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TITLE:

In vitro Transcribed RNA-based Luciferase Reporter Assay to Study Translation Regulation in Poxvirus-Infected Cells

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KEYWORDS:

In vitro Transcription, Luciferase assay, Vaccinia virus, Poxvirus, Translation, 5'-UTR, 5'-poly(A) leader

SUMMARY:

We present a protocol to study mRNA translation regulation in poxvirus-infected cells using In vitro Transcribed RNA-based luciferase reporter assay. The assay can be used for studying translation regulation by *cis*-elements of an mRNA, including 5'-untranslated region (UTR) and 3'-UTR. Different translation initiation modes can also be examined using this method.

ABSTRACT:

Every poxvirus mRNA transcribed after viral DNA replication has an evolutionarily conserved, non-templated 5'-poly(A) leader in the 5'-UTR. To dissect the role of 5'-poly(A) leader in mRNA translation during poxvirus infection we developed an In vitro transcribed RNA-based luciferase reporter assay. This reporter assay comprises of four core steps: (1) PCR to amplify the DNA template for In vitro transcription; (2) In vitro transcription to generate mRNA using T7 RNA polymerase; (3) Transfection to introduce In vitro transcribed mRNA into cells; (4) Detection of luciferase activity as the indicator of translation. The RNA-based luciferase reporter assay described here circumvents issues of plasmid replication in poxvirus-infected cells and cryptic transcription from the plasmid. This protocol can be used to determine translation regulation by *cis*-elements in an mRNA including 5'-UTR and 3'-UTR in systems other than poxvirus-infected cells. Moreover, different modes of translation initiation like cap-dependent, cap-independent, re-initiation, and internal initiation can be investigated using this method.

INTRODUCTION:

According to the central dogma, genetic information flows from DNA to RNA and then finally to protein[1,2]. This flow of genetic information is highly regulated at many levels including mRNA

translation[3,4]. Development of reporter assays to measure regulation of gene expression will facilitate understanding of regulatory mechanisms involved in this process. Here we describe a protocol to study mRNA translation using an In vitro transcribed RNA-based luciferase reporter assay in poxvirus-infected cells.

Poxviruses comprise many highly dangerous human and animal pathogens[5]. Like all other viruses, poxviruses exclusively rely on host cell machinery for protein synthesis[6–8]. To efficiently synthesize viral proteins, viruses evolved many strategies to hijack cellular translational machinery to redirect it for translation of viral mRNAs[7,8]. One commonly employed mechanism by viruses is to use *cis*-acting elements in their transcripts. Notable examples include the Internal Ribosome Entry Site (IRES) and cap-independent translation enhancer (CITE)[9–11]. These *cis*-elements render the viral transcripts a translational advantage by attracting translational machinery via diverse mechanisms[12–14]. Over 100 poxvirus mRNAs have an evolutionarily conserved *cis*-acting element in the 5'-untranslated region (5'-UTR): a 5'-poly(A) leader at the very 5' ends of these mRNAs[15,16]. The lengths of these 5'-poly(A) leaders are heterogeneous and are generated by slippage of the poxvirus-encoded RNA polymerase during transcription[17,18]. We, and others, recently discovered that the 5'-poly(A) leader confers a translation advantage to an mRNA in cells infected with vaccinia virus (VACV), the prototypic member of poxviruses[19,20].

The In vitro transcribed RNA-based luciferase reporter assay was initially developed to understand the role of 5'-poly(A) leader in mRNA translation during poxvirus infection[19,21]. Although plasmid DNA-based luciferase reporter assays have been widely used, there are several drawbacks that will complicate the result interpretation in poxvirus-infected cells. First, plasmids are able to replicate in VACV-infected cells[22]. Second, cryptic transcription often occurs from plasmid DNA[18,23,24]. Third, VACV promoter-driven transcription generates poly(A)-leader of heterogeneous lengths consequently making it difficult to control the poly(A)-leader length in some experiments[18]. An In vitro transcribed RNA-based luciferase reporter assay circumvents these issues and the data interpretation is straightforward.

There are four key steps in this method: (1) polymerase chain reaction (PCR) to generate the DNA template for In vitro transcription; (2) In vitro transcription to generate mRNA; (3) transfection to deliver mRNA into cells; and (4) detection of luciferase activity as indicator of translation (**Figure 1**). The resulting PCR amplicon contains the following elements in 5' to 3' direction: T7-Promoter, poly(A) leader or desired 5'-UTR sequence, firefly luciferase open reading frame (ORF) followed by a poly(A) tail. PCR amplicon is used as the template to synthesize mRNA by In vitro transcription using T7 polymerase. During In vitro transcription, m^7G cap or other cap analog is incorporated in newly synthesized mRNA. The capped transcripts are transfected into uninfected or VACV-infected cells. The cell lysate is collected at the desired time after transfection to measure luciferase activities that indicate protein production from transfected mRNA. This reporter assay can be used to study translation regulation by *cis*-element present in 5'-UTR, 3'-UTR or other regions of an mRNA. Furthermore, the In vitro transcribed RNA-based assay can be used to study different mechanisms of translation initiation including cap-dependent initiation, cap-independent initiation, re-initiation and internal initiation like IRES.

PROTOCOL:

1. Preparation of DNA template by PCR for In vitro **transcription**

1.1. To prepare the DNA template by PCR, design primers. When designing primers consider crucial characteristics like primer length, annealing temperature (T_m), GC content, 3' end with G or C etc.

NOTE: Discussed in detail in these literature[25–27].

1.2. Design primers to generate PCR amplicon containing the following elements in 5' to 3' direction: T7-Promoter, poly(A) leader, firefly luciferase ORF and a poly(A) tail referred hereafter to as T7_12A-Fluc. Design primers (Forward and Reverse) to encompass all the additional elements not present in the template DNA (**Figure 2A**).

NOTE: The sequence of all elements can be found in **Table 1.**

1.3. Include several extra nucleotides in forward primer (5'-3')[28], followed by T7-promoter, poly(A) leader or desired 5'-UTR sequence and approximately 20 nucleotides, adjust based on T_m, corresponding to the 5' end of the reporter gene's ORF. Ensure the corresponding region in the primer is identical to the sense strand (+ strand) of the gene.

NOTE: For long 5'-UTR, synthesize two DNA fragments: one with T7 promoter followed by long 5'-UTR and second with reporter gene's ORF. Join these two fragments using overlap extension PCR[29].

1.4. Design reverse primer (5'-3') to include poly(A) tail and approximately 20 nucleotides, adjust based on T_m, corresponding to the 3' end of the reporter gene's ORF. Ensure the corresponding region in the primer is identical to the anti-sense strand (- strand) of the gene and an in-frame stop codon is present before the poly(A) tail.

NOTE: The desired length of A's in a poly(A) leader or poly(A) tail can be customized in the primers. For example, to add 50 A's in the poly(A) tail, the reverse primer should entail 50 T's. Similarly, to add 20 A's in the poly(A) leader, the forward primer should entail 20 A's.

1.5. For internal control, design another set of primers containing the following elements in 5' to 3' direction: T7 Promoter, a random 5'-UTR coding sequence containing Kozak sequence, *Renilla* luciferase ORF and poly(A) tail referred hereafter to as T7_Kozak-Rluc.

1.6. In a PCR tube, add the reagents in the following order: DNase free water, 2x high-fidelity DNA polymerase, primers, and sequence confirmed luciferase template DNA (**Table 2**).

NOTE: Amounts of individual components in the mixture should be adjusted according to the reaction volume.

1.7. Use a standard 3-step (Denaturation, Annealing, Extension) PCR cycle to generate a DNA template as shown in **Table 3**.

NOTE: Annealing temperature X °C depends on the primer set being used and extension time T minutes depend on the PCR amplicon size and DNA polymerase used.

1.8. Detect the PCR product by running 5-10% of PCR reaction in 1% agarose Tris-acetate-EDTA (TAE) gel electrophoresis (containing 0.1µg/ml ethidium bromide) along with commercially available molecular weight standard. Visualize the gel under a UV illuminator to determine the size of the PCR product.

1.9. After determining the correct size of the PCR product, ~1.7 kb for T7_12A-Fluc and ~1.0 kb for T7_Kozak-Rluc, purify it using a commercially available PCR purification kit. Elute the DNA using 100 µL nuclease free water (**Figure 2B**).

1.10. Once purified, check the concentration of the DNA using a spectrophotometer and determine the A260/A280 ratio (~1.8-2.0 is acceptable).

1.11. Store purified DNA at -20 °C or use for In vitro transcription immediately.

2. Generate mRNA by In vitro **transcription**

2.1. Synthesize RNA from the PCR product *in vitro*, using an In vitro transcription kit (**Figure 3A**).

NOTE: T7_12A-Fluc and T7_Kozak-Rluc DNA templates are used to synthesize 12A-Fluc and Kozak-Rluc mRNAs, respectively.

2.1.1. To do this, take a microcentrifuge tube and add the reagents in the following order: DNase-RNase free water, NTP Buffer Mix, Cap Analog, Template PCR Product, T7-RNA polymerase Mix (**Table 4**).

NOTE: Other capping systems can also be used to cap RNA sequentially after In vitro transcription following the manufacturer's instruction.

2.1.2. Mix thoroughly and incubate at 37 °C for 2 h.

2.1.3. Proceed to the purification of the synthesized RNA using an RNA purification kit.

2.2. Run purified RNA in 1.5% agarose Tris-borate-EDTA (TBE) gel (containing 0.5 µg/mL ethidium bromide) to check the RNA. Visualize the gel under a UV illuminator (**Figure 3B**).

2.3. Check the concentration of the RNA using a spectrophotometer and determine the A260/A280 ratio (~1.8-2.0 is acceptable).

2.4. Aliquot the purified RNA and store at -80 °C.

3. Transfect mRNA to cells

3.1. Seed HeLa cells in a 24-well plate (to be approx. ~80-90% confluent next day) and incubate overnight in an incubator at 37 °C with 5% CO_2.

3.2. Infect HeLa cells with vaccinia virus (VACV) at a Multiplicity of Infection (MOI) of 5 or keep uninfected HeLa cells for comparison.

NOTE: MOI is the number of infectious viral particles per cell.
MOI of X= {[(Number of cells * X) / Virus Titer] * 1000} µl of virus per 1 mL medium.

3.3. After desired hours post infection (hpi) (in this experiment at 10-12 hpi), transfect mRNA (500 ng of total mRNA per well of 24-well plates) using a cationic lipid transfection reagent as shown in **Figure 3C**.

3.3.1. For one well of a 24-well plate, mix 480 ng of 12A sequence bearing firefly luciferase (12A-Fluc) mRNA and 20 ng of Kozak sequence bearing *Renilla* luciferase (Kozak-Rluc) mRNA in one microcentrifuge tube. In another microcentrifuge tube add 1.1 µL of cationic lipid transfection reagent.

3.3.2. Add 55 µL of reduced serum medium in both tubes. Mix and incubate at room temperature for 5 min.

3.3.3. After 5 min of incubation, add 55 µL cationic lipid transfection reagent containing reduced serum medium in mRNA containing tube.

3.3.4. Mix gently but thoroughly, and incubate at room temperature for 15 min.

3.3.5. During the incubation, remove the cell culture medium and add 400 µL of reduced serum medium per well of 24-well plates.

3.3.6. After incubation, add 100 µL of the mixture dropwise and evenly to one well of 24-well plates.

4. Measure luciferase activities

4.1. Five-hours post-co-transfection of 12A-Fluc and Kozak-Rluc mRNA, measure luciferase activity using a luciferase assay system capable of performing two reporter assays (e.g., Dual Luciferase Reporter assay kit).

4.2 Remove the reduced serum medium and lyse the cells by adding 150 µL 1x lysis buffer, a

component of the luciferase assay kit.

4.3. After 10 min incubation at room temperature, collect the lysate by scrapping the cells and transfer to a microcentrifuge tube.

4.4. Centrifuge the lysate at 12,000 x g for 10 min at 4 °C to pellet cell debris.

4.5. Add 30 µL of supernatant in opaque-walled 96 well white assay plate with a solid bottom.

4.6. Measure the dual luminescence using the luciferase assay kit and a multimode plate reader luminometer.

4.7. Perform the measurement using kinetics function (on a per-well basis) using the settings described in **Table 5**.

NOTE: The reading can also be taken using manual luminometer. Add an equal volume of lysate and substrate for Fluc in a cuvette. Wait for 2 s and measure for 10 s using luminometer. Following Fluc measurement, quickly take out cuvette from luminometer and add an equal volume of the substrate for Rluc manually. Again, wait for 2 s and measure for 10 s using luminometer.

4.8. Export the luminescence reading data into a desirable file format.

4.9. Determine relative translation rate from 12A-Fluc mRNA in uninfected and VACV infected HeLa cells by dividing Fluc value by internal control Rluc value.

NOTE: **Supplementary Figure 1** shows the step-by-step analysis of raw data to get relative Fluc activity.

REPRESENTATIVE RESULTS:

The four steps of In vitro transcribed RNA-based luciferase reporter assay: PCR to generate DNA template for In vitro transcription, In vitro transcription to generate mRNA, mRNA transfection, and luciferase measurement, can be seen in the schematic diagram (**Figure 1**). Designing of primers for both DNA templates (Fluc and Rluc) and the general scheme of overhang extension PCR is illustrated in the schematic (**Figure 2A**). After PCR, the correct sized PCR product was detected by TAE agarose gel electrophoresis (**Figure 2B**). Subsequently, the PCR product is used as the template to synthesize RNA In vitro (**Figure 3A**), which is purified and run in TBE gel electrophoresis to verify the size (**Figure 3B**). The purified and verified mRNA is transfected into cells using cationic lipid transfection reagent (**Figure 3C**). Primers used in this protocol are listed in **Table 6**.

The In vitro transcribed RNA-based luciferase reporter assay was developed to understand the role of 5'-poly(A) leader in mRNA translation during poxvirus infection. Using this assay, we tested the translation efficiency of a Fluc mRNA that contains a 5'-poly(A) leader (12nt) in uninfected

and VACV-infected cells. The Fluc value was normalized using Rluc value in both uninfected and VACV-infected cells to determine the relative Fluc activity (i.e. Fluc activity/Rluc activity) (**Figure 4A**). The division of Fluc by Rluc normalized the transfection efficiency and RNA stability in a particular well. Using this analysis approach, we determined that a 5'-poly(A) leader containing mRNA has a translational advantage during VACV infection (**Figure 4B**). The advantage in infected cells was not due to differential transfection efficiency or mRNA stability as the RNA level was similar in uninfected and VACV infected cells 5 hours post mRNA transfection[19].

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic of the experimental procedure. PCR is used to generate a DNA template with the desired elements. mRNA encoding a luciferase reporter gene is synthesized In vitro using a T7 RNA polymerase-based system. A Firefly luciferase (Fluc) mRNA is co-transfected with a Renilla luciferase (Rluc) mRNA into uninfected or VACV-infected cells. Luciferase activities are measured using a luminometer with dual luciferase capability.

Figure 2: Primer design and PCR-based DNA amplification. (A) The forward primer is synthesized to include an 8nt random sequence, T7 promoter followed by a desired 5'-UTR and part of the 5' end of the luciferase reporter gene, while the reverse primer includes a T-tract to generate poly(A) tail and the 3' end of the luciferase reporter gene. By overhang extension PCR using a plasmid template containing a luciferase gene, a DNA template is generated. **(B)** DNA band of the desired size from PCR reaction was detected using 1% agarose TAE gel electrophoresis.

Figure 3: mRNA synthesis and transfection. (A) Schematic of In vitro transcription. DNA amplified by PCR containing the luciferase gene downstream from the 5'-UTR of interest and the T7 promoter is used as the template. The T7 RNA polymerase is recruited to the promoter and adds ribonucleotides, shown in white, from 5' to 3' direction. Once mRNA is 25-30nt long, m^7G cap is added using an anti-reverse cap analog, ARCA. **(B)** RNA bands from In vitro transcription ware detected using 1.5% agarose TBE gel electrophoresis. **(C)** Schematic demonstrating the transfection of reporter mRNA into cells. Medium containing either the reporter mRNA or cationic lipid transfection reagent in separate tubes is allowed to equilibrate at room temperature for 5 min. The solutions are then mixed followed by incubation at room temperature for 15 min after which the RNA/transfection reagent mixture is added into cells in culture plates.

Figure 4: Increased translational efficiency of mRNA containing a 5'-poly(A) leader. (A) Fluc mRNA containing a poly(A) leader in the 5'-UTR and Rluc mRNA with the Kozak consensus sequence in the 5'-UTR are co-transfected into cells. **(B)** Fluc mRNA with 5'-poly(A) leader was transfected in uninfected and VACV-infected cells along with Rluc mRNA. Five-hours post-transfection, luciferase activity was measured using a luminometer. Rluc normalized Fluc activity is represented in uninfected and VACV-infected cells. Error bars indicate the standard deviations (SD) of at least three repeats. Student's t-test was used to determine P-values; ***P-value < 0.001.

Table 1. Sequences used in the method: The table contains the sequences of the T7 promoter, poly(A) leader, Kozak sequence, poly(A) tail.

Table 2. PCR reaction: The order and the volume of components added in the PCR reaction.

Table 3. PCR Program: The steps for PCR program along with temperature, time and cycle.

Table 4. In vitro **transcription reaction**: The order and the volume of components added for In vitro transcription reaction.

Table 5. Luciferase Measurement Settings: The steps for luciferase measurement with the recommended volume or time.

Table 6. Primers: The primers used in this method with complete sequences.

Supplementary Figure 1. Analysis of raw data: Steps for analyzing raw data to get normalized data.

DISCUSSION:

All four-core steps are critical to the success of the In vitro transcribed RNA-based luciferase reporter assay. Special attention should be given to primer design, especially for the T7 promoter sequence. T7 RNA polymerase starts transcription from the underlined first G (**G**GG-5'-UTR-AUG-) in T7 promoter added before the 5'-UTR sequence. Although the transcription start site (TSS) starts from the first G at the 5' end, decreasing the number of G's less than three in T7 promoter region decreased the RNA yield/output from In vitro transcription. During the experiment, we observed that gel purified DNA product was not the best for In vitro transcription as both yield and quality of RNA were lower. We only ran 5-10% of the PCR reaction in 1% agarose gel electrophoresis to determine the size and purified the rest 90-95%, using a PCR purification kit, to be used for In vitro transcription. In the case of non-specific amplification from PCR, cutting the desired sized band from gel and using gel-purified DNA fragment is recommended. As the yield might be low, we suggest increasing the reaction volume for the In vitro transcription reaction. Similar to other transfection-based methods, DNA/RNA may stimulate DNA/RNA sensing pathways that may globally or selectively suppress translation. Therefore, data should be interpreted with cautions, although we did not experience problems potentially caused by this issue in our experiments.

The proposed method is suitable for use in different model systems with some modifications like the method of mRNA delivery, internal control to be used, a suitable time for translation, sample preparation and analysis of data. The main limitation of this method is that it is a reporter assay to quickly test translation regulation by *cis* elements that do not completely reflect physiological conditions. Therefore, this method should be corroborated by other complementary experiments, if possible.

Compared to DNA modification, the roles of RNA modifications are less well understood.

However, with the discovery of enzymes that write, read and erase RNA modifications[30–35], it is now possible to study the influence of RNA modification in gene expression[31–36]. The In vitro transcribed RNA-based luciferase reporter assay may be modified to incorporate different RNA modifications and used to test their effects on RNA translation. For example, this method can incorporate different cap analogs that have various modifications[30,31]. Additionally, supplementing an internal RNA modifying enzyme during or after In vitro transcription can possibly incorporate internal RNA modification. Addition of a modification to cap 0, cap 1, and an internal RNA modification will provide a tool to study the roles of these RNA modifications in translation.

The In vitro transcribed RNA-based luciferase reporter assay has great potential and broad application in understanding basic biology about RNA translation. Different mechanisms for the initiation of translation, including cap-dependent initiation, cap-independent initiation, re-initiation and internal initiation such as IRES can be studied using this method. On top of these advantages, this assay can be employed to test translation regulation by *cis*-elements at 5'-UTR and 3'-UTR in an mRNA. The described protocol uses the PCR product, which provides the advantage to avoid lengthy cloning and quickly examine the effects of RNA elements on translation. To minimize potential errors during PCR, high fidelity polymerase and low PCR cycle number should be used. Alternatively, if a template is used frequently, the desired 5-'UTR and luciferase ORF can be cloned into a plasmid as the template of In vitro transcription. Together, the protocol consolidates transcription and mRNA capping in a single reaction and utilizes conventional transfection and analysis that make In vitro transcribed RNA-based luciferase reporter assay a user-friendly, quick, and straightforward method to study mechanisms of mRNA translation.

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DISCLOSURES:

The authors would like to declare no competing financial interest.

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Figure 1

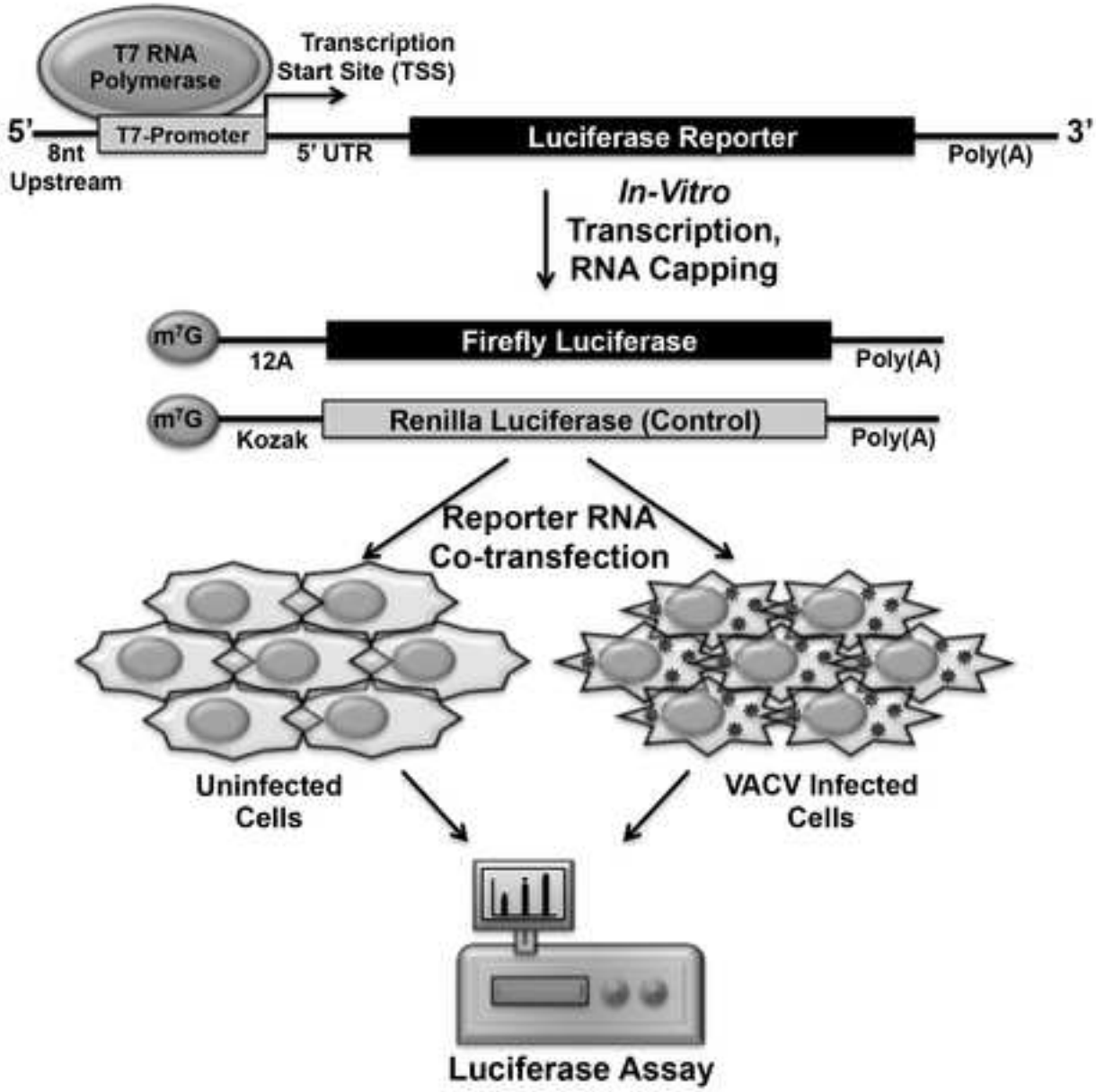

Figure 2

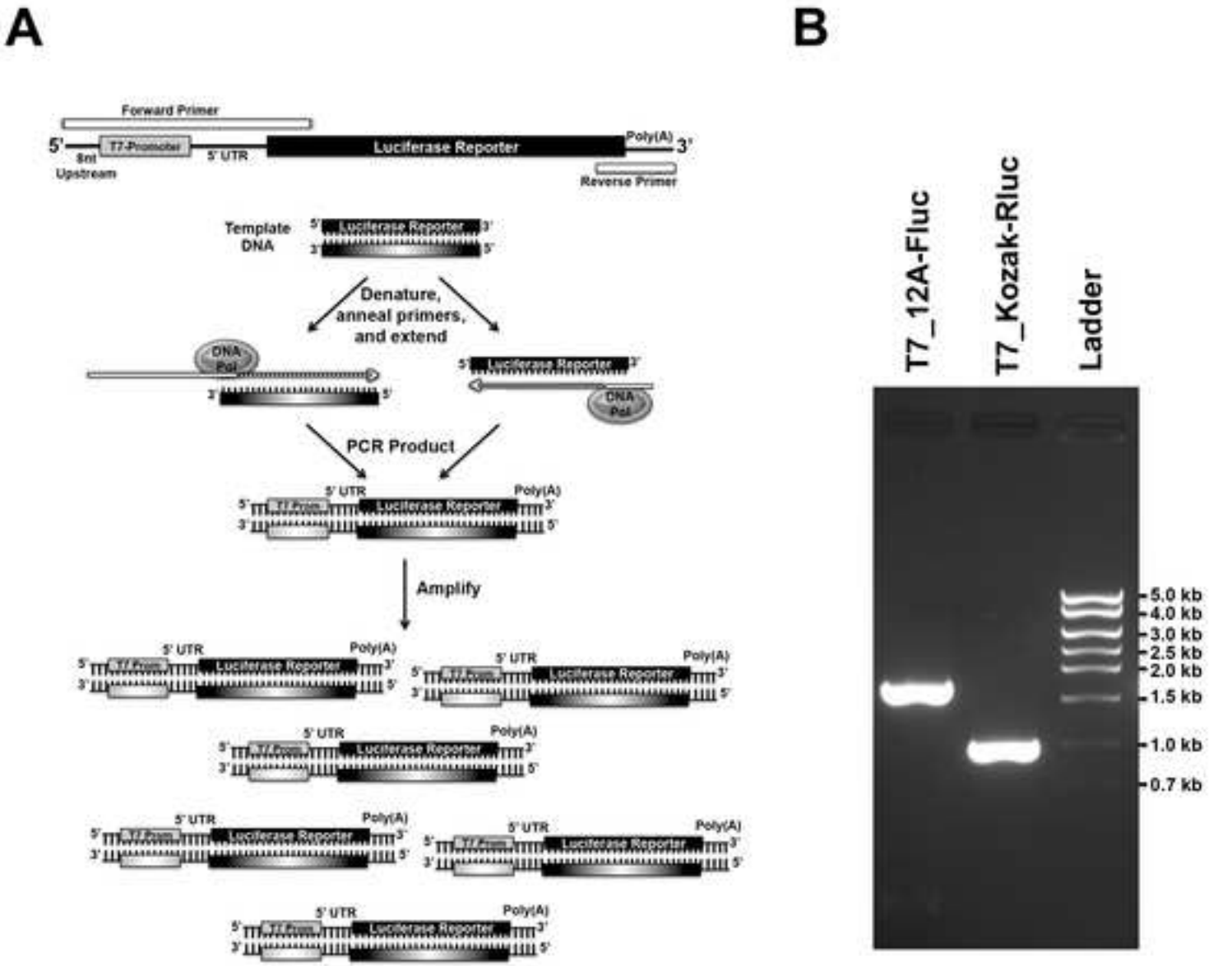

Figure 3

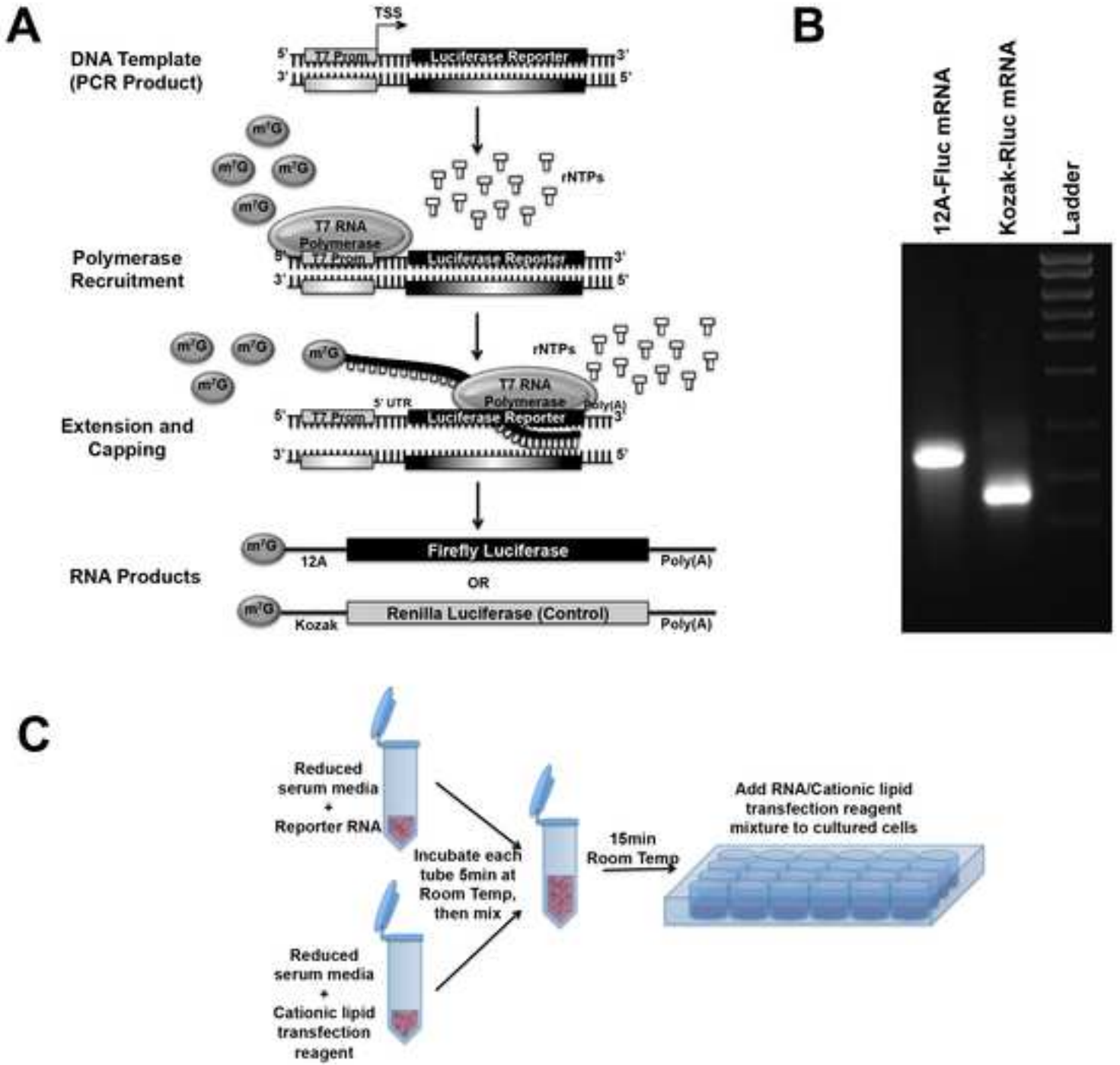

Figure 4

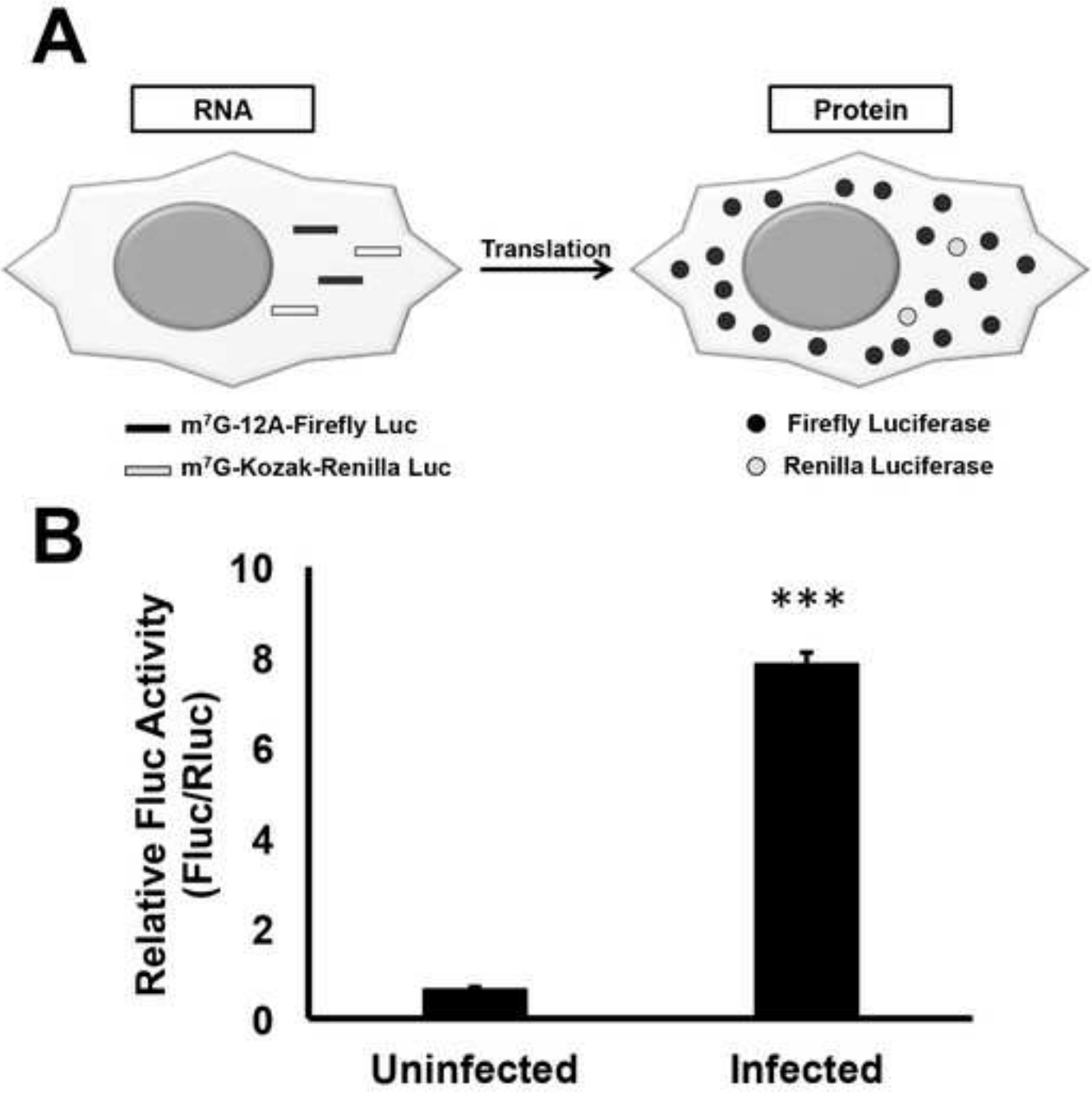

Table 1. Sequences used in the method

Elements	Sequence
T7 Promoter	TAATACGACTCACTATAGGG
Poly(A) leader	AAAAAAAAAAA, ranging from 3 to 51 As
Kozak sequence	GCCACC
Poly(A) tail	AAAAAAAAAAAAAAAAAAAAAAAAAAAAA……

Table 2. PCR reaction

Components	Volume
DNase free water:	38 µL
2X High-fidelity DNA polymerase Master mix:	50 µL
Forward Primer (10 µM):	4 µl
Reverse Primer (10 µM):	4 µl
Luciferase DNA Template (1-10 ng/µL):	4 µl
Total:	**100 µL**

Source for Fluc DNA template is pGL3-Fluc plasmid
Source for Rluc DNA template is pRL-Rluc plasmid

Table 3. PCR Program

Step	emperatu	Time	Cycle
Initial denaturation	95 °C	2 min	(1x Cycle)
Denaturation	95 °C:	15 s	(25x Cycle)
Annealing	**X °C**:	30 s	
Extension	72 °C:	**T min**	
Final Extension	72 °C:	7 min	(1x Cycle)
Hold	4 °C:	∞	

Table 4. *In-vitro* transcription reaction

Components	Volume	
DNase-RNase free water:	up to 20 µL	
NTP Buffer Mix (20 mM of each rNTP):	2 µL	
Cap Analog (40 mM):	4 µL	
Template PCR Product (400 ng)*:	**X** µL	(PCR Product Concentration dependent)
T7-RNA polymerase Mix:	2 µL	
Total:	**20 µL**	

*T7_12A-Fluc and T7_Kozak-Rluc template used to synthesize 12A-Fluc and Kozak-Rluc mRNA, respectively.

Table 5. Luciferase Measurement Settings

Steps	Volume/Time
Inject Luciferase Assay Substrate (Fluc):	30 µL
Wait / Incubation time:	2 sec
Luminescence Measurement (Fluc):	10 s
Stop & Glo Substrate (Rluc):	30 µL
Wait / Incubation time:	2 s
Luminescence Measurement (Rluc):	10 s

Table 6. Primers

Primers	Sequence
T7-12A-Flu	ATCGACGATAATACGACTCACTATAGGGaaaaaaaaaaaaATGGAAGACGCCAAAAACATAAAG
T7-12A-Flu	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTACACGGCGATCTTTCCGC
T7-Kozak-R	ATCGACGATAATACGACTCACTATAGGGatcgtagccaccATGACTTCGAAAGTTTATGATC
T7-Kozak-R	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTATTGTTCATTTTTGAGAACTCGCTC

Table of Materials:

Name of Material/ Equipment	Company
2X-Q5 Master mix	New England Biolabs
3´-O-Me-m7G(5')ppp(5')G RNA Cap Structure Analog	New England Biolabs
Corning 96 Well Half-Area white flat bottom polystyrene microplate	Corning
Dual-Luciferase Reporter Assay System	Promega
E.Z.N.A. Cycle Pure Kit	OMEGA BIO-TEK
GloMax Navigator Microplate Luminometer	Promega
HiScribe T7 Quick High Yield RNA synthesis Kit	New England Biolabs
Lipofectamine 2000	Thermo Fisher Scientific
NanoDrop2000	Thermo Fisher Scientific
Opti-MEM	Thermo Fisher Scientific
Purelink RNA Mini Kit	Thermo Fisher Scientific
Vaccinia Capping System	New England Biolabs

Catalog Number

M0492

S1411L

3693
E1960
D6492
GM2010
E2050S
11668019
ND-2000
31985070
12183018A
M2080

Comments/Description

High-Fidelity DNA Polymerase used in PCR

Anti reverse Cap analog or ARCA

Opaque walled 96 well white plate with solid bottom
Dual-Luciferase Assay Kit (DLAK)
PCR purification kit
Referred as multimode plate reader luminometer
In-Vitro transcription kit
Cationic lipid transfection reagent
Used to measure DNA and RNA concentration
Reduced serum media
RNA purification kit
Capping system

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Author(s):	Pragyesh Dhungel, Fernando Cantu, Candy Hernandez, Zhilong Yang

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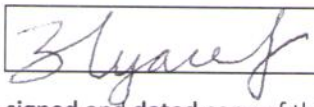
Signature:	
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Response to reviewers (*Revision for manuscript number JoVE59626****):***

We thank the editor and reviewers for their meticulous reading of the manuscript and thoughtful comments. We have modified the manuscript accordingly. Below are the point-by-point responses.

Editorial comments:

Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Response: We greatly appreciate the editor's critical comments. We are certain this process has made our manuscript better. We have proofread the manuscript and ensured, to the best of our knowledge, that there are no spelling or grammar issues.

2. Please define all abbreviations before use.

Response: We have made sure to define all abbreviations before use.

3. Please rephrase the Short Abstract/Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …"

Response: We have added broader application of this protocol in the summary section. Please see the mark up document (Line 27).

4. Please leave a single line space between each step, substep and note in the protocol section.

Response: We have left a single line space in all the suggested section of the protocol. Please see revised manuscript's protocols section.

5. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Q5 mastermix, HiScribe T7 Quick High Yield RNA Synthesis Kit, Vaccinia Capping System, Dual-Luciferase Reporter Assay System, excel, etc

Response: We have ensured there are no commercial languages in the manuscript. We changed Q5 to high-fidelity DNA polymerase, vaccinia-capping system to capping system, dual-luciferase reporter assay system to dual-luciferase assay kit and excel to desirable file format. Further, we removed HiScribe T7 quick high yield RNA synthesis kit from the manuscript.

6. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes.

Response: We have followed the suggested format and removed bullets or dashes in protocol.

7. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."

Response: We made sure to use imperative tense throughout the protocol, while, suggestive text is written in the note section.

8. Please only have one note section following each step.

Response: We have updated text to include no more than one note in each step. Please see the revised protocol section.

9. The Protocol should contain only action items that direct the reader to do something.
10. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections.
11. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Please ensure that the protocol is written exactly how you would perform it in a stepwise fashion providing all specific details with respect to your experiment.

Response: Regarding comment 9, 10 and 11, we changed the protocol to include only instructive actions, minimized the note section and included details to answer how to perform each step. Please see edits in protocol.

12. 1.1: What are the crucial characteristics to look for?

Response: We have included the key characteristics and directed readers to referenced literature for detailed information. Please find the changes in lines 95-98.

13. 1.2, 1.3? how do you do so?

Response: We have moved step 1.4 and 1.5 immediately after 1.2. Now all the instruction for primer design are located together. Also, these two steps help answer how to design forward and reverse primers in detail for both experimental and internal control.

14. 1.6, 1.7, 2.2, 4.7: Please consider making this a table and upload separately as .xlsx file. Else this can also be written as a sentence. Where did you get the luciferase DNA template from?

Response: We moved all these steps to make tables 2-5 and will be uploaded as *.xlsx* file. Commercially available pGL3-Fluc vector expressing Fluc and pRL-Rluc vector expressing Rluc were used as luciferase DNA template.

15. 1.9: What is the correct size in your experiment. Please do not generalize. What volume is the elution performed and what solution?

Response: The size of the PCR product and eluting solution with volume is added in lines150-152.

16. 3.1: Which cells are used?

Response: HeLa cells are used in these experiments and has been added in line 187.

17. 3.2: Expand what is VACV and explain what is mock in your case? How do you calculate MOI= 5?

Response: VACV stands for Vaccinia virus and we have explained it in the protocol. Mock has been changed to uninfected HeLa cells for clarification and consistency. We have defined MOI and shown how to calculate MOI *via* an equation. Please see lines 193-194.

18. 4.1: Fluc and Rluc are not explained before. Please be as clear as possible with respect to your experiment.

Response: We have defined 12A-Fluc and Kozak-Rluc mRNA in note after step 2.1 and we are consistent in using the defined term thereafter.

19. 4.9: how do you analyze the data?

Response: We have expanded step 4.9 to explain the way we analyze the data to determine relative translation. We added table 7 showing step-by-step analysis of raw data. Please see step 4.9 in lines 248-249 and 252.

20. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Response: Excluding notes, the protocol is less than 2.75-page limit. Because of this reason, we didn't highlight the protocol.

21. Figure 3: Please provide results in this case… how did your RNA look on the gel? RIN values etc? Please provide transfection results.

Response: Purified *in-vitro* transcribed RNA was run in 1.5% agarose TBE gel (Shown in added figure 3B). DNA ladder was used for a quick estimation of size of the RNAs. The RNA synthesized *in-vitro* using T7-polymerase do not contain 28S and 18S rRNA and hence, RNA integrity number (RIN) cannot be calculated. Due to variability in transfection efficiency

between wells and experiments, we show normalized firefly luciferase activities by dividing experimental firefly luciferase activities by internal control *Renilla* luciferase activities.

22. Figure 4: where is the result with respect to VACV and in vitro transcribed RNA? What does the error bar signify? Where is the mock result?

Response: In figure 4B, we show relative Fluc activity determined by normalizing *in-vitro* transcribed Fluc RNA activity to Rluc RNA activity. Mock has been changed to uninfected to make it consistent. VACV infected sample is indicated by "infected" only. Error bars indicate the standard deviations (SD) of at least three biological replicates. Student's t-test was used to determine P-values; *** indicates P value < 0.001.

23. Please remove the embedded Tables(s) from the manuscript. All figures should be uploaded separately to your Editorial Manager account. Each figure must be accompanied by a title and a description after the Representative Results of the manuscript text.

Response: Suggested changes has been made, please see revised protocol section. Updated figures with larger text and additional figure 3B have been uploaded separately to editorial manager account. All figures have title and description in the manuscript text. Please see figures and table legends section

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Response: The figures used in this manuscript have not been published yet.

25. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

Response: All the suggested details can be found in discussion section.

26. Please expand the journal title in the reference section of the manuscript.

Response: The journal titles have been expanded in reference section, please see tracked changed update in reference section.

27. Please alphabetically sort the materials table.

Response: The material table is alphabetically sorted

28. Please print and sign the attached Author License Agreement (ALA). Please then scan and upload the signed ALA with the manuscript files to your Editorial Manager account.

Response: We have signed the ALA.

Reviewers' comments:

Reviewer #1:

Dhungel et al describe a PCR-based and in vitro transcription method to monitor reporter mRNA translation in mock and infected cells. The approach of using an in vitro transcribed reporter RNA to monitor translation has been well-documented and there are numerous methods and papers on this approach, especially in the IRES translation field (Gilbert W, JBC 2010; Thompson SR, Wiley Interdiscip Rev RNA 2012; Wang QS et al Methods 2013). Moreover, I was also able to find Avci-Adali M et al JOVE 2014 describing a similar method. The main difference is that Dhungel describes transfection in poxvirus-infected cells, otherwise, the description of the methods is the same as other methods described. I understand that the criteria for publication in JOVE is not on novelty but this manuscript describes a very simple and routinely used method. I am left wondering how this manuscript is better suited for a methods video rather than the traditional written methods report. Maybe I am not understanding of the goals of JOVE but I feel that this manuscript is too simplistic and basic.

Major comments or additions:

1) PCR based methods always run into issues with potential errors, regardless of the polymerase used. The alternative is to clone the PCR 5'UTR-luciferase into a plasmid, thus ensuring accuracy of the 5'UTR after sequencing. In my opinion, the latter is preferred but this should be provided as an alternative nonetheless.

Response: PCR based methods may have potential errors. To avert the potential errors, we used high-fidelity polymerase and low PCR cycle number. PCR based method allows user to synthesize large quantity of diverse templates in short time. We also recommend the suggested alternative, see lines 369-371.

2) After in vitro transcription, it is imperative that the cleaned up RNA is run out on a gel to ensure integrity. ~200-300 ng of the RNA should suffice.

Response: Please see the response to editor's comment #21. Briefly, *in-vitro* transcribed RNA was run in TBE agarose gel and measured for A260/280 ratio.

3) Cleaned up RNA should be aliquoted and stored in -80C to reduce freeze-thaws.

Response: We have made necessary changes as seen in Step 2.7 line 184.

4) After transfection and lysis, the lysates need to be measured by Bradford assay or equivalent to ensure equal amounts of protein lysates are being measured.

Response: The sophistication in this protocol comes from the use of internal control mRNA. We normalize the value of experimental Fluc by the internal control Rluc to negate differential transfection efficiency, mRNA stability and other effects on general translation. In addition, poxvirus infection causes global protein synthesis shutoff that affects the total amounts of proteins in virus-infected cells. Therefore, we think normalizing using internal control *Renilla* mRNA is a better normalization method. In fact, using same amount of protein of the lysates do not normalize transfection efficiency.

Reviewer #2:

Manuscript Summary:
Dhungel et al. described the methodology of examining the effect of RNA cis-elements to translation with a simple but comprehensive approach. As an example of the RNA cis-element, authors used poxvirus 5'-poly(A) leader to demonstrate the working process. The presence of 5'-poly(A) leader in mRNA can significantly improve mRNA translation, which is a novel and important phenomenon especially for cellular translation regulation. The manuscript provides important details for others to apply the protocol and it is a helpful guide for researchers who are interested in investigating RNA elements of interest. Minor but important revisions are recommended.

Response: We greatly appreciate reviewer #2's recognition of the importance of our method manuscript.

Major Concerns:
No major concerns on rationale, principles, or information disclosed.

Minor Concerns: The MS is well written, and following suggestions are provided to improve the MS:

1. The organization of "section 1" may be improved by moving the content of "1.4" (lines 102-109) and "1.5" (lines 111-122) under "1.2" (lines 95-98). This way the instructions on primer design can be clustered under the general guideline related to the experimental group.

Response: We have moved the sections accordingly and it is better organized now. Please see lines 107-125.

2. Image quality of Fig 2A and 3 can be improved. Details of these figures are illiterate even after printing them on a A4 paper

Response: We have increased the size of the text in figures 1, 2A and 3 to make it more readable. Please see updated figures 1-3.

3. At the lines 100-101 where "a random 5'-UTR coding sequence containing Kozak sequence" is mentioned. It will be helpful to provide examples of the sequences that are commonly used in authors' lab. One example is shown in the table of the last page and it will be helpful to mark and annotate corresponding sequence here. If different random 5'-UTR sequences have been proven to work, it will be helpful to list them in "Table 1". Or if certain sequences were proven not to work, it will also be helpful to describe them along with discussion. Another issue is associated with the last table; if it meant to be "Table 2", it will need to be labeled in the last page and in the section right above "Discussion".

Response: That's a very good suggestion. As we were trying to use sequence which is required for optimal translation in eukaryotic cell, we decided to use Kozak sequence. The last table has been renamed table 6 and is described in section above discussion.

4. At the lines 153-154, a possibility of conducting gel purification may be necessary in the case of nonspecific amplification from PCR. It is understandable for the reason in the discussion to avoid gel purification, but it will be helpful to provide some suggestions to overcome the hurdle under circumstances.

Response: We have added a recommendation in lines 339-342.

5. At the lines 155-156 and 179, perhaps for generally accepted DNA quality it is acceptable to be ~1.8-2.0 A260/280 ratio. Clarify that in the parenthesis.

Response: We have made the recommended changes, which can be seen in lines 154-155, and 181.

6. At the lines 174-175, please provide specific guideline of the alternative way of generating capped RNA. If no specific info is required other than following the manual of the kit, please say so.

Response: As per recommendation, we have added necessary text in lines 170-171.

7. At the lines 183-184, provide info on what cell line was used in this protocol and whether any concern on the choice of cells for this protocol. Take into consideration that whether transfecting large quantity of RNA with potential hairpin structure may stimulate RNA sensing event that can potentially shut down translation selectively or globally.

Response: We used HeLa cells for this protocol. However, we have also used primary cell line HFF, monkey kidney cell line BSC-1, and rabbit kidney cell line RK-13 for transfection experiments. The reviewer is correct that transfection of DNA or RNA may stimulate DNA/RNA sensing pathway, therefore data should be interpreted with cautions like other experiments using DNA/RNA transfection. We did not experience problems potential caused by this issue in our experiments. We added discussion about this issue in our manuscript. See lines 342-345.

8. At the lines 192, 195, and 198, if Opti-MEM was the choice of medium, replace "reduced serum media" with "Opti-MEM".

Response: The JOVE policy does not allow using commercial language; hence, we used more generic term.

9. At the line 199, replace "mix" with "mixture".

Response: We made the change.

10. At the lines 209-210, provide Cat# for the 96-well-plate used for the bioluminescent readout. A solid bottom plate is used in this protocol, and appropriate clear bottom plates can also be used. Thus, address as such and provide suggestions (e.g., cat # for products).

Response: We added detail for 96-well plate with Cat#3693 in the Table of material section.

11. At the lines 212-221, consider to provide an alternative guideline for researchers who may want to manually add substrates for the dual luciferase readout.

Response: We have included manual option using luminometer. See lines 240-244.

12. At the lines 235-242, description on how data was processed and final results are shown. It will be helpful to include an example of raw data in the table format to show the magnitude of readings for both experimental and internal control. It will be very helpful for audience who will conduct this experiment and possibly troubleshoot for their own experiments to compare the raw data and then corresponding end result (Fig 4B).

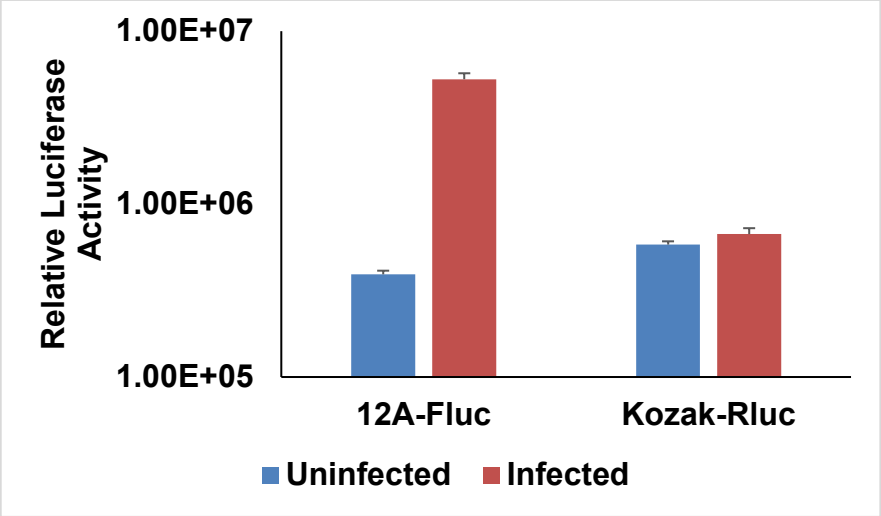
Response: We have included the raw data and how we analyzed it step-by-step. The raw value and calculated normalized values are depicted in Table 7, analysis of raw data section.

2018.05.10 Diff time (12 hpi)

	Uninfected-1	Uninfected-2	Uninfected-3	Infected-1	Infected-2	Infected-3
12A-Fluc	3.84E+05	4.15E+05	3.79E+05	4.77E+06	5.55E+06	5.50E+06
Kozak-Rluc	5.80E+05	6.11E+05	5.59E+05	6.12E+05	7.22E+05	6.77E+05

RAW DATA:

	Average		Stdev		
	Uninfected	Infected	Uninfected	Infected	T-test
12A-Fluc	392300.00	5273333.33	19731.19	436615.77	0.0000421
Kozak-Rluc	583200.00	670266.67	25948.41	55385.95	0.0692731



RELATIVE DATA:

	Uninfected-1	Uninfected-2	Uninfected-3	Infected-1	Infected-2	Infected-3
12A-Fluc/Kozak-Rluc	0.66	0.68	0.68	7.80	7.69	8.12

	Average		Stdev		
	Uninfected	Infected	Uninfected	Infected	T-test
12A-Fluc/Kozak-Rluc	0.67	7.87	0.0099	0.2243	0.0000006