Video Article

In vitro Transcription and Capping of *Gaussia* Luciferase mRNA Followed by HeLa Cell Transfection

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URL: https://www.jove.com/video/3702
DOI: doi:10.3791/3702

Keywords: Genetics, Issue 61, *In vitro* transcription, Vaccinia capping enzyme, transfection, T7 RNA Polymerase, RNA synthesis

Date Published: 3/26/2012

Citation: Jani, B., Fuchs, R. *In vitro* Transcription and Capping of *Gaussia* Luciferase mRNA Followed by HeLa Cell Transfection. *J. Vis. Exp.* (61), e3702, doi:10.3791/3702 (2012).

Abstract

In vitro transcription is the synthesis of RNA transcripts by RNA polymerase from a linear DNA template containing the corresponding promoter sequence (T7, T3, SP6) and the gene to be transcribed (**Figure 1A**). A typical transcription reaction consists of the template DNA, RNA polymerase, ribonucleotide triphosphates, RNase inhibitor and buffer containing Mg^{2+} ions.

Large amounts of high quality RNA are often required for a variety of applications. Use of *in vitro* transcription has been reported for RNA structure and function studies such as splicing[1], RNAi experiments in mammalian cells[2], antisense RNA amplification by the "Eberwine method"[3], microarray analysis[4] and for RNA vaccine studies[5]. The technique can also be used for producing radiolabeled and dye labeled probes[6]. Warren, *et al.* recently reported reprogramming of human cells by transfection with *in vitro* transcribed capped RNA[7]. The T7 High Yield RNA Synthesis Kit from New England Biolabs has been designed to synthesize up to 180 µg RNA per 20 µl reaction. RNA of length up to 10kb has been successfully transcribed using this kit. Linearized plasmid DNA, PCR products and synthetic DNA oligonucleotides can be used as templates for transcription as long as they have the T7 promoter sequence upstream of the gene to be transcribed.

Addition of a 5' end cap structure to the RNA is an important process in eukaryotes. It is essential for RNA stability[8], efficient translation[9], nuclear transport[10] and splicing[11]. The process involves addition of a 7-methylguanosine cap at the 5' triphosphate end of the RNA. RNA capping can be carried out post-transcriptionally using capping enzymes or co-transcriptionally using cap analogs. In the enzymatic method, the mRNA is capped using the *Vaccinia* virus capping enzyme[12,13]. The enzyme adds on a 7-methylguanosine cap at the 5' end of the RNA using GTP and S-adenosyl methionine as donors (cap 0 structure). Both methods yield functionally active capped RNA suitable for transfection or other applications[14] such as generating viral genomic RNA for reverse-genetic systems[15] and crystallographic studies of cap binding proteins such as eIF4E[16].

In the method described below, the T7 High Yield RNA Synthesis Kit from NEB is used to synthesize capped and uncapped RNA transcripts of *Gaussia* luciferase (GLuc) and *Cypridina* luciferase (CLuc). A portion of the uncapped GLuc RNA is capped using the Vaccinia Capping System (NEB). A linearized plasmid containing the GLuc or CLuc gene and T7 promoter is used as the template DNA. The transcribed RNA is transfected into HeLa cells and cell culture supernatants are assayed for luciferase activity. Capped CLuc RNA is used as the internal control to normalize GLuc expression.

Video Link

The video component of this article can be found at https://www.jove.com/video/3702/

Protocol

RNases are commonly present on skin, hair, dust, laboratory surfaces, solutions, etc. Wear gloves and clean bench surface, pipettes etc. thoroughly before use to avoid RNase contamination. Use of nuclease- free pipette tips, tubes, glassware and reagents is strongly recommended.

1. *In vitro* Transcription using the T7 High-yield RNA Synthesis Kit

The DNA template for the transcription reaction is a linearized plasmid containing the *Gaussia* luciferase gene and a T7 promoter upstream of the coding sequence (**Figure 1B**).

1. Thaw the components from the T7 High Yield RNA Synthesis kit and keep on ice.
2. For uncapped RNA, set up the reaction at room temperature in the following order:

Component	Volume (µl)	Final

Nuclease free water*	X	
10X Reaction Buffer	2	1X
ATP (100 mM)	2	10mM
CTP (100 mM)	2	10mM
UTP (100 mM)	2	10mM
GTP (100 mM)	2	10mM
Template DNA *	X	1 µg
T7 RNA Polymerase Mix	2	
Total	**20**	

*Add 1 µg DNA and make up the total reaction volume to 20 µl with nuclease free water. The amount of water to be added will vary based on the concentration of the template DNA.

3. For capped RNA: Set up the reaction at room temperature in the following order:

Component	**Volume (µl)**	**Final**
Nuclease free water*	X	
10X Reaction Buffer	2	1X
ATP (100 mM)	2	10mM
CTP (100 mM)	2	10mM
UTP (100 mM)	2	10mM
GTP (20 mM)	2	2mM
3´-0-Me-m^{7}G(5')ppp(5')G cap analog (ARCA) (40mM)	4	8mM
Template DNA*	X	1 µg
T7 RNA Polymerase Mix	2	
Total	**20**	

*Add 1 µg DNA and make up the total reaction volume to 20 µl with nuclease free water. The amount of water to be added will vary based on the concentration of the template DNA.

4. Mix well by vortexing and incubate the reactions at 37 °C for 2 hours in a dry air incubator.
5. Using steps 1.3-1.4, synthesize capped transcripts of *Cypridina* luciferase (CLuc) from a linearized plasmid containing the CLuc gene. This mRNA will be co-transfected with the capped and uncapped GLuc mRNA for normalization of GLuc expression.

2. Removal of Template DNA

The transcription reactions are treated with DNase I to remove the DNA template before proceeding with purification.

1. Add 70 µl nuclease free water to the transcription reactions followed by 10 µl of DNase I reaction buffer.
2. Add 2 µl DNase I to the reactions.
3. Incubate at 37 °C for 15 minutes in a dry air incubator.

3. Column Purification of Capped and Uncapped RNA

1. Purify the capped and uncapped RNA using the MEGAclearKit as per the manufacturer's instructions (any spin column based RNA purification kit may be used).
2. Quantify the RNA using a NanoDrop Spectrophotometer.
3. As per the manufacturer's instructions, assess RNA sample quality using the Agilent RNA 6000 Nano Kit and Agilent 2100 Bioanalyzer.

4. RNA Capping using the *Vaccinia* Capping System

1. Take 10 µg of the purified uncapped GLuc mRNA.

A standard capping reaction can cap up to 10 µg (100nt or larger) RNA. If a larger amount of RNA needs to be capped the reaction can be scaled up accordingly.

2. Increase the volume to 15 µl using nuclease free water.
3. Heat the RNA at 65 °C for 10 minutes to remove secondary structures. Place on ice for 5 minutes.

4. To set up the capping reaction, add 2 µl 10X Capping Buffer, 1 µl GTP (10 mM), 1 µl freshly diluted S-adenosyl methionine (2 mM) and 1 µl (10 units) Vaccinia capping enzyme to the 15 µl denatured RNA.
5. Mix well by gently vortexing and incubate the reaction at 37 °C for 30 minutes in a dry air incubator.

5. Column Purification of Capped RNA

1. Purify the capped RNA using the MEGAclear Kit (any spin column based RNA purification kit may be used).
2. As per the manufacturer's instructions, assess RNA sample quality using the Agilent RNA 6000 Nano Kit and Agilent 2100 Bioanalyzer.

6. HeLa Cell Transfection using *Trans*IT mRNA Transfection Kit

1. Plate HeLa cells in a 48-well plate in 150 µl Dulbecco's Modified Eagle's Medium (DMEM), High Glucose, supplemented with 10% fetal bovine serum (0.7-2.3 × 10^5 cells/well).
2. Incubate for 16-24 hours at 37 °C such that the cells are 60-90% confluent.
3. Dilute the GLuc (capped and uncapped) and CLuc RNA to 100 ng/µl concentration.

Each transfection is done in replicates of 8.

4. Set up 8 eppendorf tubes. To each tube add 1 µl uncapped GLuc RNA, 1 µl capped CLuc RNA (for normalization) and 25 µl of serum free DMEM. Mix well.
5. Set up 8 eppendorf tubes. To each tube add 1 µl capped GLuc RNA (cap analog), 1 µl capped CLuc RNA (for normalization) and 25 µl of serum free DMEM. Mix well.
6. Set up 8 eppendorf tubes. To each tube add 1 µl capped GLuc RNA (*Vaccinia*), 1 µl capped CLuc RNA (for normalization) and 25 µl of serum free DMEM. Mix well.
7. Add 0.2 µl mRNA Boost Reagent to the first set of 8 tubes and mix.
8. Add 0.2 µl *Trans*IT mRNA Reagent to the first set of 8 tubes and mix.
9. Incubate at room temperature for 2-5 minutes. (Do not incubate longer than 5 minutes).
10. Add the transfection mixture drop wise to the wells containing HeLa cell cultures (refer **Figure 3** for plate set-up).
11. Repeat steps 7 through 10 with the 2nd and 3rd sets of 8 tubes.
12. Incubate overnight (16 hours) at 37 °C in 5% CO_2.

7. *Gaussia* Luciferase (GLuc) Assay

Assay the supernatant from each well for GLuc activity using the BioLux *Gaussia* Luciferase Assay Kit as follows:

1. Prepare fresh assay solution by adding 15 µl of BioLux GLuc Substrate to 1.5 ml of BioLux GLuc Assay Buffer (50 µl assay solution required per sample). Prepare enough for samples and for priming luminometer injector (refer to manufacturer's instructions).
2. Mix well by inverting the tube several times (do not vortex).

Luminescence is measured using the Centro LB 960 microplate luminometer from Berthold Technologies.

3. Set the luminometer to 50 µl injection volume and 2-10 seconds integration.
4. Add 10 µl of the cell culture supernatant from each well into a 96-well black plate.
5. Prime the injector with the assay solution and proceed with measurement of luminescence.
6. Values obtained from untransfected wells will be used as negative controls.

8. *Cypridina* Luciferase (CLuc) Assay

Assay the supernatant from each well for CLuc activity using the BioLux *Cypridina* Luciferase Assay Kit as follows:

1. Prepare fresh reconstituted substrate (100x) according to instructions given in the kit manual.
2. Thaw the BioLux *Cypridina* Luciferase Assay Buffer and mix well (protect from light).
3. To prepare the CLuc assay solution, add 15 µl of the reconstituted substrate (100x) to 1.5 ml of BioLux *Cypridina* Luciferase Assay Buffer (50 µl assay solution required per sample). Prepare enough for samples and for priming luminometer injector (refer to manufacturer's instructions).
4. Mix well by inverting the tube several times (do not vortex).
5. Keep the solution at room temperature for 30 minutes (protect from light).

Luminescence is measured using the Centro LB 960 microplate luminometer from Berthold Technologies.

6. Set the luminometer to 50 µl injection, 1-2 seconds delay and 2-10 seconds integration.
7. Add 10 µl of the cell culture supernatant from each well into a 96-well black plate.
8. Prime the injector with the CLuc assay solution and measure luminescence.
9. Values obtained from untransfected wells will be used as negative controls.

9. Representative Results

The T7 High Yield RNA Synthesis Kit can produce up to 180 µg uncapped RNA and 40 to 50 µg of capped RNA per 20 µl reaction. When analyzed on the Agilent 2100 Bioanalyzer, good quality, intact RNA should show a single, sharp peak representing the RNA transcript. A broad peak or multiple peaks indicate RNA degradation. It is also important to verify the size of the RNA. RNA transcripts of a longer length than expected may be due to incomplete digestion of the template plasmid DNA. RNA degradation and lower yield are usually a result of contaminants introduced into the reaction from the template DNA. **Figure 2** is an example of the electropherogram and gel image obtained after running high quality, intact RNA on the Bioanalyzer.

Transfection of HeLa cells with both capped and uncapped GLuc RNA is followed by incubation and assaying cell culture supernatants for luciferase activity. The 5' cap structure is important for protecting the RNA against exonuclease degradation[8] and for promoting translation initiation[9] of the mRNA. Therefore, RNA capping is an essential step for transfection experiments. The co-transcriptional (cap analog) method yields approximately 40 µg RNA that is 80% capped. The protocol utilizing Vaccinia capping enzyme caps up to 10 µg RNA (100 nt or larger) per reaction with nearly 100% efficiency.

Figure 4 depicts the difference in luciferase expression between cultures transfected with capped and uncapped RNA samples. It can be clearly seen that cell cultures transfected with capped RNA show much higher expression of luciferase as compared to those transfected with uncapped RNA (which show no luciferase activity). In addition, **Figure 4** also validates that both capping techniques, using cap analog and using the Vaccinia capping enzyme, successfully produce functional, capped RNA transcripts that can be translated into protein. The two tailed p-value obtained from the t-test was 0.2583 which indicates that there is no significant statistical difference between the luminescence data from the two capping methods.

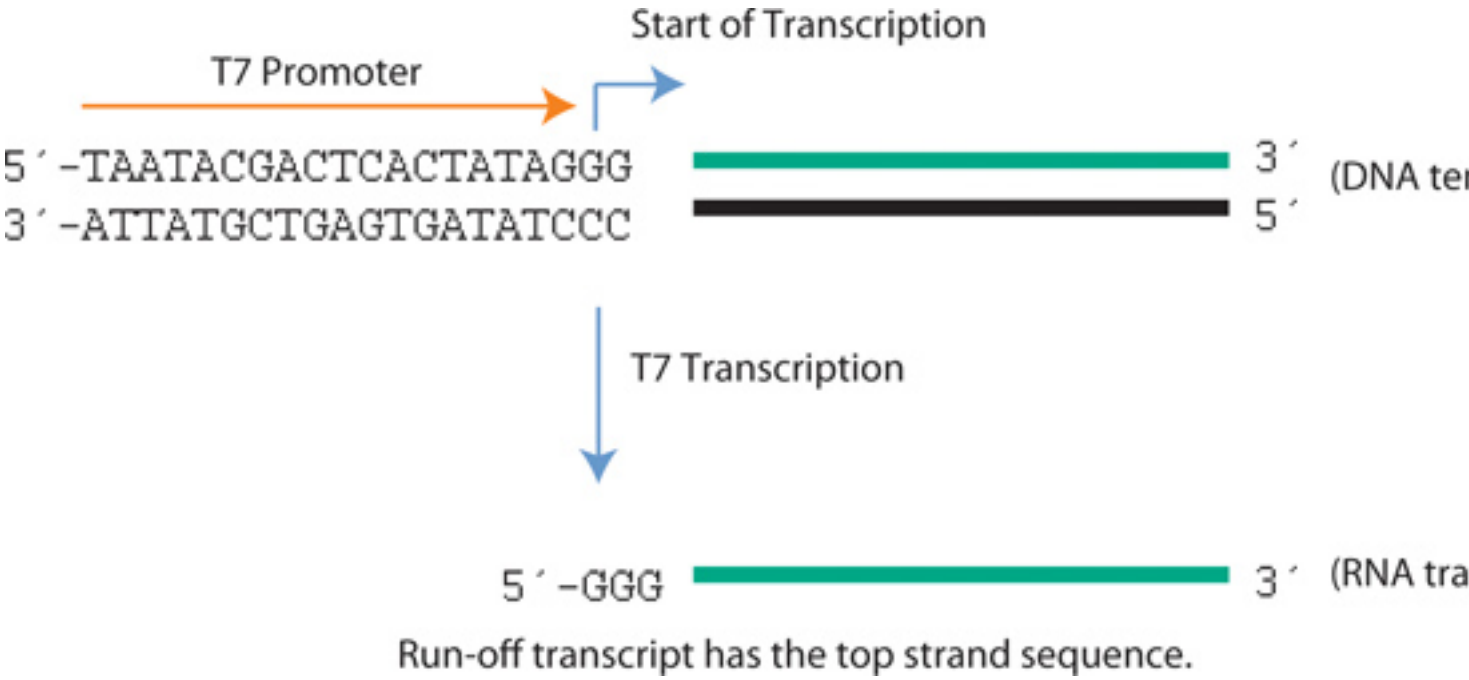

Figure 1A. RNA Synthesis by T7 RNA Polymerase

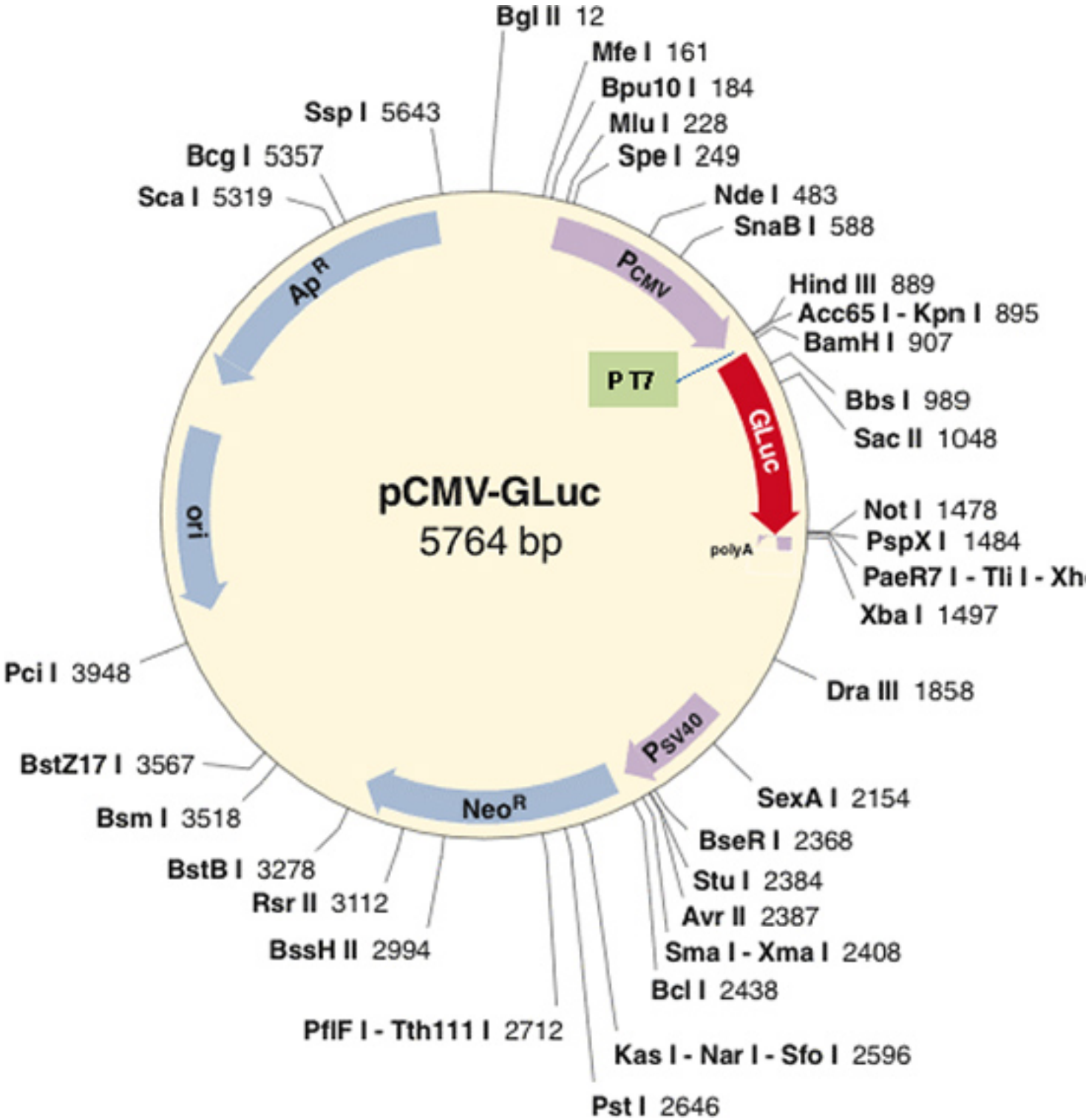

Figure 1B. pCMV-GLuc vector containing GLuc gene and T7 promoter.

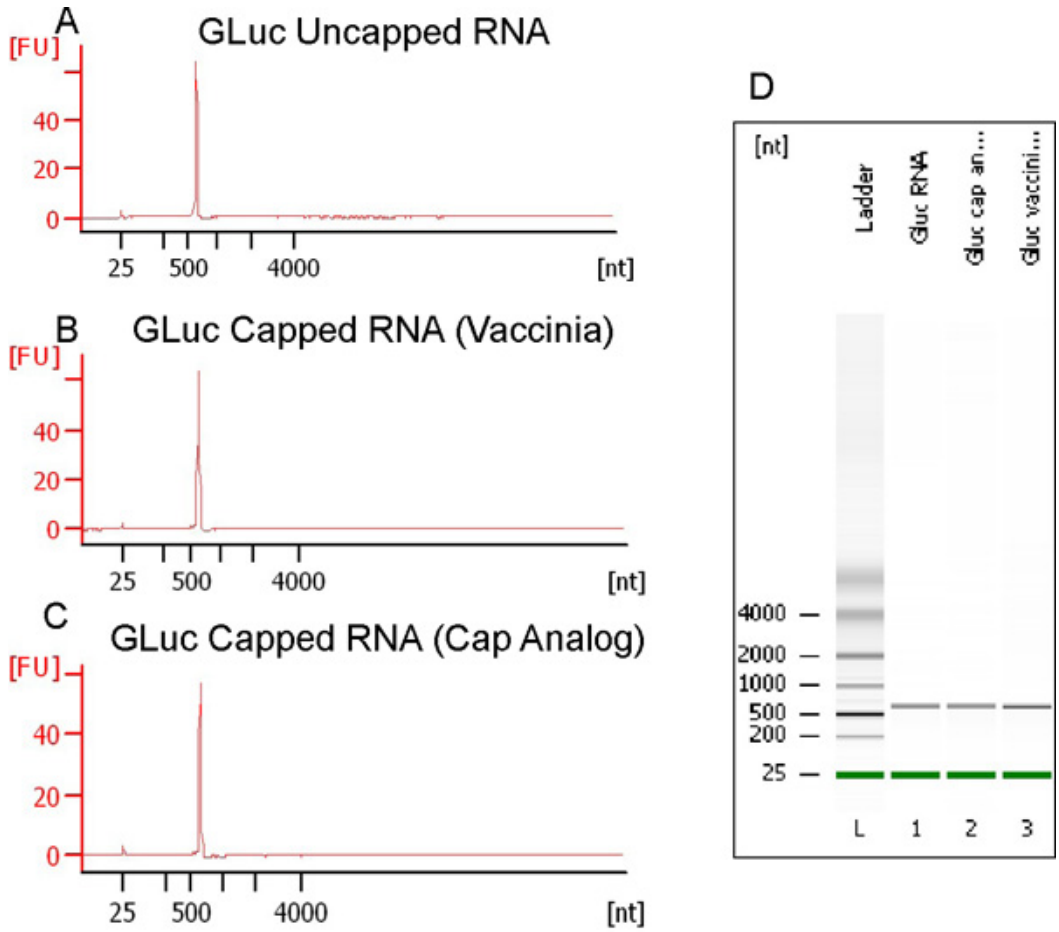

Figure 2. Bioanalyzer electropherograms and gel- image of capped and uncapped RNA . The X-axis is the size of the RNA in nucleotides (nt) and the Y-axis represents fluorescence units (FU). A) Electropherogram of GLuc mRNA (uncapped) after purification. B) Electropherogram of GLuc mRNA (capped using *Vaccinia* capping enzyme). C) Electropherogram of GLuc mRNA (capped co-transcriptionally using cap analog). D) Gel-like image generated by the Bioanalyzer. The 'L' lane is the RNA ladder, Lane 1 is uncapped GLuc mRNA, Lane 2 is GLuc mRNA capped using the cap analog and Lane 3 is the GLuc RNA capped using *Vaccinia* capping enzyme.

	1	2	3	4	5	6	
A	Uncapped GLuc & CLuc	Capped GLuc (Cap Analog) & CLuc	Capped GLuc (Vaccinia) & CLuc	Untransfected Cells	Uncapped GLuc & CLuc	Capped GLuc (Cap Analog) & CLuc	Cap (V
B	Uncapped GLuc & CLuc	Capped GLuc (Cap Analog) & CLuc	Capped GLuc (Vaccinia) & CLuc	Untransfected Cells	Uncapped GLuc & CLuc	Capped GLuc (Cap Analog) & CLuc	Cap (V
C	Uncapped GLuc & CLuc	Capped GLuc (Cap Analog) & CLuc	Capped GLuc (Vaccinia) & CLuc	Untransfected Cells	Uncapped GLuc & CLuc	Capped GLuc (Cap Analog) & CLuc	Cap (V
D	Uncapped GLuc & CLuc	Capped GLuc (Cap Analog) & CLuc	Capped GLuc (Vaccinia) & CLuc	Untransfected Cells	Uncapped GLuc & CLuc	Capped GLuc (Cap Analog) & CLuc	Cap (V

Figure 3. Example of plate set-up for HeLa cell transfection . Each well consists of plated HeLa cells that are 60-90% confluent. The transfection mix consisting of the appropriate GLuc RNA (uncapped, capped with cap analog or capped with *Vaccinia* capping enzyme), capped CLuc RNA (for normalization), mRNA Boost Reagent and *Trans*IT mRNA Reagent is added to each well. Each transfection is done in replicates of 8 (same colored wells in the figure).

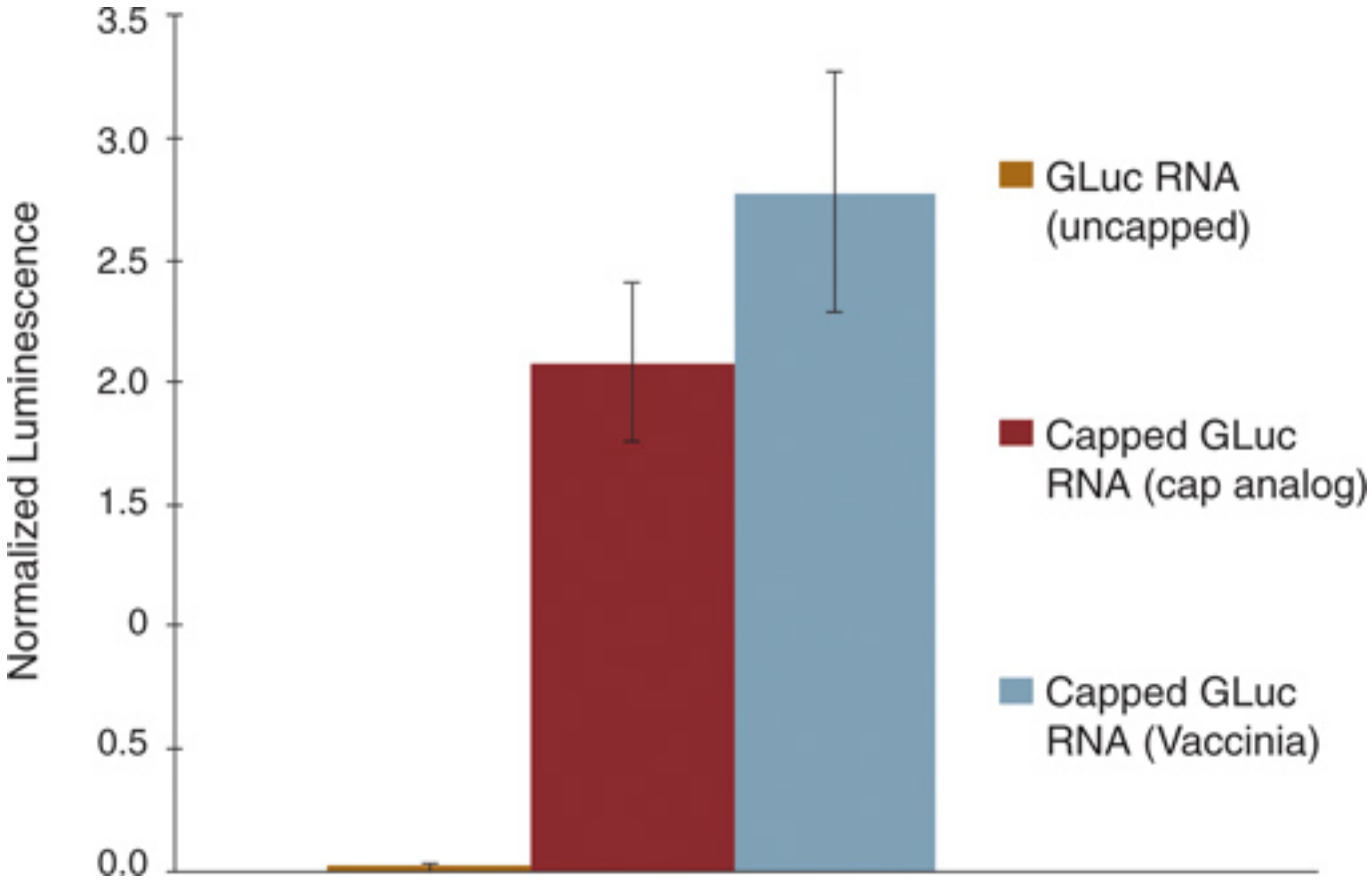

Figure 4.GLuc expression in HeLa cells. Purified capped and uncapped GLuc mRNA was transfected into HeLa cells and incubated overnight (16 hrs) at 37 °C. Cell culture supernatants from each well were assayed for GLuc and CLuc activity and luminescence values were recorded. The GLuc luminescence values were normalized to the luminescence values of capped CLuc RNA.

$$\text{Normalized GLuc Luminescence*} = \frac{\text{GLuc luminescence value (RLU)}}{\text{CLuc luminescence value (RLU)}}$$

* Luminescence from untransfected cells was subtracted from GLuc and CLuc values before normalization.

Trademarks

BioLux is a trademark of New England Biolabs, Inc.

*Trans*IT is a registered trademark of Mirus Bio LLC.

MEGAclear is a trademark of Ambion.

Discussion

In vitro transcription is a useful method for obtaining high yields of RNA for a variety of applications. The major advantage of using the T7 High Yield RNA Synthesis Kit is that its formulation has been optimized to achieve high yields of RNA. In addition, the reagents provided in the kit are free of contaminating nucleases, resulting in synthesis of high quality, intact RNA transcripts. The kit is designed for high stability and flexibility such that it can be used for synthesizing mRNA, dye labeled RNA, high specific activity radiolabeled probes and capped RNA. The kit manual includes a list of specific protocols and additional materials required for each of these applications. It also includes technical information on using PCR products and synthetic DNA oligonucleotides as templates for transcription. It is important to note that the transcription kit can only utilize DNA templates containing a T7 promoter.

The method described in this article demonstrates post-transcriptional RNA capping using Vaccinia capping enzyme, as well as co-transcriptional capping using RNA cap structure analog. We have shown that both methods synthesize capped RNA that is functionally active post-transfection. The 5' cap structure improves stability of the RNA and translation efficiency, and hence is important for microinjection[11] and transfection[7] experiments. The co-transcriptional capping method generates 40-50 µg of ~80% capped RNA. It is important to note that using 3'-0-Me-m^7G(5')ppp(5')G RNA cap analog (ARCA), which is blocked at the 3'-hydroxyl of the m^7G ensures incorporation of the cap in the correct orientation[17]. To cap larger quantities of RNA it is possible to scale up the standard reactions for both capping methods. However, using the Vaccinia Capping System would be a more feasible option cost-wise since co-transcriptional capping utilizes cap analog, which is a relatively expensive component. The Vaccinia Capping System is also a better method since it has capping efficiency of almost 100%.

The RNA capping methods described above synthesize RNA with a cap 0 structure at the 5' end. Cap 1 structure involves additional methylation at the 2'-O position of the ribose sugar of the first nucleotide at the ' end of the RNA. The cap 1 structure has been reported to enhance RNA translation efficiency[18] and can be incorporated by using 2'-O- Methyltransferase[20, 21].

The method described in this paper can be used to transcribe, cap and transfect any desired mRNA. The luciferases GLuc and CLuc were specifically used in this protocol due to their many advantages. They are directly secreted into the cell medium, therefore avoiding need for cell lysis. Also, they generate high bioluminescent signal intensity, and the activity assays are highly sensitive and easy to perform. It is important to note that the GLuc expression in the cells can be influenced by various factors other than the functionality of the RNA itself. Some examples are pipetting errors, varying cell numbers per well, transfection efficiency etc. Data normalization is used to account for these factors. In our protocol, we co-transfect CLuc RNA as an internal control with the GLuc RNA. When GLuc luminescence is divided by the CLuc control values, it gets normalized for the errors and variability in transfection[19].

Disclosures

All authors are employees of New England Biolabs, Inc.

Acknowledgements

Dongxian Yue, Brett Robb, George Tzertzinis, Tanya Bhatia, Breton Hornblower.

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