Novel *In Vitro* Method for Screening Inhibitors of Protein Translation

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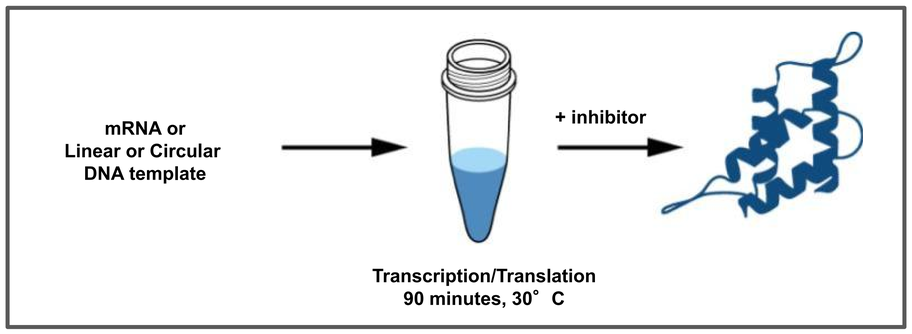
Abstract

Inhibitors of protein synthesis represent an emerging class of anti-viral and anti-cancer therapeutics. *In vitro* translation (IVT) or cell-free expression offers a convenient and powerful assay to screen for translational inhibitors that regulate both cellular as well as viral mRNA expression. We have developed a simple method to identify inhibitors of cap-dependent and cap-independent protein translation using expression of luciferase mRNA (5'-cap-TuboLuc luciferase mRNA and IRES-Red Firefly luciferase mRNA) in the Thermo Scientific 1-Step Human Coupled IVT system. To facilitate detection of both cap-dependent and cap-independent translation in the same well, we developed a 2-step luciferase assay reagent for TurboLuc and Firefly luciferases. Rapid assay read-out (<90 min), ability for miniaturization, and insensitivity to compound toxicity make the *in vitro* translation format an attractive alternative to cell-based screens for HTS identification of novel inhibitors of protein synthesis.

Figure 1. Novel Method for Screening for Translational Inhibitors Using an IVT-based format.

Inhibitors of protein synthesis represent a current and recurring interest of Pharma and academic researchers for anti-viral and anti-cancer therapeutics. *In vitro* translation offers a unique and powerful assay to screen for translational inhibitors because it provides rapid protein expression and is amenable to miniaturization for HTS. Compounds specific for both cap-dependent and cap-independent (IRES) translation mechanisms can be screened & identified using IVT reactions containing two different mRNAs, one capped mRNA encoding for TurboLuc luciferase and another uncapped (IRES) mRNA encoding for Red Firefly luciferase. The levels of both luciferases are measured following a 90 min IVT reaction using a newly developed 2-Step TurboLuc/RFF dual assay reagent. Theoretical cap-dependent inhibitor screening results are illustrated.



A. *In vitro* protein expression is a rapid, convenient system to study protein translation in a *cell-free* format

- Protein produced in <60 min
- Can screen toxic compounds
- Amenable to HTS

B. Simultaneously monitor cap-dependent and cap-independent (IRES) translation in IVT reaction

- Capped TurboLuc (Tluc) luciferase mRNA
- IRES Red Firefly (RFF) luciferase mRNA

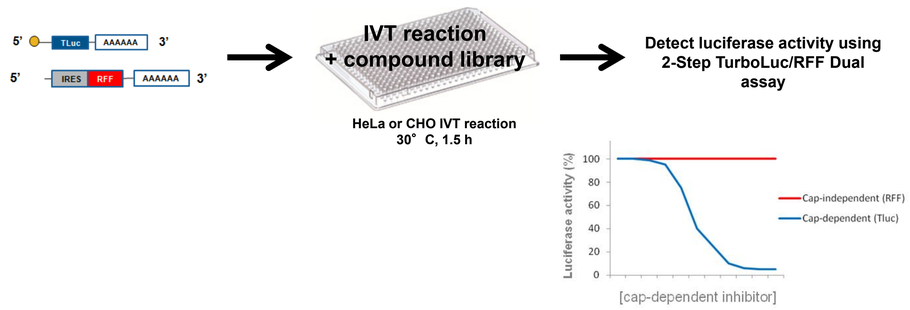


Figure 2. Optimization of mRNA capping for IVT.

mRNA capped co-transcriptionally with ARCA by using T7 TranscriptAid High Yield Transcription kit (Thermo Scientific) or by using NEB and CellScript enzymatic capping kits after transcription with GTP and S-adenosylmethionine. IVT reactions were run in HeLa cell lysate and luminescence detected with TurboLuc™ Luciferase One-Step Glow Assay Kit (Thermo Scientific). The yellow circle represents the mRNA cap structure. Results showed that ARCA capping of mRNA is more efficient than enzymatic capping, thus ARCA capped mRNA was used in all further experiments.

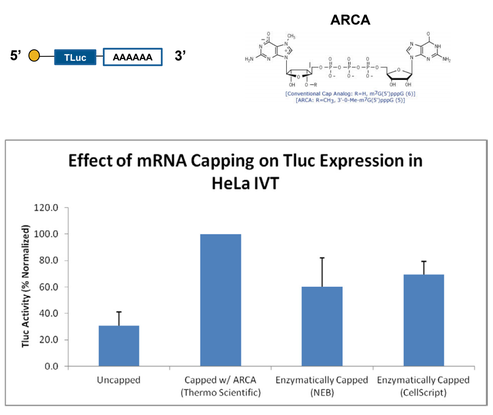


Figure 3. Evaluation of different mRNA configurations for screening inhibitors specific for cap-dependent and cap-independent translation in IVT.

Panel A demonstrates expression of capped-TurboLuc luciferase (Tluc) mRNA in IVT. This format can be used to obtain identify inhibitors of cap-dependent translation. Inhibition of translation is observed with either cap specific m7GDP (0.1 mM) or any of the general inhibitors of translation such as Cyclohexamide (CHX, 0.1 mM) and Puromycin (0.1 mM). In Panel B, where capped-Tluc and IRES- Red Firefly (RFF) mRNA's were simultaneously expressed in IVT, TLuc expression was again inhibited by m7GDP, whereas RFF expression was unaffected, suggesting specificity of m7GDP inhibition towards capped mRNA's. IRES-RFF mRNA expression in these experiments could potentially serve as internal control making sure the IVT expression system is functional. In Panel C, instead of having two separate mRNA's as in Panel B, a single dual-luciferase mRNA containing both Tluc and RFF on the same construct was expressed. Again, m7GDP specifically affected the expression of Tluc but not the IRES. 7-Methylguanosine 5′-diphosphate (m7GDP) is a eukaryotic mRNA cap inhibitor that binds to cap binding protein eIF4E and prevents its function. Cyclohexamide and Puromycin inhibit general protein synthesis by either blocking elongation step or inducing premature termination of protein synthesis, respectively. Ribavirin is a RNA nucleoside analogue known to block viral RNA replication *in vivo*, and its mechanism is not clearly understood.

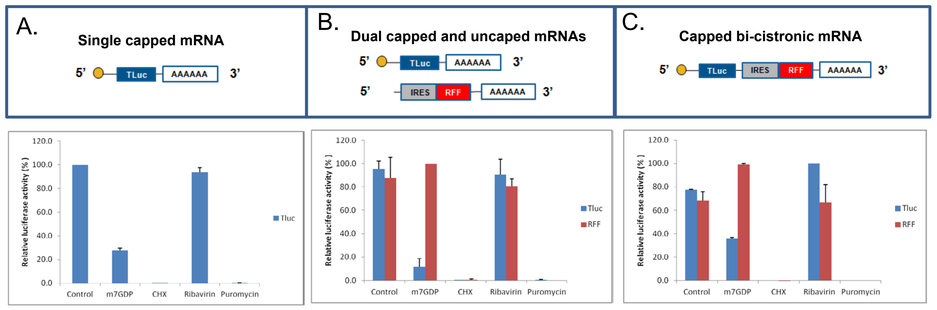
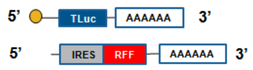


Figure 4. Demonstration of both selective and general inhibition of translation using dual mRNA luciferase assay in both HeLa and CHO IVT.

mRNAs for cap-dependent expressing Turbo-luc and cap independent expressing Red Fire Fly luciferase were expressed in both HeLa and CHO IVT reactions where various inhibitors where added and luciferase activity was assayed and quantified as percent control. Either IVT lysate (HeLa or CHO) can be used for screening depending on needs/preferences.



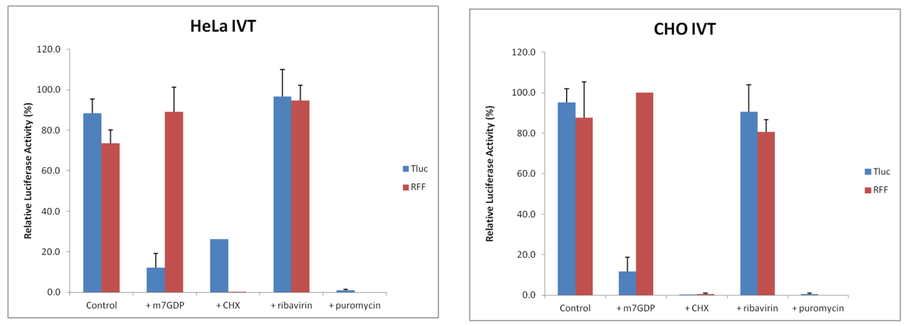
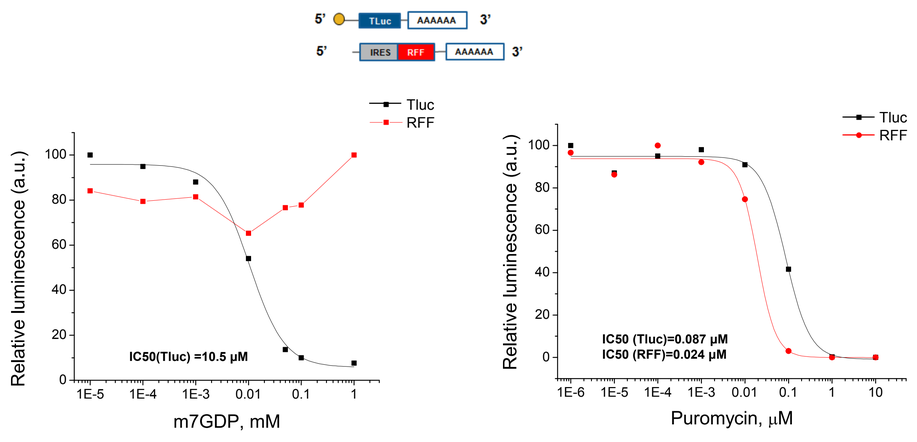


Figure 5. Selective inhibition of cap-dependent translation (m7GDP) and non-selective inhibition of translation with puromycin detected with dual mRNA IVT assay.

Specific titration of m7GDP and puromycin in HeLa IVT system using both cap-dependent-Turboluc and cap-independent-RFF mRNAs. Inhibition of cap-dependent translation (Tluc) is seen with addition of m7GDP but not cap-independent translation (RFF). Puromycin inhibits both cap-dependent and cap-independent translation.



Summary

- ***In vitro* translation systems based on HeLa and CHO lysate were successfully used to demonstrate a rapid *in vitro* assay for screening protein synthesis inhibitors in a miniaturized, fast and HTS compatible format.**
- **Simultaneous monitoring of cap-dependent and IRES-dependent translation using two distinct luciferase mRNA templates differentiates cap-dependent vs. cap-independent translation mechanisms. A single assay reagent can further be used to measure both the luciferases sequentially in the same microplate well.**
- **The Anti-Reverse Cap Analog (ARCA) from Thermo Scientific produces at least 70% more translatable capped transcripts than capping kits from other suppliers.**