Cellular Translational Reporter Assay

Taishi Kimura and Kiyoshi Takeda*

Department of Microbiology and Immunology, Graduate School of Medicine, Osaka University, Suita, Japan

*For correspondence: ktakeda@ongene.med.osaka-u.ac.jp

[Abstract] The method described here allows measuring the effect of exogenously introduced modifications to *in vitro*-transcribed mRNA on the translation in cells. Using cells derived from knockout mice and control littermates, this method enables to compare the results in the presence or absence of specific gene products. In our lab, we used this protocol to check whether the exogenous addition of 5' capping and 2'-O methylation to *in vitro*-mRNA affects the translational efficiency. Here we describe the details of our experiments.

Materials and Reagents

1. Mouse embryonic fibroblasts (prepared from day 14.5 embryos)
2. Vector pGL4.14 (*Luc2* encoding vector) (Promega Corporation, catalog number: E6691)
3. Primers (Invitrogen custom DNA primers)
   a. 5'-TAATACGACTCACTATAGGCCACCATGGAAGATGCCAAAAA-3' (the T7 class III promoter sequence is underlined)
   b. 5'-TACCACATTTGTAGAGGTTTTACTTGCTTT-3'
4. rTaq DNA polymerase (TOYOBO, catalog number: TAP-211)
5. Agarose gel
6. Ethidium bromide
7. Illustra GFX PCR and Gel Band Purification Kit (GE Healthcare, catalog number: 28-9034-71)
8. MEGAScript *In vitro* transcription Kit (Life Technologies, Ambion®, catalog number: AM1333)
   Note: Nuclease-free Water and LiCl Precipitation Solution are included in the kit.
9. 80% ethanol
10. ScriptCap m7G capping system (EpiCentre, catalog number: SCCE0610)
11. ScriptCap 2'-O-Methyltransferase Kit (EpiCentre, catalog number: SCMT0610)
12. RNeasy Mini Kit (QIAGEN, catalog number: 74104)
13. Opti-MEM I Reduced Serum Medium (Life Technologies, Gibco®, catalog number: 31985-070)

14. D-PBS(-) (Nacalai Tesque, catalog number: 14249-95)
15. Lipofectamine 2000 DNA Transfection Reagent (Life Technologies, catalog number: 11668-019)
16. Dual-luciferase reporter assay system (Promega Corporation, catalog number: E1960)
17. BCA Protein Assay Reagent (Thermo Fisher Scientific, catalog number: 23227)

Equipment

1. Cell scraper
2. GeneAmp PCR system 9700 (Applied Biosystems®)
3. Centrifugal Concentrator CC105 (TOMY)
4. Lumat LB 9507 Luminometer (Bertjold Technologies)
5. Model 680 Microplate Reader (Bio-Rad Laboratories)

Procedure

A. Subcloning of *Luc2* cDNA
1. Prepare the polymerase chain reaction mix.
   Mix following components on ice:
   0.2 µl of rTaq DNA polymerase
   5 µl of 10x Buffer (+Mg) for rTaq
   5 µl of dNTPs (2 mM each)
   4 µl of primers (10 mM each of Forward and Reverse primers)
   1 µl of vector pGL4.14 (100 ng)
   35 µl of water
2. Run the polymerase chain reaction.
   Place the reaction mix to the GeneAmp PCR system 9700.
   PCR program:
   Step 1: 94 °C 5 min
   Step 2: 94 °C 30 sec
   Step 3: 60 °C 2 min
   Step 4: 74 °C 1 min (repeat steps 2-4 for 35 times)
   Step 5: 74 °C 10 min
3. Purification of PCR amplified fragments.
   a. Electrophorese the PCR products on 1% of agarose gel.
   b. Stain Gel with Ethidium Bromide.
   c. Cut the gel region including an amplified DNA fragment (approximately 1.6-kbp).

d. Purify PCR amplified DNA from gels using illustra GFX PCR and Gel Band Purification Kit.

Note: In this process, we elute DNA fragment with 30 µl of Nuclease-free Water (8 µl aliquots of the eluent are used as a template DNA for following in vitro transcription).

B. *In vitro* transcription of Luciferase mRNA under the control of T7 promoter

1. Prepare the *in vitro* transcription mix.
   Mix following components at room temperature:
   8 µl of Template DNA
   2 µl of 10x Reaction Buffer
   2 µl of 10 mM ATP
   2 µl of 10 mM CTP
   2 µl of 10 mM UTP
   2 µl of 10 mM GTP
   2 µl of T7 Enzyme Mix
   Mix thoroughly with pipetting.
2. Incubate at 37 °C, 4 h.
3. Add 2 µl of TURBO DNase (a component of MEGAScript) to digest the rest template DNA and incubate 15 min at 37 °C on the block incubator.
4. RNA extraction with lithium chloride (LiCl) precipitation.
   a. Add 30 µl of Nuclease-free Water and 30 µl of LiCl Precipitation Solution to the *in vitro* transcription products.
   b. Mix thoroughly with vortex mixer. Chill for 1 h at -20 °C.
   c. Centrifuge at 4 °C for 15 min at 15,000 rpm.
   d. Discard the supernatants, and wash the pellet with 1 ml of 80% ethanol.
   e. Centrifuge at 4 °C for 15 min at 15,000 rpm.
   f. Discard the supernatants, and dry the pellet with centrifugal concentrator CC105 for 5 min.
   g. Resuspend the RNA in 20 µl of Nuclease-free Water, and stored at -80 °C.

C. Enzymatic modification of RNA 5' end

1. Add m7G cap, or m7G cap and 2'-O methylation to the *in vitro*-transcribed RNA with ScriptCap system.
   a. Adjust the volume of transcribed RNA (50 µg) to 67 µl with Nuclease-free Water.
   b. Denature the RNA at 65 °C for 10 min, then transfer the tube immediately to ice.
   c. While the RNA is denaturing, mix following components:
      10 µl of 10x ScriptCap Capping Buffer

10 µl of 10 mM GTP

2.5 µl of 20 mM SAM

2.5 µl of ScriptGuard RNase Inhibitor

4 µl of ScriptCap 2'-O-Methyltrasferase (100 U/µl) for 2'-O methylated 5' capped RNA or Nuclease-free Water for 2'-O unmethylated 5' capped RNA

d. Add 4 µl of ScriptCap Capping Enzyme (10 U/µl) and 67 µl of denatured RNA to the reaction cocktail from step C1c.
e. Incubate at 37 °C for 2 h.
f. Reaction products were purified with an RNeasy Mini Kit.

D. RNA transfection with mouse embryonic fibroblasts.

1. Seed a 35 mm diameter dish with 2 x 10^5 mouse embryonic fibroblasts in 1 ml DMEM, and incubate 24 h.

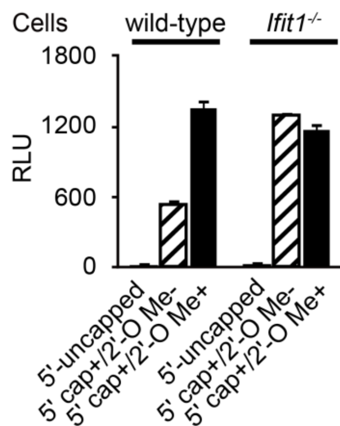

Figure 1. Luciferase activity of introduced RNAs. Wild-type and *Ifit1*-deficient (*Ifit1*$^{-/-}$) mouse embryonic fibroblasts were transiently transfected with three different types of luciferase mRNAs (5'-uncapped, 5' capped but 2'-O unmethylated; 5' cap+/2'-O Me-, 5' capped and 2'-O methylated; 5' cap+/2'-O Me+). Luciferase activities are shown as relative light units (RLU), and the numbers of RLU were normalized by the concentrations of proteins determined in step E1d. Data are shown as means ± SDs of triplicate samples. These data show that Ifit1 selectively inhibits the translation of 5' capped but 2'-O unmethylated (5' cap+/2'-O Me-) luciferase mRNA.

2. Transfect RNA using Lipofectamine 2000.
   a. Dilute 10 µl of Lipofectamine 2000 DNA Transfection Reagent in 240 µl of Opti-MEM medium per sample.
   b. Dilute 2 µg of luciferase RNA in 250 µl of Opti-MEM medium.

c. Add 250 µl of diluted Lipofectamine 2000 DNA Transfection Reagent to 250 µl of diluted Luciferase RNA. Mix thoroughly.
d. Incubate at room temperature for 15 min.
e. Add RNA-lipid complex to the mouse embryonic fibroblasts, and incubate for 6 h.

E. Luciferase translational reporter assays
1. Preparation of cell lysates.
a. After incubation for 6 h, wash the RNA-transfected mouse embryonic fibroblasts with D-PBS(-) twice.
b. Add 100 µl of 1x passive lysis buffer and harvest cells with cell scraper.
c. Centrifuge at 4 °C for 15 min at 15,000 rpm.
d. Determine the protein concentration in 5 µl aliquots of the supernatants by BCA Protein Assay.
e. Add equal protein amount (determined by BCA protein assay) of aliquots of the supernatants to 50 µl of Luciferase Assay Reagent II (LAR II).
f. Immediately, measure the relative luciferase units (RLUs) on Lumat LB 9507 Luminometer according to the manufacturer's instruction.

Acknowledgments

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References

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