APPLICATION NOTE

A Novel, High Sensitivity, Quantitative Hepatitis C Virus Assay

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AMPIPROBE® HCV ASSAY KIT (ENZ-GEN200)

INTRODUCTION

More than 170 million individuals are chronically infected with Hepatitis C Virus (HCV), and it is a major cause of hepatocellular carcinoma, liver cirrhosis, and liver-related mortality. Successful HCV treatment results in sustained virologic response (SVR), defined as undetectable levels of HCV RNA in the blood 12 or more weeks after completing treatment. A sensitive method to quantify HCV RNA is paramount to the management of patients undergoing antiviral therapy.

A quantitative HCV viral load assay using novel probe technology was validated for use in the quantitative detection of HCV RNA. The AMPIPROBE HCV Assay is a real-time reverse transcription quantitative polymerase chain reaction assay that incorporates probe detection technology in primer design. It is intended for the quantitative detection of HCV RNA, genotypes 1 through 6, in plasma or serum.

The limit of detection (LOD) of the assay was determined using spiked plasma or serum specimens with Acrometrix® reference materials calibrated by using the World Health Organization HCV RNA standard per guidelines described in CLSI EP17-A. The LOD of HCV RNA in EDTA plasma was determined to be as low as 0.90 log IU/mL (7.9 IU/mL) and in serum as low as 0.74 log IU/mL (5.5 IU/mL), both with a positive rate greater than 95%. For both plasma and serum, the lower limit of quantification was 1.0 log IU/mL (10 IU/mL) via 95% hit rate analysis. A reference panel was utilized to confirm that the AMPIPROBE HCV Assay was able to detect genotypes 1a, 1b, 2a, 3a, 4acd, 5a, and 6 at 15 IU/mL or greater at a 95% hit rate. The linear range was evaluated in accordance with the methods recommended in the CLSI guideline EP06-A. The linear range was determined to be from 0.7 to 7.4 log IU/mL (5 to 25,000,000 IU/mL).

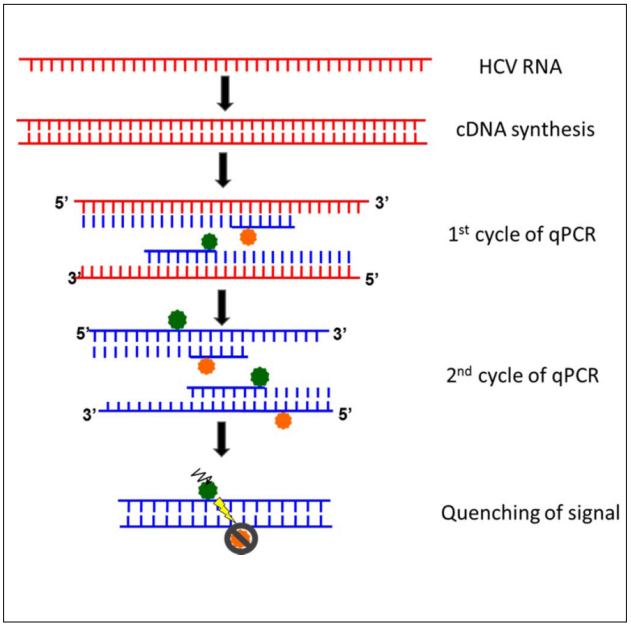
The LOD and LOQ determined for the AMPIPROBE HCV Assay are lower than that of comparable HCV viral load assays, making it a more sensitive assay suitable for monitoring viral load during antiviral therapy.

AMPIPROBE® HCV ASSAY VALIDATION

- The AMPIPROBE HCV Assay Kit results presented here were evaluated for use with the QIAGEN® QIAsymphony® SP and Rotor-Gene® Q systems.
- All validation studies were performed with an input volume of 500 µL of either EDTA plasma or serum (600 µL onboard volume).
- The validation package is currently undergoing review in the New York State Department of Health's comprehensive test approval process.
- The AcroMetrix® HCV-S panel was used for the creation of all test sample dilutions. To verify the accuracy of the panel's calibrated values it was verified with the 4th World Health Organization HCV RNA standard per guidelines described in CLSI EP17-A.

ENZO'S AMPIPROBE TECHNOLOGY

- Real-time reverse transcription quantitative PCR
- Incorporates probe detection technology in primer design
  - Fluorescent reporter-labeled primers
  - Quencher-labeled primers
- When free in solution, fluorescent primers generate a signal. However, as the primers are incorporated into amplified DNA, the quencher and the fluorophore are brought within close proximity and exhibit Förster resonance energy transfer (FRET).
- Enzo's AMPIPROBE Assay Kits provide the following benefits:
  - Compatible with open qPCR platforms
  - Smaller sample input allows remaining extracted samples to be used in other tests
  - Smaller reaction volume consumes less reagent



MATERIALS AND METHODS

AMPIPROBE HCV ASSAY (ENZ-GEN200) Kit Components
AMPIPROBE HCV Primer Mix
Negative Control
HCV High Control
HCV Medium Control
HCV Low Control
Internal Control

Other Reagents Used:

- Acrometrix® HCV-S
- SeraCare HCV Accuset™ Panel
- QIAsymphony® DSP Virus/Pathogen Midi Kit
- Qiagen QIAsymphony®
- Qiagen One-Step RT-PCR Kit
- Qiagen Rotor-Gene® Q

Sensitivity – Limit of Detection

To determine plasma or serum LOD, seven dilutions of spiked plasma samples with low concentrations (LOD1 to LOD7, 0.0 – 1.70 log IU/mL) of HCV RNA were prepared by spiking known quantity of HCV RNA (Acrometrix® 5E4) into HCV-negative plasma specimens. For plasma, nine separate extractions and PCR runs were performed on nine different days using three different reagent lots throughout testing. Four replicates of each dilution level were run each day, resulting in 36 replicate data points for each dilution. For serum, three separate extractions and PCR runs were performed on three different days using three different reagent lots each day. Three replicates of each dilution level were run each day, resulting in 6-9 replicate data points for each dilution.

Genotype Inclusivity

Genotype verification of the LOD was performed using one reference sample member from the SeraCare HCV Worldwide AccuSet™ Performance Panel for each different genotype/subtype represented in the panel (1a, 1b, 2a, 3a, 4acd, 5a, 6) for a total of seven different genotypes tested. Each panel member was used to create plasma spike in samples at 3 different concentrations (5, 15, and 45 IU/mL). Each concentration for all genotypes was performed in duplicate across 3 days using 3 different lots of all reagents.

Linear Range

The linear range was evaluated in accordance with the methods recommended in the CLSI guideline EP06-A. Nine levels of HCV RNA dilutions, ranging from 0.7 to 7.4 log IU/mL or 5 to 25,000,000 IU/mL, were prepared using a reference panel member (Acrometrix 2.5E7) straight for the highest dilution level and then creating eight other serial dilution levels into HCV RNA-negative plasma or serum. The samples were run on three separate days in triplicate each day with three different lots of reagents.

SENSITIVITY – LIMIT OF DETECTION

Probit analysis was employed to determine the plasma and serum LOD of the assay by using SPSS Software. These studies demonstrate that the AMPIPROBE HCV Assay can detect HCV RNA in EDTA plasma as low as 0.90 log IU/mL (7.9 IU/mL) and in serum as low as 0.74 log IU/mL (5.5 IU/mL), both with a positive rate greater than 95%. For both plasma and serum, the lower limit of quantification was 1.0 log IU/mL (10 IU/ mL) via 95% hit rate analysis.

Plasma Limit of Detection (LOD) of the AMPIPROBE HCV Assay

DILUTION	EXPECTED HCV RNA (Log IU/mL)	EXPECTED HCV RNA (IU/mL)	NO. OF SAMPLE TESTED	NO. OF SAMPLE POSITIVE	POSITIVITY (%)	PROBABILITY*
LOD1	1.70	50.0	36	36	100.0	1.000
LOD2	1.40	25.0	36	36	100.0	1.000
LOD3	1.18	15.0	36	36	100.0	1.000
LOD4	1.00	10.0	36	35	97.2	0.992
LOD5	0.70	5.0	36	25	69.4	0.719
LOD6	0.40	2.5	36	20	55.6	0.370
LOD7	0.00	0.0	36	0	0.00	0.106

* LOD: 0.898 log IU/mL (7.91 IU/mL) via 95% Probit analysis
LOQ: 1.00 log IU/mL (10 IU/mL) via 95% hit rate analysis

Serum Limit of Detection (LOD) of the AMPIPROBE HCV Assay

DILUTION	EXPECTED HCV RNA (Log IU/mL)	EXPECTED HCV RNA (IU/mL)	NO. OF SAMPLE TESTED	NO. OF SAMPLE POSITIVE	POSITIVITY (%)	PROBABILITY*
LOD1	1.70	50.0	9	9	100.0	1.000
LOD2	1.40	25.0	9	9	100.0	1.000
LOD3	1.18	15.0	9	9	100.0	1.000
LOD4	1.00	10.0	9	9	100.0	1.000
LOD5	0.70	5.0	9	8	88.9	0.911
LOD6	0.40	2.5	9	4	44.4	0.390
LOD7	0.00	0.0	9	0	0.00	0.028

* LOD: 0.737 log IU/mL (5.46 IU/mL) via 95% Probit analysis
LOQ: 1.00 log IU/mL (10 IU/mL) via 95% hit rate analysis

GENOTYPE INCLUSIVITY

For genotype inclusivity, the AMPIPROBE HCV Assay detected the seven different genotypes/subtypes at concentrations of 15 IU/mL or greater with a hit rate of ≥ 95%.

Plasma Limit of Detection (LOD) Across HCV Genotypes

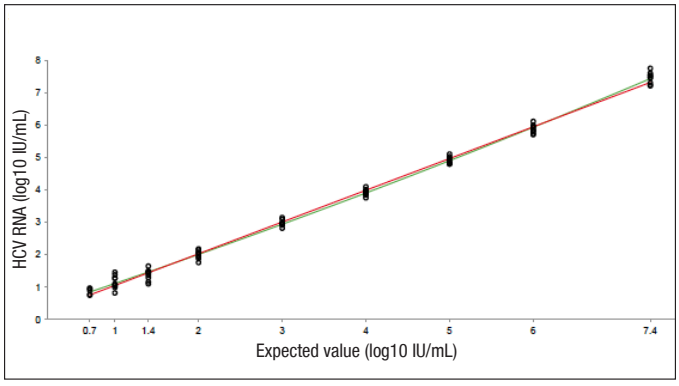
	5 IU/mL*			15 IU/mL*			45 IU/mL*		
GENOTYPE	No. of Replicates	No. of Positive	Hit Rate	No. of Replicates	No. of Positive	Hit Rate	No. of Replicates	No. of Positive	Hit Rate
1a	6	5	83%	6	6	100%	6	6	100%
1b	6	3	50%	6	6	100%	6	6	100%
2a	6	6	100%	6	6	100%	6	6	100%
3a	6	5	83%	6	6	100%	6	6	100%
4acd	6	4	67%	6	6	100%	6	6	100%
5a	6	4	67%	6	6	100%	6	6	100%
6	6	3	50%	6	6	100%	6	6	100%

* HCV RNA concentration for spiking was initially determined by AMPIPROBE HCV Assay.

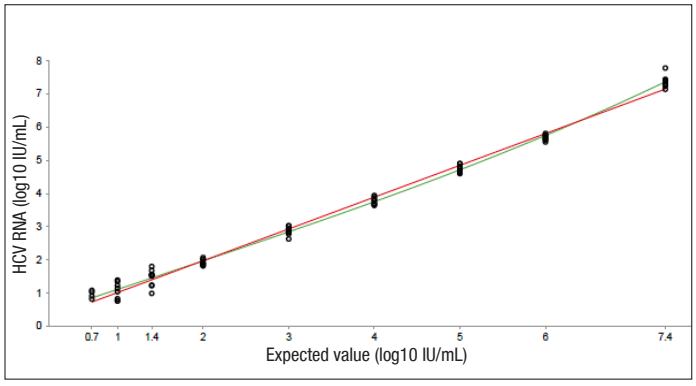
LINEAR RANGE

The linearity of plasma and serum was analyzed using the CSLI StatisPro™. The AMPIPROBE HCV Assay demonstrated a linear response from 0.7 to 7.4 log IU/mL (5 to 25,000,000 IU/mL).

AMPIPROBE HCV Assay Plasma Linearity Plot



AMPIPROBE HCV Assay Serum Linearity Plot



CONCLUSION

A sensitive, real-time reverse transcription polymerase chain reaction assay is recommended to detect HCV RNA levels during treatment with direct-acting antiviral agents. Compared to HCV viral load assays available, the LOD and LOQ determined for the AMPIPROBE HCV Assay are lower, making it a more sensitive assay suitable for monitoring viral load during antiviral therapy.

Visit www.enzolifesciences.com for more information about our AMPIPROBE HCV Assay Kit.

NOTES

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