CELLSCRIPT™
RNA for Translation in Cells

EZ-QC™ mRNA Capping Efficiency Assay Kit

Cat. No. **ACE240910**

INTRODUCTION

The EZ-QC™ mRNA Capping Efficiency Assay Kit allows one to quantitate the capping efficiency (percent capped RNA content) of their mRNA production product. 5'-cap structures can be added co-transcriptionally or post-transcriptionally to *in vitro* transcribed (IVT) RNA to produce Cap 0 and/or Cap 1 structures as a step in the mRNA synthesis process. Since only capped RNAs will be expressed in cells, it is important to have the highest possible percentage of capped RNAs present in one's sample. The EZ-QC mRNA Capping Efficiency Assay Kit allows one to forego tedious and expensive capping efficiency determination methods such as HPLC and mass spectrometry, instead allowing for determination based upon standard polyacrylamide gel electrophoresis (PAGE) methodology.

Primary transcript (uncapped) IVT RNA lacks a cap structure.

(5') $pppN_1pN(pN)_x$-OH (3')

Capped RNAs (Cap 0 and Cap 1) contain an additional N7-methylated guanosine linked by a 5' to 5' triphosphate bridge to the 5'-end of the IVT RNA.

m^7G(5')ppp(5')$N_1pN(pN)_x$-OH (3')

Where m^7G represents the 7-methylguanosine cap nucleoside, ppp represents the triphosphate bridge between the 5' carbons of the cap nucleoside and the first nucleotide of the primary RNA transcript, and $N_1pN(pN)_x$-OH (3') represents the primary RNA transcript, of which N_1 is the most 5' nucleotide. In Cap 1 RNA, this nucleotide contains a 2'-O-methyl group on the ribose moiety.

The EZ-QC mRNA Capping Efficiency Assay utilizes a chimeric RNA-DNA-RNA Targeting Oligonucleotide **(user provided)** which hybridizes to the 5'-end region 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 PAGE and quantified by gel band analysis allowing for the calculation of the percent capped RNA content of the sample. The EZ-QC mRNA Capping Efficiency Assay does not differentiate between Cap 0 and Cap 1 capped RNAs, only between capped (Cap 0 + Cap 1) and uncapped RNAs. Also available is the EZ-QC **XBG** mRNA Capping Efficiency Assay Kit for use on Xenopus β-globin (XBG) 5' UTR (untranslated region)-containing mRNAs **(Targeting Oligonucleotide is provided in the kit)**. For determination of Cap 0 vs Cap 1 content, CELLSCRIPT also offers the EZ-QC mRNA Cap 1 Efficiency Assay Kit as well as the EZ-QC mRNA Poly(A) Tail Length Assay Kit for complete mRNA characterization.

726 Post Road, Madison, WI 53713 • 888-826-5586 (U.S. only) • (608) 442-6484 • Fax (608) 442-6187

MATERIALS

Materials Supplied

Important Store at –20°C in a freezer without a defrost cycle. Do not store at –70°C.

EZ-QC™ mRNA Capping Efficiency Assay Kit Contents (10 reactions) Sufficient for 10 experimental and 10 control reactions.	
Kit Component	Reagent Volume
EZ-QC™ RNase H in 50% glycerol, 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM dithiothreitol (DTT), 0.1 mM EDTA and 0.1% Triton® X-100.	20 µl
10X EZ-QC™ RNase H Reaction Buffer 0.2 M Tris-acetate, pH 7.9, 0.5 M potassium acetate, 0.1 M magnesium acetate and 0.01 M DTT.	20 µl
ScriptGuard™ RNase Inhibitor, 40 U/µl in 50% glycerol, 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM DTT, 0.1 mM EDTA and 0.1% Triton X-100.	10 µl
Stop/Loading Buffer 95% formamide, 10 mM EDTA, pH 7.5, 0.01% Bromophenol Blue and 0.01% Xylene Cyanol.	200 µl
RNase-Free Water	500 µl

Materials Required, but not Supplied

- Purified capped mRNA/RNA
- EZ-QC mRNA Capping Efficiency Targeting Oligonucleotide (**see design considerations on page 3**).
- Materials for polyacrylamide gel electrophoresis.
- Materials for polyacrylamide gel visualization, imaging and quantification.
- Optional: molecular weight marker (e.g., ssDNA 10/60 Ladder [IDT])

SPECIFICATIONS

Unit Definitions

One standard reaction from the EZ-QC mRNA Capping Efficiency Assay Kit treats 5-10 pmoles of RNA.

One unit of ScriptGuard RNase Inhibitor results in 50% inhibition of 5 ng of RNase A.
RNase H is not inhibited by ScriptGuard RNase Inhibitor.

Functional Testing

The EZ-QC mRNA Capping Efficiency Assay Kit is functionally tested by treatment of capped and uncapped 55-nt RNAs resolved on a 20% polyacrylamide (19.5% acrylamide, 0.5% Bis-acrylamide), 8 M Urea, TBE gel, and quantified using GeneTools software of the captured gel image using a Syngene® G:Box.

Contaminating Activity Assays

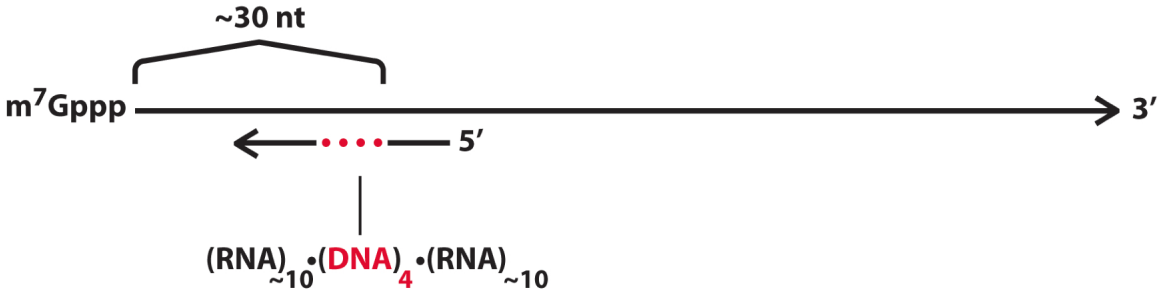
All components of the EZ-QC mRNA Capping Efficiency Assay Kit are free of detectable RNase and DNase activities except for the inherent activity of the EZ-QC RNase H.

For more information, consult the appropriate safety data sheet (SDS) at www.cellscript.com.

EZ-QC mRNA Capping Efficiency Targeting Oligonucleotide Design Considerations (User Provided)

EZ-QC mRNA Capping Efficiency Targeting Oligos should be designed to release an mRNA 5'-end fragment of 25-40 nucleotides in length. This released fragment(s), +/– the cap nucleotide, is to be resolved via PAGE analysis. RNase H cleavage will occur in the mRNA strand across from the 5'-most DNA base in the Targeting Oligo.

Figure 1 Example Schematic



Example capped mRNA/Targeting Oligonucleotide hybrid

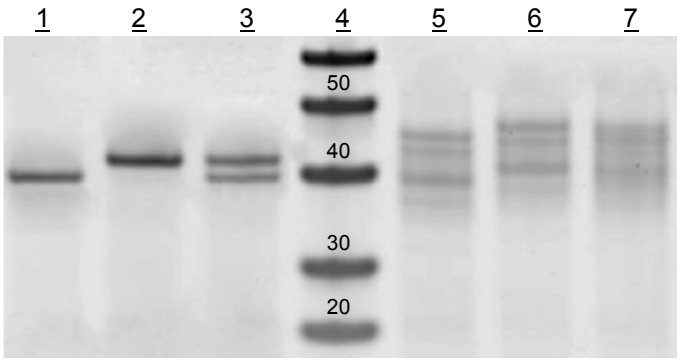
5′ GpppGGGCCAΨΨAΨΨCAACGGGAAACGΨCACGCACGAGGCCGCGAΨΨAAAΨΨCCAA...
GCCCUUUGCAGTGCGUGCUCCGGC 5′

Example of possible released mRNA fragments

5′ GpppGGGCCAΨΨAΨΨCAACGGGAAACGΨCACG Capped fragment

5′ pppGGGCCAΨΨAΨΨCAACGGGAAACGΨCACG Uncapped fragment

Figure 2 Comparative Targeting Oligonucleotide Gel



A Chimeric RNA-DNA-RNA Targeting Oligo was utilized in reactions 1-3.

A standard DNA oligo of the same sequence was utilized in reactions 5-7.

Lanes 1 & 5) 100% uncapped RNA
Lanes 2 & 6) 100% capped RNA
Lanes 3 & 7) 50%/50% capped and uncapped RNA mixture

Lane 4) ssDNA Molecular Weight Marker 10/60 Marker (IDT)

Considerations

- Optimal oligo T_m is 50-68°C.
- Oligo RNA arms are roughly symmetrical in length.
- RNase H cleavage is inhibited by the hybridization of any of the oligo's DNA bases to pseudouridine or N1-methyl-pseudouridine. As such, design the oligo such that the four DNA bases do not hybridize to any modified nucleosides.
- Design the oligo to be a different size than the expected excised RNA fragment to avoid co-migration during PAGE. In the example above, the excised fragments are 28 & 29 nucleotides long and the Targeting Oligo is 24 nucleotides long. Commonly, the excised fragments would be 5-20 nucleotides longer than the Targeting Oligo (see example on page 6).
- Store the master stock tube (high concentration) of EZ-QC mRNA Capping Efficiency Targeting Oligo at –70°C. Multiple small-volume aliquots of the Targeting Oligo may be made at the working concentration of 5 μM and stored at –20°C or –70°C.

BEFORE YOU START: IMPORTANT TIPS

♦ **RNA Source**:

RNAs can be capped co-transcriptionally using a cap analog molecule during the IVT reaction, or post-transcriptionally (enzymatically) using a Capping Enzyme (e.g., CELLSCRIPT's ScriptCap™ Cap 1 Capping System, T7 mScript™ Standard mRNA Production System or INCOGNITO™ T7 mScript™ Ψ- or N1meΨ-mRNA Production Systems) after mRNA production. **It is important to retain and assay enough of the uncapped IVT RNA product to serve as the uncapped control reaction, along with the experimental capped RNA product.**

♦ **Cap 0- vs. Cap 1-mRNA**:

The difference between Cap 0- and Cap 1-mRNA is the addition of a methyl group at the 2'-O position of the penultimate (second from the 5' end) nucleotide of the transcript in Cap 1-mRNA. This methylation is part of the natural capping process in higher eukaryotic cells and usually improves *in vivo* translation versus the corresponding Cap 0-mRNA. The EZ-QC mRNA Capping Efficiency Assay does not differentiate between Cap 0 and Cap 1 capped RNAs, only between capped (Cap 0 + Cap 1) and uncapped RNAs. For determination of Cap 0 vs Cap 1 content, CELLSCRIPT also offers the EZ-QC mRNA Cap 1 Efficiency Assay Kit.

♦ **Maintaining an RNase-Free Environment**:

Highly stable RNases are ubiquitous, including on human skin.

Creating an RNase-free work environment and maintaining RNase-free solutions is critical for successful work with RNA.

It is strongly recommended to:

– Use RNase-free tubes and pipette tips.

– Always wear gloves when handling kit components or samples containing RNA and change gloves frequently, especially after touching potential sources of RNase contamination such as doorknobs, pens, pencils and human skin. Do not touch any kit component or tube containing RNA with an ungloved hand.

– Keep all kit components tightly sealed when not in use. Keep all tubes containing RNA tightly sealed during the incubation steps.

PROCEDURE

A. EZ-QC RNase H Treatment Step

1. For each reaction, **treat both**, some of the **uncapped** (IVT) RNA that was used to make the capped mRNA, as well as the **capped** mRNA itself. The uncapped RNA sample serves as a necessary control for the analysis.

   Combine the following reagents at room temperature in the order indicated below:

EZ-QC mRNA Capping Efficiency Assay Reaction	
Component	Amount
RNase-Free Water	x µl
10X EZ-QC RNase H Reaction Buffer	1 µl
5 µM EZ-QC mRNA Capping Efficiency Targeting Oligo (user provided)	1.2 µl
ScriptGuard RNase Inhibitor	0.5 µl
5-10 pmoles * capped mRNA, **and in a separate control reaction**, uncapped IVT RNA	y µl
EZ-QC RNase H	1 µl
Total Volume	10 µl

   * Estimating amount of input RNA/mRNA

   pmol/µg ssRNA = 10^6 / (estimated length of the RNA in nucleotides x 330)

   for example, for a 1,700-b RNA: 10^6 / (1,700 x 330) = 1.8 pmol/µg

2. Incubate at 37°C for 30 minutes, then add 10 µl Stop/Loading Buffer to each reaction.

3. While the reactions are incubating, prepare a 20% acrylamide/8 M Urea/1X TBE gel.
   a) Blow out unpolymerized acrylamide from the wells immediately after comb removal.
   b) Pre-run the gel at 300 volts constant voltage for 20-30 minutes.
   c) Blow out the excess urea from the wells just prior to sample loading.

4. Pre-heat denature the samples at 65–70°C for 5 minutes just prior to gel loading.
   a) Load 5 µl of each stopped reaction mix to the appropriate lanes for PAGE.
   b) Load the capped sample adjacent to the uncapped control sample.
   c) Running a molecular weight marker, while not necessary for analysis, is optional.

5. Run the gel at 300 volts (constant voltage) for 2-3 hours.
   a) The Stop/Loading Buffer contains two dyes; bromophenol blue (a darker blue that migrates faster) and xylene cyanol (a lighter blue that migrates more slowly).
   b) The gel should be run until the xylene cyanol dye front is approximately 1 cm from the end of the gel; the bromophenol blue dye will have run off the end of the gel.

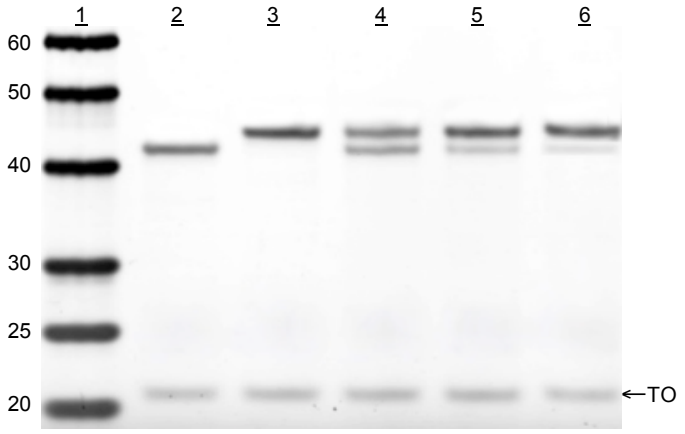
6. Stain the gel with a dye appropriate for your gel visualization system (e.g., SYBR® Gold Nucleic Acid Gel Stain [Invitrogen]).

7. Visualize/image the gel. Quantitate relative band intensities (system specific).

Example Data:

Figure 3A

20% acrylamide, 8 M Urea, 1X TBE gel stained with SYBR-Gold (5 µl per 50 ml water).

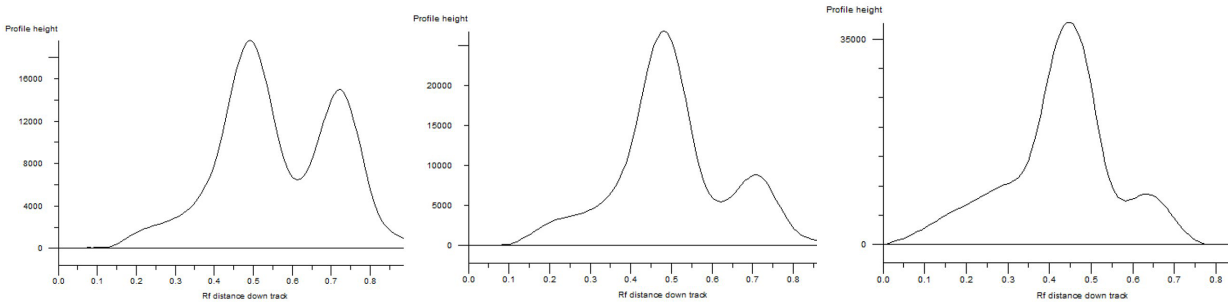


1) IDT ssDNA 10/60 Ladder
2) 100% Uncapped RNA (34-nt ssRNA fragment)
3) 100% Capped mRNA (35-nt ssRNA fragment)
4) 60% Capped/40% Uncapped mRNA Mixture
5) 75% Capped/25% Uncapped mRNA Mixture
6) 90% Capped/10% Uncapped mRNA Mixture

TO) unhybridized EZ-QC mRNA Capping Efficiency Targeting Oligo (18-nt)

Figure 3B

Densitometric data from lanes 4, 5 & 6 from the above PAGE gel using a Syngene G:Box Gel Documentation System.



Lane 4 Lane 5 Lane 6

Lane	Sample	Percent Capped	Percent Uncapped
4	60:40 Mix	58.1	41.9
5	75:25 Mix	77.2	22.8
6	90:10 Mix	91.2	8.8

TROUBLESHOOTING

Symptom	Solution
More than two bands present in the capped/uncapped region of the gel	The Targeting Oligo hybridized in multiple locations. • Redesign the Targeting Oligo.
	A DNA-only Targeting Oligo was used. • Use a chimeric RNA:DNA:RNA Targeting Oligo.
	Targeting Oligo migrates within the capped/uncapped excised fragment range. • Redesign Targeting Oligo to either be of a different size than the capped/uncapped excised fragments or to hybridize in a different position such that the capped/uncapped excised fragments are of a different size than the Targeting Oligo.
Faint capped/uncapped bands	mRNA 5'-end secondary structure is preventing efficient hybridization of the Targeting Oligo. • Preanneal the mRNA and Targeting Oligo, via mixing, heating and cooling, before adding the rest of the reaction components. • Redesign the Targeting Oligo to hybridize to a different region of the mRNA. Sometimes shifting the RNase H cut site over by as little as one base can be helpful.
	One or more of the four DNA bases in the chimeric Targeting Oligo are hybridizing to a pseudouridine or N1-methyl-pseudo-uridine base on the mRNA. • Redesign Targeting Oligo to hybridize to a different region where the DNA bases of the Targeting Oligo will hybridize only to non-pseudouridine or non N1-methyl-pseudouridine bases.
	A molar excess of Targeting Oligo was not used in the reaction. Unhybridized Targeting Oligo should be visible on the gel. See image on page 6. • Recheck experimental stoichiometry. • Add more Targeting Oligo to the reaction.
	Load more completed reaction sample per well.
	Increase the reaction incubation time to 60 minutes.
Near full lane of many bands	RNase contamination. • Be sure to use ScriptGuard RNase Inhibitor in the reaction. • Decontaminate tubes, pipet tips and surfaces. Always use gloved hands.
	Working stock of Targeting Oligo is degrading. • Use or make a different working concentration aliquot of the Targeting Oligo.
Fuzzy bands	Problems with the PAGE system (e.g., gel not totally polymerized, gel ran too hot, urea wasn't blown out of the wells just prior to sample loading, buffer issue). • Rerun the gel.
Capped and uncapped bands unresolved	A <20% polyacrylamide gel was used. • Rerun the gel using 20% polyacrylamide. Commercially-available pre-poured 15% gels most often do not resolve the bands adequately.

RELATED PRODUCTS

– Cap-Clip™ Acid Pyrophosphatase
– EZ-QC™ mRNA Cap 1 Efficiency Assay Kit
– EZ-QC™ mRNA Poly(A) Tail Length Assay Kit
– EZ-QC™ XBG mRNA Capping Efficiency Assay Kit
– INCOGNITO™ T7 mScript™ Complete N1meΨ-mRNA Production System
– INCOGNITO™ T7 mScript™ Complete Ψ-mRNA Production System
– INCOGNITO™ T7 mScript™ N1meΨ-mRNA Production System
– INCOGNITO™ T7 mScript™ Ψ-mRNA Production System
– ScriptCap™ Cap 1 Capping System
– ScriptCap™ m^7G Capping System
– ScriptCap™ 2'-O-Methyltransferase
– T7 mScript™ Complete Standard mRNA Production System
– T7 mScript™ Standard mRNA Production System V2

The performance of this product is guaranteed for one year from the date of purchase.

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