlay of 5′-end capping determination gel lane traces for Lane 4 (uncapped standard; blue trace) and Lane 5 (NIST FLuc mRNA, red trace).

5′-Cap Structure / Cap 1 Efficiency Determination

The EZ-QC™ mRNA Cap 1 Efficiency Assay showed that 92.4% of the 5′-capped NIST FLuc mRNA is Cap 1. The remaining 7.6% was assigned to Cap 0, although its migration is more consistent with an unmethylated cap (GpppN, lacking the N7-methyl group) than with canonical Cap 0. It was counted as Cap 0 to give a conservative Cap 1 value.



A minor species migrating consistently with an unmethylated cap (lacking the N7-methyl group). It has been included in the calculations as Cap 0. If it had not been included in the calculations as Cap 0, the NIST FLuc mRNA sample would have tested as 100% Cap 1.

Figure 3A. 5′-Cap 1 Efficiency determination gel.

Lane assignments: (1) Fluorescently-labeled cap from Cap 0 RNA standard; (2) Fluorescently-labeled cap from Cap 1 RNA standard; (3) Fluorescently-labeled caps from mixture of fluorescently-labeled 80% Cap 1/20% Cap 0 RNA standard; (4) Fluorescently-labeled cap from NIST FLuc mRNA.

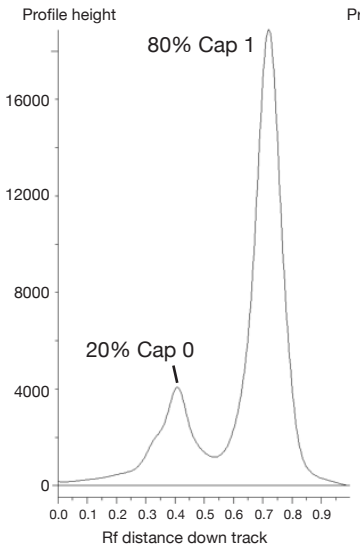


Figure 3B

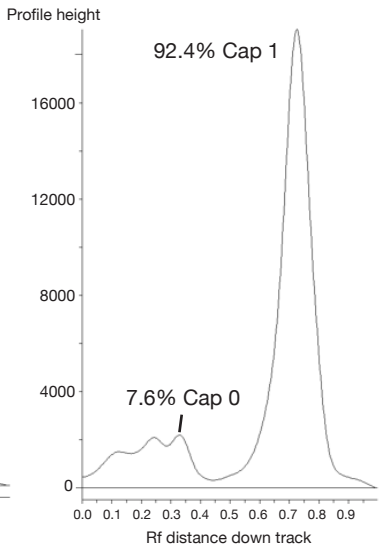


Figure 3C

Figure 3B and 3C. Gel lane traces for Lane 3 (80% Cap 1/20% Cap 0 RNA standard) and Lane 4 (NIST FLuc mRNA) of the Cap 1 Efficiency Assay gel shown in Figure 3A.

Poly(A) Tail Length

The EZ-QC™ Poly(A) Tail Length Assay results were as expected in that EZ-QC™ RNase A digestion of untailed RNA did not result in products that would be visible on a 10% polyacrylamide gel whereas the enzymatically polyadenylated control RNA showed an average poly(A) tail length of 183-nt. When subjected to the EZ-QC™ assay, the NIST FLuc mRNA resulted in two bands determined to be 75- and 32-nt via interpolation from the relative gel mobility of the Poly(A) 20-mer Ladder. These results are consistent with the NIST-provided sequence for the standard DNA template, which encodes discontinuous 3′ poly(A) stretches of 31- and 70-nt.

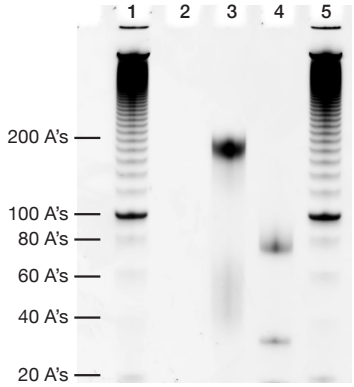


Figure 4. Poly(A) tail length determination gel.

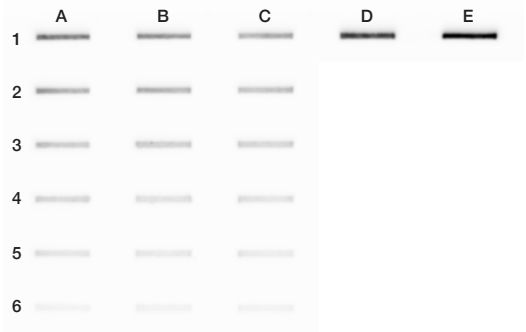
Lane assignments: (1) Poly(A) 20-mer Ladder; (2) Untailed control standard; (3) Enzymatically polyadenylated control standard; (4) NIST FLuc mRNA; (5) Poly(A) 20-mer Ladder.

dsRNA Content

The J2 antibody-based immunoblot for dsRNA showed that the NIST FLuc mRNA contained ~0.05% dsRNA by mass, as determined by extrapolation from the standard curve. Successful recovery of standard in a "spike-in" positive control where 100 pg dsRNA standard was added into the test sample demonstrates reliability of the quantitative result.

Test Sample	Calculated pg dsRNA in 1 µg RNA loaded	% dsRNA	% Spike Recovery
NIST FLuc mRNA	462*	0.046	N/A
NIST FLuc mRNA + 100 pg dsRNA Standard	568*	N/A	106

*These values are outside the range of the standard curve, so quantitative results are products of extrapolation.



A1, B1, C1. 250 pg dsRNA each	A5, B5, C5. 75 pg dsRNA each
A2, B2, C2. 175 pg dsRNA each	A6, B6, C6. 50 pg dsRNA each
A3, B3, C3. 125 pg dsRNA each	D1. 1 µg NIST FLuc mRNA
A4, B4, C4. 100 pg dsRNA each	E1. 1 µg NIST FLuc mRNA + 100 pg dsRNA standard

Figure 5. Quantification of dsRNA content by J2 antibody dot blot.

Well assignments: J2 antibody dot blot of dsRNA standard curve (50–250 pg, with triplicates averaged) resulted in $R^2 = 0.994$ (A-C, rows 1-6). J2 antibody dot blot of 1 µg NIST FLuc mRNA (D1) and 1 µg NIST FLuc mRNA + 100 pg dsRNA standard (E1).

Discussion

This study provides an analytical profile of the NIST FLuc mRNA standard using CELLSCRIPT™ EZ-QC™ mRNA quality control kits. The NIST material demonstrated:

- **High RNA integrity,** as shown by a sharp band on a formaldehyde agarose gel.
- **Complete capping,** with 92.4% Cap 1 structure and no detectable uncapped transcripts.
- **Poly(A) tails** of ~30- and ~70-nt, consistent with template sequence.
- **dsRNA content** of ~0.05%.

The data set presented here can serve as a benchmark for analytical method development, quality control, and inter-laboratory harmonization in mRNA research and manufacturing.