

Dual luciferase assay to assess the replication of the hepatitis C virus subgenomic replicon

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Application Note 172

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- DLR™ assay utilized to monitor early stage replication events of Hepatitis C virus
- Measurements performed on DLR™ certified FLUOstar OPTIMA
- Potential inhibitors can be easily detected

Introduction

Hepatitis C virus (HCV) is a global health problem affecting an estimated 170 million people worldwide. Chronic infection can lead to the development of cirrhosis resulting in end-stage liver failure or hepatocellular carcinoma. There is no vaccine for HCV and the current therapy is only effective in around 50 % of individuals. Therefore, improved understanding of the viral life cycle and systems for screening potential viral inhibitors are required.

The development of a subgenomic replicon system, encoding only the viral non-structural proteins required for RNA replication, allows the study of replication of the virus, without the release of infectious particles and the necessary containment facilities required for this work. Recently a subgenomic replicon has been generated from the efficiently replicating genotype 2a strain of HCV (JFH1), incorporating the firefly luciferase gene under the control of the HCV internal ribosome entry site element.¹ This has allowed monitoring of early stage replication events of HCV in the absence of any selective pressure, and furthermore investigation of the effects of compounds on these events.

This application note describes a method used for directly assessing the effects of potential inhibitors simultaneously on both the RNA replication of HCV, and overall translation within human hepatoma cells using the BMG LABTECH's FLUOstar OPTIMA microplate reader. As an indicator for overall translation in the cell, a capped RNA transcript from the pRLTK plasmid was used. This plasmid contains a *Renilla* luciferase gene, preceded by a T7 promoter.



Fig. 1: BMG LABTECH's multimode microplate reader FLUOstar OPTIMA

Materials and Methods

- BMG LABTECH's FLUOstar OPTIMA microplate reader
- MEGAscript® T7 Kit, AM1333, Ambion (Applied Biosystems)
- Cap Analog (m7G(5')ppp(5')G), AM8048, Ambion (Applied Biosystems)
- Bio-rad Gene Pulser Xcell Eukaryotic System electroporator
- Gene Pulser/MicroPulser Cuvettes, 0.4 cm gap, 165-2081, Bio-rad
- 96 Well Tissue Culture (TC) Treated Plate with Lid, White, Sterile, 655083, Greiner Bio-One Ltd
- Dual-Luciferase® Reporter Assay System, E1910, Promega²

In addition, tissue culture consumables, pipette tips etc were used as needed from various manufacturers.

Dual luciferase Replication and Translation Assay

In vitro transcribed (IVT) replicon RNA was made from a linearised template of the JFH1 luciferase subgenomic replicon (SGR-JFH1-Luc) DNA using the MEGAscript® T7 Kit. *In vitro* transcription of linearised pRLTK plasmid DNA² was as above but in the presence of a cap analogue. Four million PBS washed human hepatoma (Huh-7.5) cells were electroporated in 0.4 cm cuvettes with 5 µg of SGR-JFH1-Luc RNA and 5 µg of RTLK RNA simultaneously at 270 V and 950 µF. Cells were then resuspended in standard cell media and seeded at a density of 1000 cells per well in 96 well plate. Cells were incubated as normal at 37°C. Cells were lysed directly in 96 well plates by carefully removing media and washing twice in PBS before adding 20 µL of 1 x Passive Lysis Buffer (Promega), and placing on a rocking platform (with gentle rocking to ensure even coverage of the cell monolayer) at room temperature for 15 minutes.

The 96 well plate containing the lysed cells was then either stored at -20°C (for up to a month) or read directly in the microplate reader using the following parameters:

Read mode: Luminescence, well mode kinetics
Optics: 3 mm combination optic
Positioning delay: 0.2 sec
Measurement start time: 0.0 sec
No of intervals: 48
Interval time: 0.50 sec
Emission Filter: empty
Recommended gain: 3800
Injection speed: 260 µL/sec
Injection start time: 1 and 13 sec

For data calculation, the relative luminescence units were averaged over two ranges:

- Range 1 – Firefly luminescence (cycles 5-26 or 2 -12.5 secs)
- Range 2 – *Renilla* luminescence (cycles 29-48 or 14-23.5 secs)

Results and Discussion

Figure 2 shows a typical signal curve of the experiment.

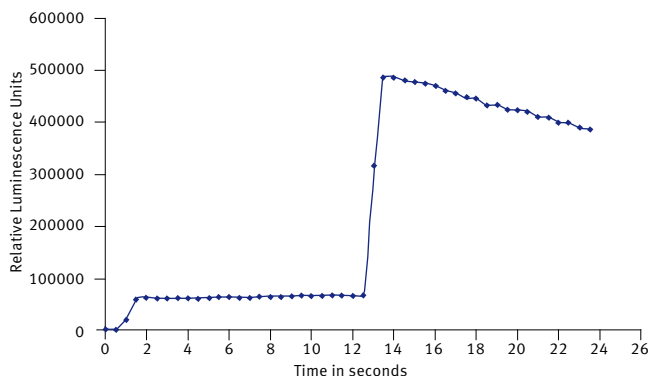


Fig. 2: Typical signal curve for the DLR assay. The substrate for the Firefly luciferase was injected in cycle 1, whereas the substrate for the *Renilla* enzyme was injected after 13 seconds.

Cells were harvested at 4, 8 and 24 hours post-electroporation, and analysed for Firefly and *Renilla* luciferase activity.³ Readings taken at 4 hours allowed establishment of input levels of RNA (Figs. 3 and 4).

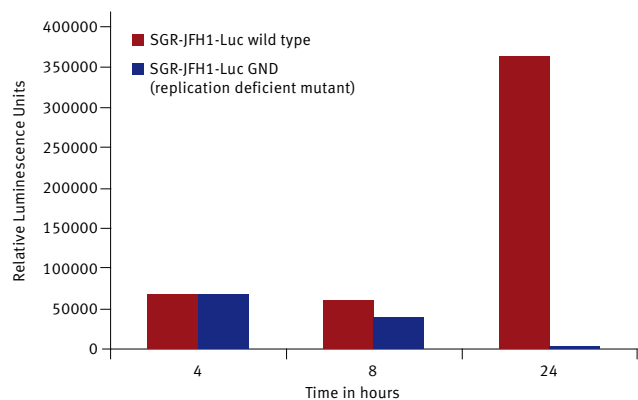


Fig. 3: Time course of SGR-JFH1-Luc wild type and replication deficient mutant (GND) luciferase levels over 24 hours

For the first 4-8 hours translation of input SGR-JFH1-Luc RNA takes place, however, between 8 and 24 hours levels of luciferase increased 6 fold, indicating replication of the wild type replicon RNA, contrasting with luciferase levels of a replication deficient mutant (GND) which shows a decrease in luciferase activity after 8 hours (Fig. 3). Translation of RLTK RNA took place throughout the 24 hour time period but due to lack of replication and degradation

of the RNA, the luciferase signal dropped over time (Fig. 4). A signal, however, was detectable at 24 hours allowing assessment of cellular translation.

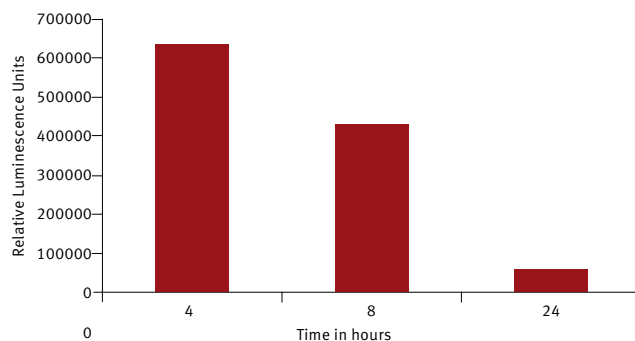


Fig. 4: Time course of RLTK RNA translation over 24 hours

In order to assess the effects of various compounds on replication rather than translation, compounds were added 8 hours after electroporation and cells were incubated for a further 16 hours. Untreated cells were harvested at 4 hours (for normalisation of input RNA) and both treated and untreated cells were harvested at 24 hours to detect effects on replication (data not shown).

Conclusion

This assay allows evaluation of the effects of compounds on early stage replication events and on cellular translation. The 96 well plate format allows a number of compounds of varying concentrations to be tested simultaneously against cells derived from a single electroporation event.

The assay was successfully performed on the FLUOstar OPTIMA. All OPTIMA instruments are certified by Promega for the Dual Luciferase Reporter (DLR™) gene assay.

References

- 1) Targett-Adams, P., and McLauchlan, J. (2005) Development and characterization of a transient-replication assay for the genotype 2a hepatitis C virus subgenomic replicon. *J. Gen Virol* **86**, 3075-3080.
- 2) Promega, Corp. Dual Luciferase Reporter Assay Technical Manual (TM046) (8/06)
- 3) BMG LABTECH, Dual Luciferase Reporter (DLR) Assay Certification on the Omega Series of Readers

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