

A HIGHLY SENSITIVE REAL-TIME PCR ASSAY FOR THE DETECTION OF HCV RNA IN CELLS

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Abstract

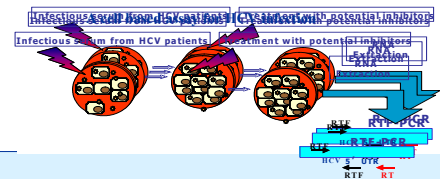
In vitro cultures of human cell lines capable of supporting HCV propagation are critical for understanding viral infection and pathogenesis, as well as for evaluating anti-HCV compounds. We took the approach of infecting human hepatoma cell-lines with HCV sera derived from HCV patients. Currently, viral infection is monitored by measuring total cellular RNA, followed by qualitative detection of HCV RNA via end point RT-PCR.

The need for specific detection of low abundance RNA and quantitative analysis directed us to develop a highly sensitive real-time PCR assay specific for HCV. The assay is based on TaqMan method utilizing specific primers for the 5' untranslated region of HCV and a Fam-Tamra labeled probe, which covers most of the HCV genotypes. Reagents that constitute the reaction mixture were optimized using cells spiked with HCV. For standardization of the extraction protocol and PCR amplification we included a house keeping gene analysis; human β -actin, which was performed alongside the HCV reaction. In order to determine absolute viral copy number, cells were spiked with a calculated HCV viral titer and a standard curve was generated for each experiment. The dynamic range of the optimized assay was between 10^0 - 10^6 viral RNA copies. This facilitates the detection of very few viral copies in cells infected with HCV sera relative to cells incubated with normal human sera.

This rapid and sensitive technique was utilized to analyze and compare infectivity of different human sera as well as to test activity of anti HCV compounds. Our experimental results demonstrate that real-time PCR can serve as a convenient and powerful tool for determination of HCV RNA in cell lysates and for the evaluation of potential anti HCV agents.

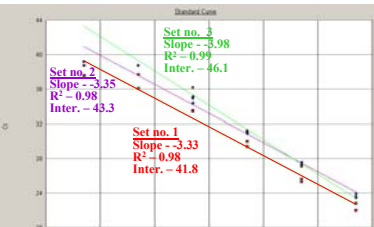
Introduction

XTL has developed a unique cell-based HCV assay in cell lines of human hepatic origin. Sera from HCV patients are used for infection of the cells: the virus binds and enters the cells, replicates and forms new infective copies capable of infecting naïve cells. This system thus mimics the in vivo life cycle of the virus and as such presents a unique opportunity for studying the virus as well as for testing potential inhibitors. In order to best utilize this system we had to develop an accurate and quantitative method for measuring HCV levels. After thorough calibration, real-time RT-PCR allows rapid and sensitive quantitation of HCV RNA. The calibration and validation of the assay are presented in this work.

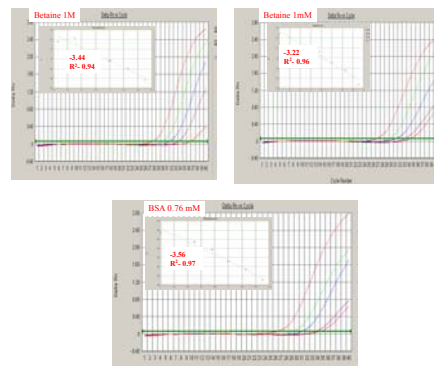


Assay calibration

Primer name	Sequence	Location
C1F	5' TGCGGAACCGGTGAGTACA 3'	5' UTR 137-155
C1R	5' CCGGTGATCCAAGAAAGGA 3'	198-179
C1P	5' CGGAATGCCAGGACACCGG 3'	156-177
C2F	5' CTGTCTCACGCAAGAGCG 3'	46-65
C2R	5' CACTCGAAGCACCTATCA 3'	301-282
C2P	5' CATGGGTTAGTATGAGTGTCTGC 3'	72-97
C3F	5' AACTACTGCTCTCACGAGAAAGC 3'	41-64
C3R	5' CCCAACACTACTCGGCTAG 3'	256-238
C3P	5' TGCGGTAGTATGAGTGTCTGC 3'	74-97



Primer optimization – Optimal detection of HCV viral RNA on cellular background required comparison between various primer-probe sets. The best set combination was no. 1 which consists of C1F, C1R and C1P as probe. This combination gives an optimal slope and the best sensitivity over the entire tested range (5×10^5 - 5×10^0). The other two combinations tested in this experiment utilized C2F and C1R, resulting in a 150 bp fragment, where either C2P (#2) or C3P (#3) served as probes. Both mixes proved less sensitive than the elected combination.



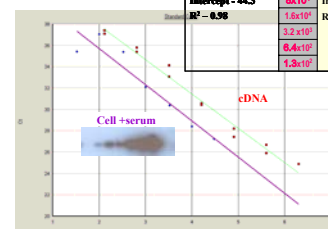
Carrier optimization - The need for a protein carrier in the reaction was tested as part of the optimization of the assay. Several carriers were tested, among them BSA, betaine and α crystalline. The experiment shown here evaluates BSA and Betaine and compares the best concentration found for BSA with two concentrations of betaine. This experiment illustrates that use of betaine was superior to BSA and that 1M of betaine in the reaction mix yielded the most efficient reaction (values of ΔR_n). As all other parameters were quite comparable between the two concentrations of betaine, further experiments were carried out with 1M betaine.

Stock Reagents	Final Concentration
PCR buffer (10x Promega)	1X
Taq-Pol (Promega)	2.5 units
MgCl ₂	4.5 mM
Betaine	1M
C1F	50 pmol
C1R	50 pmol
C1P	18 pmol
T6-ROX	600 nM
dNTP	0.4 mM each
H ₂ O	As needed

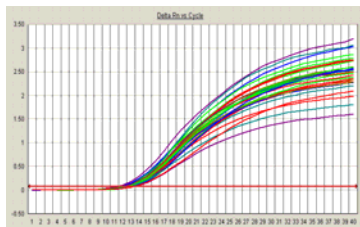
10 μ l RT sample + 40 μ l PCR mix
Cycler program (ABI-7000)
Step #1 - 5 min 95°C.
Step #2 (x40) 30 sec. 95°C and 60 sec. 60°C

Optimal concentrations of other reagents in the reaction mix were tested on cDNA curves as well as on cells spiked with diminishing amounts of HCV-positive serum, which were subjected to RNA extraction. The optimal reaction mix depicted above yielded the best slope on cDNA, with a dynamic range of 1×10^5 - 2×10^0 . The range of the serum based curve is narrower (3×10^5 - 3×10^0) however, the slope as well as other parameters tested were comparable to cDNA. There is a good correlation with results from end point PCR (utilizing a different set of primers) followed by Southern blot analysis.

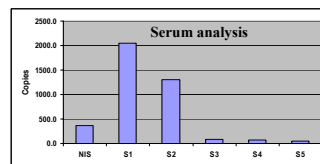
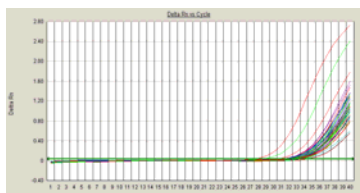
cDNA	Cells + Serum
Copies	2x10 ⁶
Slope - 3.2	4x10 ⁶
Intercept - 44.3	8x10 ⁶
R ² - 0.98	1.6x10 ⁶
	3.2x10 ⁶
	6.4x10 ⁶
	1.3x10 ⁷



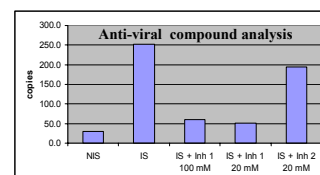
Assay validation



Cells were infected according to the protocol illustrated on top and parallel analysis of HCV viral RNA and human β -actin (ABI) was carried out. Typical amplification plots of the various samples of both β -actin and HCV reactions are presented this figure



Results of an experiment comparing infection of a hepatoma cell-line by different human sera. Two different sera were found to be infective in this experiment, while the other tested were not different then the NIS (none infective sera).



Activity of anti HCV compounds is demonstrated in this experiment. Two different doses of an anti-HCV compound reduced the viral copy number following infection while a different compound had a minor effect.

Summary

• RTF PCR provides a convenient and rapid assay for the detection of HCV RNA in cells

• It is an accurate, sensitive and quantitative assay, by which minute amounts of HCV in cells can be detected.

• The assay can serve for screening HCV positive sera for their ability to infect Hepatoma cells. It can potentially be utilized for viral detection in other cell lines.

• RTF PCR can serve as a detection method for the assessment of potential HCV inhibitors. Its high sensitivity allows differentiation and selection among various compounds.