

In vitro research method for screening inhibitors of protein translation

Use of mammalian *in vitro* translation (cell-free) systems for high-throughput screening of inhibitors

Introduction

Inhibitors of protein synthesis represent an emerging class of antiviral and anticancer therapeutics. Protein production involves a large number of factors and regulatory components that assemble the ribosomes, mRNA templates, and aminoacylated tRNAs. Deregulation of protein synthesis has been identified as a major contributor to cancer initiation [1]. As such, protein synthesis represents a potential target for chemotherapeutic intervention. However, current techniques to search for new inhibitors of eukaryotic protein synthesis can be long and arduous.

In vitro translation (IVT), or cell-free expression, offers a unique and powerful research tool to screen for translational inhibitors that regulate both cellular and viral protein expression. In this study we have developed a simple, quick method to identify inhibitors of both cap-dependent and cap-independent protein translation, using the Thermo Scientific™ 1-Step Human Coupled IVT Kit. Rapid assay readout (60–90 min), amenability for miniaturization, and insensitivity to compound toxicity make *in vitro* translation an attractive alternative to cell-based

screens for high-throughput screening (HTS) of novel inhibitors of protein synthesis.

The 1-Step Human Coupled IVT Kit is a cell-free system using the cellular transcription and translation machinery from a modified HeLa cell line. The *in vitro* protein expression is rapid, with protein production occurring within 60–90 minutes, making it an effective alternative to cell-based protein expression methods that require extensive expertise and infrastructure. The 1-Step Human Coupled IVT Kit accommodates transcription and translation of DNA templates or direct translation of mRNA transcripts containing key elements, including the encephalomyocarditis virus (EMCV) IRES element and other mRNA-stabilizing elements [2]. Using the 5'-capped mRNA for Thermo Scientific™ TurboLuc™ luciferase, and red firefly (RFF) luciferase mRNA with an IRES element, we successfully demonstrate a dual assay for simultaneous monitoring of both cap-dependent and cap-independent translation. This assay provides a simple, sensitive readout of protein synthesis inhibition (Figure 1).

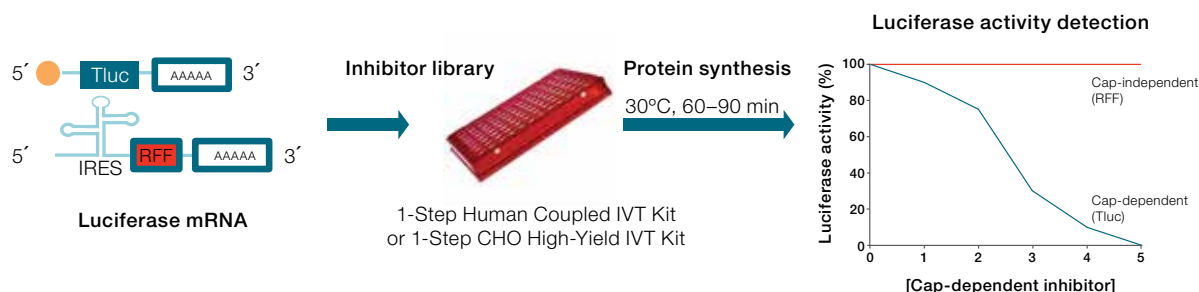


Figure 1. Novel research method for screening translational inhibitors using TurboLuc (Tluc) and red firefly (RFF) luciferase in a cell-free, *in vitro* translation-based format.

Results

Optimization of mRNA translation

To monitor protein synthesis we utilized two luciferase systems, TurboLuc luciferase and red firefly luciferase. Luciferase was chosen due to its ease in monitoring, requiring only a one-step or two-step reagent addition, high sensitivity, and dynamic range, as well as high signal-to-noise ratio.

Most eukaryotic mRNAs have a modified nucleotide structure (7-methylguanosine, m⁷G), known as a cap, present on the 5' end. *In vivo*, the m⁷G cap is added enzymatically to mRNA produced by RNA polymerase II. However, the standard cap analog used in cell-free systems can be incorporated into the RNA in the forward [m⁷G(5')pppG(pN)] or the reverse orientation [G(5')pppm⁷G(pN)], leading to synthesis of two forms of isomeric RNAs. RNAs with 5' caps in the reverse orientation are not efficiently translated.

To optimize cap-dependent protein synthesis of TurboLuc luciferase in our IVT system, a novel cap analog, Anti-Reverse CAP Analog (ARCA), was added to our transcript. In ARCA, one of the 3'-OH groups is eliminated from the cap analog and is substituted with -OCH₃ (Figure 2).

This modification forces RNA polymerases to initiate transcription with the remaining -OH group in guanosine and thus synthesize RNA transcripts capped exclusively in the correct orientation. Substitution of the traditional cap analog with ARCA allows for synthesis of capped RNAs that are 100% functional, in contrast to transcription reactions using traditional cap analogs where only half of the cap analog is incorporated in the correct orientation.

IVT reactions were performed using the 1-Step Human Coupled IVT Kit, and translation was monitored by luminescence detection with the Thermo Scientific™ TurboLuc™ Luciferase One-Step Glow Assay Kit (Cat. No. 88263) (Figure 2). Results showed that ARCA capping of mRNA produced the highest levels of translation.

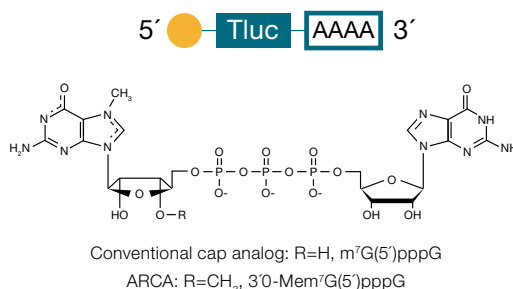
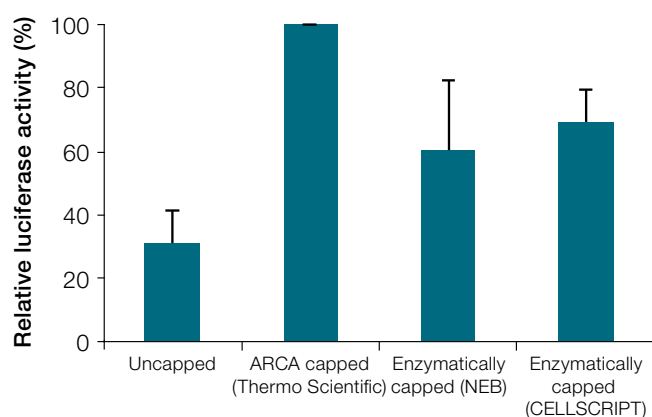


Figure 2. Effect of capping TurboLuc luciferase mRNA on translation efficiency with the 1-Step Human Coupled IVT Kit. Translation of TurboLuc luciferase was monitored using the TurboLuc Luciferase One-Step Glow Assay Kit. The yellow circle represents the mRNA cap structure.

Optimization of mRNA configuration for dual screening

Ideally, to test new inhibitors of eukaryotic protein synthesis, the effects on both cap-dependent and cap-independent protein synthesis should be evaluated simultaneously. To optimize our IVT expression system for dual screening, several mRNA configurations were evaluated.

Capped TurboLuc luciferase mRNA and uncapped red firefly mRNA containing an IRES element were added together to the 1-Step Human Coupled IVT Kit reaction, and alternatively the two transcripts were combined into a bicistronic capped mRNA (Figure 3). As seen in Figure 3, addition of the second transcript did not negatively affect translation of TurboLuc luciferase, indicating that a dual screen for inhibitors of both cap-dependent and cap-independent translation can be performed. However, when the transcripts were combined into a bicistronic capped mRNA, translation was slightly decreased, as seen by the decrease in TurboLuc luciferase activity.

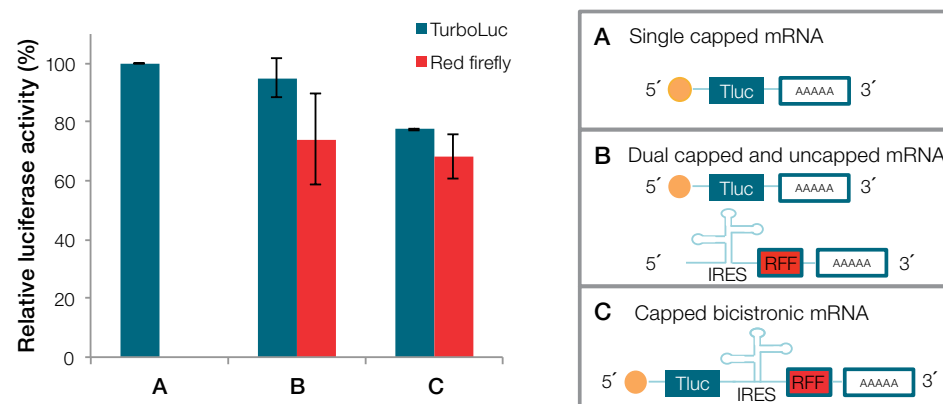


Figure 3. Translation of both TurboLuc luciferase mRNA and red firefly luciferase mRNA containing an IRES element in a single IVT reaction.

Inhibition of translation using a dual-mRNA luciferase assay of human and CHO coupled IVT kits

Cap-dependent expression of TurboLuc luciferase mRNA and cap-independent expression of red firefly luciferase mRNA were carried out using both the 1-Step CHO High-Yield IVT and 1-Step Human Coupled IVT systems. Luciferase activity was assayed and quantified in the presence or absence of inhibitors of protein synthesis. Cap-specific inhibitor 7-methylguanosine 5'-diphosphate (m⁷GDP), general inhibitors cycloheximide (CHX) and puromycin, and viral replication inhibitor ribavirin were

evaluated for the system's ability to measure inhibition of protein synthesis (Figure 4). Selective inhibition was observed with m⁷GDP, whereas general inhibition was observed for protein synthesis of both TurboLuc and red firefly luciferase in the presence of CHX and puromycin. Ribavirin had little to no effect on translation. This demonstrates that either IVT lysate system (HeLa or CHO) can be used to screen both selective and general inhibitors of protein synthesis for research purposes.

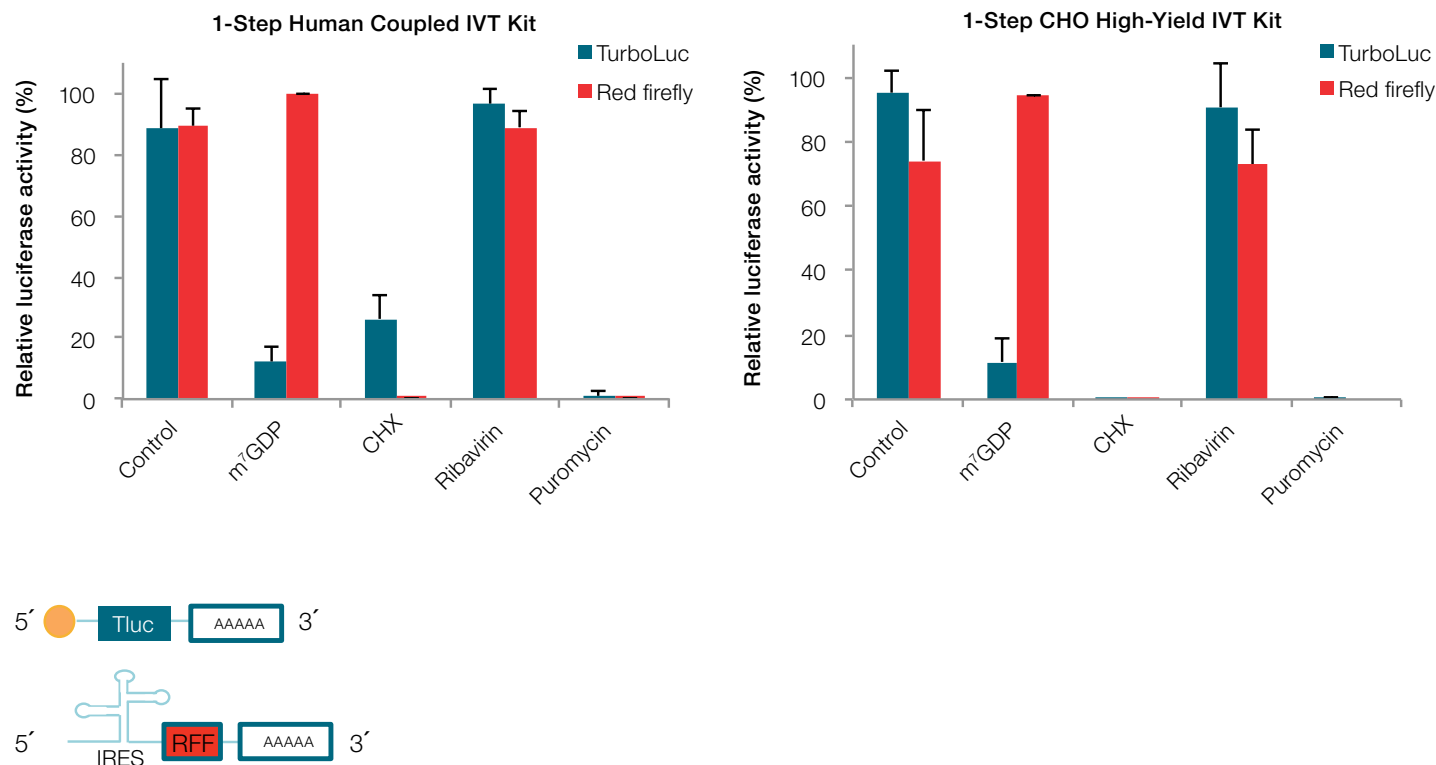


Figure 4. Selective and general inhibition of translation using the dual-mRNA luciferase assay with the 1-Step Human Coupled IVT Kit (HeLa lysate) and the 1-Step CHO High-Yield IVT Kit.

To demonstrate the sensitivity of the dual-mRNA IVT assay, titration of m⁷GDP and puromycin was performed with the 1-Step Human Coupled IVT Kit. Inhibition of cap-dependent translation (Tluc) but not cap-independent translation (RFF) is seen with addition of m⁷GDP even at the highest levels of m⁷GDP (1 mM). Puromycin, on the other hand, inhibits both cap-dependent and cap-independent translation (Figure 5).

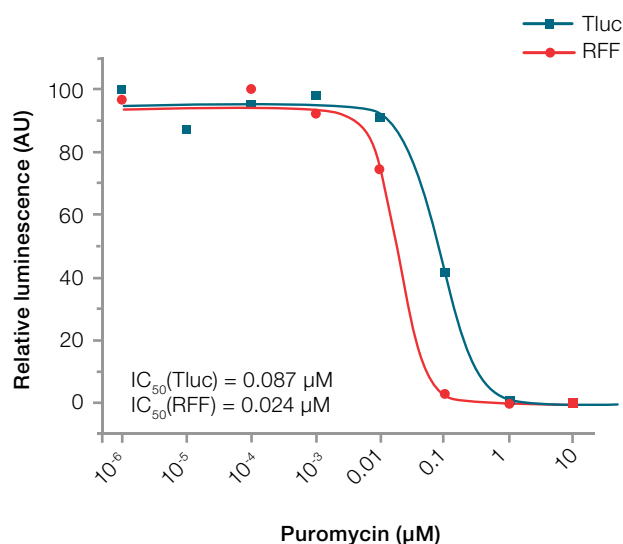
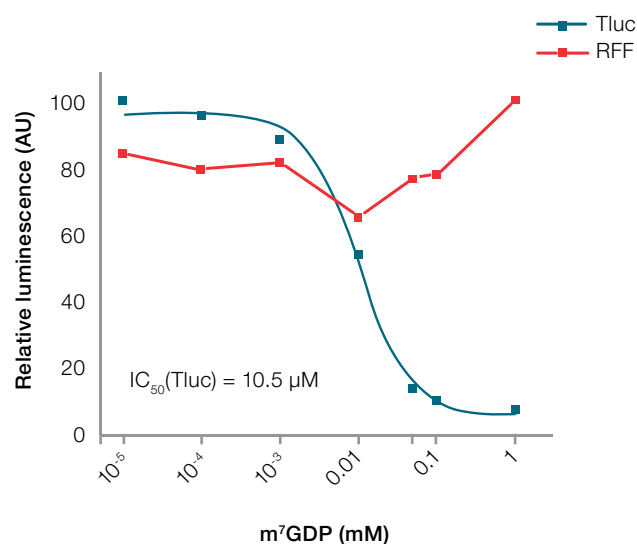


Figure 5. Selective inhibition of cap-dependent translation with m⁷GDP, and nonselective inhibition of translation with puromycin, detected with a dual-mRNA IVT assay.

Conclusions

In vitro translation systems offer a novel approach to studying protein synthesis inhibition. In this study we demonstrated that our 1-Step Human Coupled IVT Kit can be utilized for high-throughput screening of protein synthesis inhibitors in a rapid, miniaturized format. Simultaneous monitoring of cap-dependent and cap-independent (IRES-dependent) translation can be performed reliably using capped TurboLuc luciferase mRNA and uncapped red firefly luciferase mRNA containing an IRES element. The dual luciferase assay provides several unique advantages: (1) Inhibitors specific for regulation of cap structure can be easily identified, via TurboLuc luciferase expression, while evaluating their effect on general translation by monitoring the expression of cap-independent red firefly luciferase. (2) IRES structure, which is very specific to viruses, can be used in research as a target for screening viral inhibitors. (3) Lastly, the flexibility of the system enables replacement of the EMCV IRES with other IRES elements such as the cricket paralysis virus or hepatitis C virus IRES [3].

Methods

Capping mRNA transcripts

TurboLuc luciferase mRNA was capped with anti-reverse CAP analog (ARCA) using the Thermo Scientific™ TranscriptAid™ T7 High Yield Transcription Kit (Cat. No. K0441) and the Thermo Scientific™ pMCS Tluc16-DD vector (Cat. No. 88231) according to manufacturer instructions. 7-Methylguanylate caps were incorporated at the 5' end of the mRNA using the Vaccinia Capping System (New England BioLabs) or the ScriptCap m⁷G Capping System (CELLSCRIPT) according to manufacturer instructions.

Cell-free expression of TurboLuc luciferase and red firefly luciferase

Protein synthesis of capped TurboLuc luciferase mRNA and IRES-containing red firefly luciferase mRNA was performed in 10 μL cell-free expression reactions in a 384-well plate, following instructions provided in the 1-Step Human Coupled IVT Kit (Cat. No. 88881). Lysates were incubated at 30°C for 60–90 min. TurboLuc luciferase and red firefly luciferase activity was measured as described below.

TurboLuc and red firefly luciferase dual assay

Luminescence was measured using the TurboLuc Luciferase One-Step Glow Assay Kit (Cat. No. 88263) and the Thermo Scientific™ Pierce™ Firefly Luciferase Glow Assay Kit (Cat. No. 16176). Briefly, at the completion of the cell-free reaction, the 384-well plate was removed from the incubator and 20 μ L of TurboLuc™ Glow Assay Working Solution was added per well. The plate was shaken on a plate shaker at a medium speed for 10 min. TurboLuc luciferase luminescence was read using the Thermo Scientific™ Varioskan™ Flash Multimode Reader (Cat. No. 5250040). Subsequently, 40 μ L of red firefly luciferase assay working solution was added per well. The plate was shaken on a plate shaker at medium speed for 10 min. Red firefly luciferase luminescence was read using the Varioskan Flash Multimode Reader. TurboLuc Glow Assay Working Solution was prepared by diluting 50X TurboLuc™ One-Step Substrate 1:50 into TurboLuc™ One-Step Assay Buffer. The red firefly luciferase substrate pellet was reconstituted in 1 mL of ultrapure water. Red Firefly Luciferase Glow Assay Working Solution was prepared by diluting 100X Red Firefly Luciferase Substrate Solution 1:100 in Red Firefly Luciferase Assay Buffer.

Inhibition of protein synthesis

Capped TurboLuc luciferase mRNA and uncapped red firefly mRNA were added to the 1-Step Human Coupled IVT HeLa lysate or the 1-Step CHO High-Yield IVT lysate with or without the following inhibitors of protein synthesis: m⁷GDP (0.1 mM), cycloheximide (0.1 mM), puromycin (0.1 mM), or ribavirin (0.1 mM). Where necessary, some inhibitors were initially dissolved in DMSO; further dilutions were carried out in water and added to the cell-free reactions, ensuring the final DMSO concentration did not exceed 1% (v/v). Reactions were conducted in 10 μ L in a 384-well plate and incubated for 60–90 minutes at 30°C. TurboLuc luciferase and red firefly luciferase activity were measured as previously described. With the 1-Step CHO High-Yield IVT Kit, the dialysis buffer was omitted and the assay was performed in a batch assay, coupled format in a similar approach to the 1-Step Human Coupled IVT Kit.

Titration of m⁷GDP and puromycin

Capped TurboLuc luciferase mRNA and uncapped red firefly luciferase mRNA containing an IRES element were added to the 1-Step Human Coupled IVT lysate with increasing amounts of m⁷GDP (10 nM to 1 mM) or puromycin (1 pM to 10 μ M). TurboLuc luciferase and red firefly luciferase activity was measured as previously described.

References

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2. Mikami S, Kobayashi T, Masutani M et al. (2008) A human cell-derived *in vitro* coupled transcription/translation system optimized for production of recombinant proteins. *Protein Expr Purif* 62(2):190–198.
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