Product Manual

AMPIPROBE® HCV Assay Kit

Catalog #: ENZ-GEN200-0100

100 Assays

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Product Manual

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Please read entire booklet before proceeding with the assay.

Carefully note the handling and storage conditions of each kit component.

Please contact Enzo Life Sciences Technical Support if necessary.

DESCRIPTION

The AMPIPROBE® HCV Assay is a quantitative reverse transcription polymerase chain reaction (RT-qPCR) assay for the quantitative detection of human hepatitis C virus (HCV) RNA in plasma or serum. The proprietary primer mix included in the kit is specific for HCV genotypes 1 through 6. The kit includes titered high, medium, and low HCV controls which enable virus quantitation in IU/mL.

INTRODUCTION

HCV is a single-stranded, positive sense RNA virus with a genome of approximately 9,500 nucleotides coding for 3,000 amino acids[1]. HCV exists as six closely related, yet distinct genotypes[2].

HCV infects about 4 million individuals in the United States[3] and about 170 million individuals worldwide[4]. Acute HCV infection will most often progress to chronic infection. If left untreated, the disease may progress to liver fibrosis, cirrhosis, decompensated liver disease, and death. In addition, 25% of all cirrhotic patients will also develop hepatocellular carcinoma (HCC)[5,6].

Quantifying HCV RNA is a well-established method for measuring baseline viral loads and response to treatment. HCV RNA can be detected in plasma or serum by extraction and amplification of nucleic acid. The AMPIPROBE® HCV Assay Kit uses a novel probe system to accurately quantify HCV RNA.

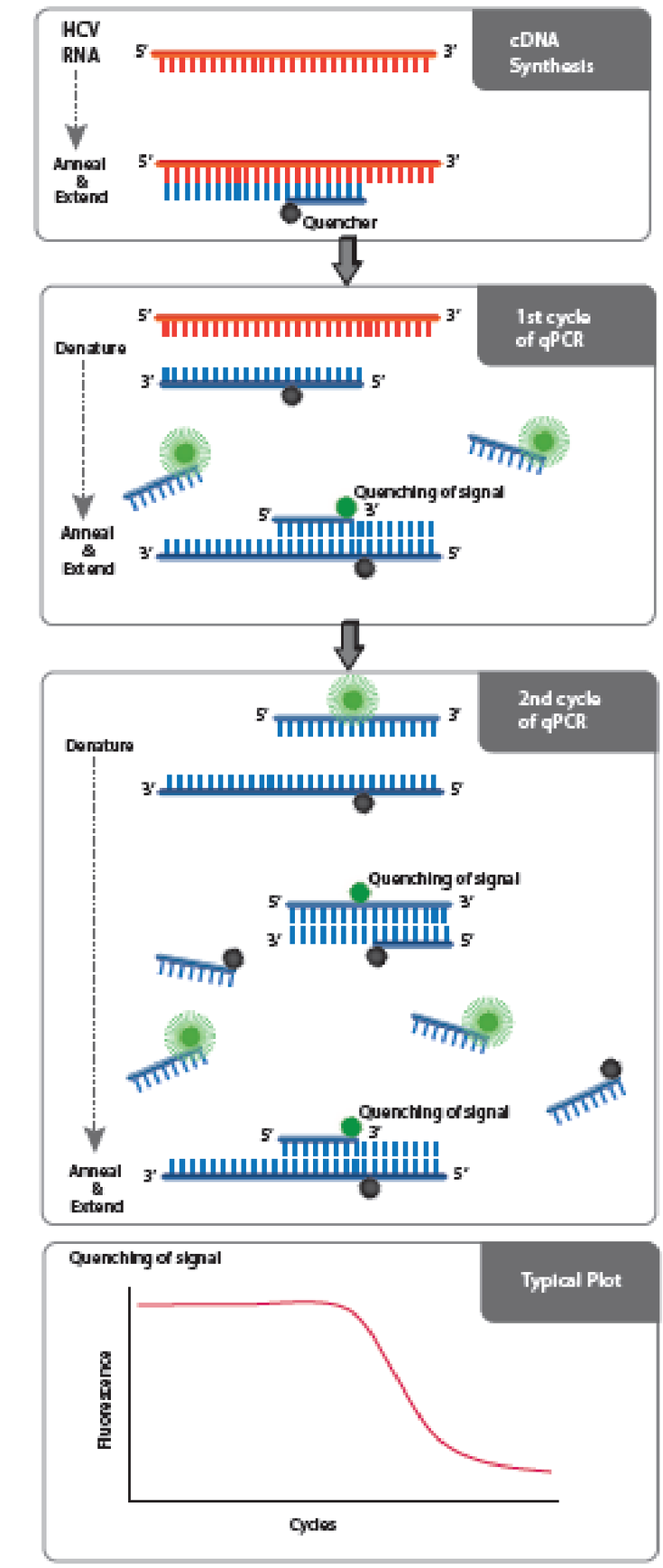
AMPIPROBE® TECHNOLOGY

Enzo's AMPIPROBE® technology incorporates probe detection technology in primer design. It employs a combination of fluorescent reporter-labeled primers and quencher-labeled primers to amplify DNA, akin to traditional PCR. 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). This causes a logarithmic decay of signal with respect to the number of amplification cycles of DNA. Once the signal decays to a defined threshold, a value is generated with respect to the corresponding cycle. The threshold cycle is indicative of the amount of target RNA or DNA in the sample.

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 reagents

AMPIPROBE® HCV ASSAY



PRINCIPLE OF AMPIPROBE® HCV ASSAY

The AMPIPROBE® HCV Assay utilizes quantitative reverse transcription PCR. In the first step of the reaction, the viral RNA template is used to create complimentary DNA (cDNA) through reverse transcription. Once the cDNA template is created, PCR amplification occurs using primers that target sequences within the highly conserved region of the 5'-untranslated region of the HCV genome. The nucleotide sequence of the primers has been optimized to yield amplification of HCV genotypes 1 through 6.

The HCV primers have been designed using AMPIPROBE® technology which incorporates reporter and quencher dyes into the primers. The detection of amplification is based on fluorescence decay. The corresponding cycle number at which fluorescence decays below a defined threshold is known as the threshold cycle, **Ct**. This is used to quantify HCV RNA with respect to a set of standards with predefined viral load.

An internal control serves as an extraction and amplification control for each individually processed sample.

SAFETY WARNINGS & PRECAUTIONS

FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.

- The AMPIPROBE® HCV Primer Mix is photosensitive and direct light should be avoided whenever possible.
- Some components of this kit may contain hazardous substances. Reagents can be harmful if ingested or absorbed through the skin and may cause irritation to the eyes. Reagents should be treated as possible mutagens and should be handled with care and disposed of properly.
- RNases are found in all cell types and generally have high specific activity. In order to limit RNase contamination wear gloves at all times. Change gloves frequently, especially after using them to touch potential RNase contaminated surfaces such as keyboards and door knobs.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas. All blood components and biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

MATERIALS SUPPLIED

Reagent	Quantity	Vial Cap Color
AMPIPROBE® HCV Primer Mix	200 µL	Clear
Negative Control	4 x 600 µL	Green
HCV High Control	4 x 600 µL	Red
HCV Medium Control	4 x 600 µL	Yellow
HCV Low Control	4 x 600 µL	Blue
Internal Control	1215 µL	Purple

STORAGE

All components of this kit are stable at -80°C until the kit's expiration date.

OTHER MATERIALS NEEDED

1. QIAsymphony DSP Virus/Pathogen Midi kit (QIAGEN, Cat# 937055) or similar
2. QIAGEN QIAsymphony or similar
3. One-Step RT-PCR kit (QIAGEN, Cat# 210212) or similar (AMPIGENE® qPCR 1-Step RT-PCR Kit, Enzo Cat# ENZ-KIT105)
4. PCR tubes, 0.2 mL (QIAGEN Cat# 981005) or similar
5. Loading Block, 96 x 0.2 mL tubes (QIAGEN Cat# 9018905) or similar
6. 36-Well Rotor (QIAGEN Cat# 9018907) or similar
7. 36-Well Rotor Locking Ring (QIAGEN Cat# 9018906) or similar
8. QIAGEN Rotor-Gene Q or similar
9. DNase/RNase free tubes
10. DNase/RNase free pipet tips

PROCEDURAL NOTES

Do not mix components from different kit lots or use reagents beyond the kit expiration date.

SAMPLE COMPATIBILITY

AMPIPROBE® HCV Assay Kit is compatible with HCV RNA extracted from plasma or serum samples.

RNA ISOLATION

The procedure described below is optimized using the QIAsymphony DSP Virus/Pathogen Midi kit (QIAGEN 937055). Any commercial RNA/DNA isolation kit can be used and optimization may be required.

1. Equilibrate samples and controls to room temperature for 30 minutes. Transfer 0.6 mL serum or plasma from each sample into a labeled 2 mL sample tube.
2. Thaw previously frozen carrier RNA solution or make fresh carrier RNA solution by dissolving the contents of one vial of carrier RNA in 1.35 mL AVE solution. Unused carrier RNA solution may be stored at -80°C.
3. Prepare sufficient internal control for up to 12 samples in a 2 mL DNase/RNase free tube according to the volumes of reagents given in **Table 1**. If more than 12 samples are being run, use more than one 2 mL tube to prepare the internal control. Include excess reagents equivalent to 3 samples per tube to allow the QIAsymphony to easily pipette. For example, for 24 samples, prepare sufficient internal control for 30 isolations.

   Vortex gently and spin down the liquid, the tube will be loaded as internal controls in nucleic acid isolation run.

Table 1. Preparation of Internal Control

Reagent	Volume per sample	Total volume required*
AVE Buffer	106 µL	106 µL x (N+3)
Carrier RNA	5 µL	5 µL x (N+3)
Internal Control	9 µL	9 µL x (N+3)

** Total volume required for N + 3 reactions, where N is the number of samples including controls.*

4. Carry out nucleic acid extraction according to the manufacturer's instructions. If using QIAsymphony DSP Virus/Pathogen Kit, refer to QIAsymphony Cellfree500 DSP Protocol Sheet. This protocol sheet is available at www.QIAGEN.com/goto/dsphandbooks.

REAGENT PREPARATION

1. Thaw the following **QIAGEN One-step RT-PCR Kit** reagents at room temperature (15 - 25ºC): OneStep RT-PCR Buffer, dNTP Mix, and RNase-free Water. Once thawed, vortex briefly and spin down to collect the contents at the bottom of the tubes. Keep tubes on ice until use.
2. Thaw the **AMPIPROBE® HCV Primer Mix** at room temperature while avoiding light exposure. Once thawed, keep tube on ice and avoid exposure to light until use.
3. Pre-cool the loading block at 4°C for at least one hour.

RT-PCR SETUP

The procedure described below uses QIAGEN Rotor-Gene Q instrument, but other real-time PCR machines may also be used.

For each assay run, include the following controls:

- Negative Control
- HCV High Control
- HCV Medium Control
- HCV Low Control

1. Immediately prior to use, prepare sufficient **RT-PCR Master Mix** for the RNA samples and controls in a labeled, DNase/RNase free tube according to the volumes given in **Table 2**, adding the reagents to the tube in the order listed. Include reagents for 2 extra reactions to allow for any pipetting variance.

   The **RT-PCR Master Mix** contains all of the components needed for the PCR reaction except the RNA template (sample).
2. Thoroughly mix the **RT-PCR Master Mix** by inverting the tube for 15 times. If glycerol swirls are still visible, spin briefly and pipette up and down to mix. Centrifuge briefly to bring contents to the bottom of the tube, avoiding light exposure.
3. Dispense 25 µL of **RT-PCR Master Mix** and 25 µL of extracted sample or control into each of 0.2 mL QIAGEN PCR tubes. Mix by pipetting up and down 10 times. Ensure there are no bubbles at the bottom of the tubes as this may interfere with readings.
4. Insert the tubes in the thermal cycler.

Table 2. Preparation of RT-PCR Master Mix*

Reagent	Volume per reaction	Total volume required*
RNase-free Water	9 µL	9 µL x (N+2)
QIAGEN OneStep RT-PCR Buffer (5x)	10 µL	10 µL x (N+2)
dNTP mix	2 µL	2 µL x (N+2)
AMPIPROBE® HCV Primer Mix	2 µL	2 µL x (N+2)
QIAGEN OneStep RT-PCR Enzyme Mix	2 µL	2 µL x (N+2)
TOTAL	**25 µL**	**25 µL x (N+2)**

** Total volume required for **N + 2** reactions, where **N** is the number of samples including controls per assay run. When preparing the RT-PCR Master Mix, prepare enough for 2 extra reactions to allow for any pipetting variance.*

RT-PCR RUN

Create a temperature profile on your instrument as summarized in **Table 3** (RT-PCR Program Parameters). The listed parameters in the table have been verified using a QIAGEN Rotor-Gene Q instrument.

Table 3. RT-PCR Program Parameters

Step	Parameter	Temp [ºC]	Cycles	Hold [mm:ss]
Hold #1	Reverse Transcription	50	1	30:00
Hold #2	Enzyme Activation	95	1	15:00
Cycling	Amplification	95	45	00:15
		65		01:00 (fluorescent signal detection)

Fluorescence is detected at the end of the 2nd segment in the Cycling Step (65°C) using FAM/Green and Cy5/Red channels.

INSTRUMENT SETTINGS

The following settings are recommended for use with the QIAGEN Rotor-Gene Q. Perform gain optimization based on a negative control according to **Table 4**. Set the instrument to perform optimization before 1st acquisition.

Table 4. Gain Optimization Settings

Channel	Min Reading	Max Reading	Min Gain	Max Gain
FAM/Green	55Fl	65Fl	-10	10
Cy5/Red	55Fl	65Fl	-10	10

DATA ANALYSIS

The fluorescent signal intensity is detected in two channels:

- The signal from the HCV amplification product is detected in the FAM/Green channel.
- The signal from the Internal Control amplification product is detected in the Cy5/Red channel.

The results are interpreted with the instrument software by generating a cycle number at which the threshold line crosses the output amplification curve.

Using QIAGEN Rotor-Gene Q software, invert the raw data and convert to linear display. See **Table 5** for cycle elimination setting.

Table 5. Cycle Elimination Setting

Channel	Eliminate Cycles Before	Threshold	Slope Correct
FAM/Green	10	0.0	Off
Cy5/Red	10	0.01	Off

INTERPRETATION OF RESULTS

- A standard curve is drawn using lot-specific titer values associated with the HCV High, Medium, Low controls. The viral titer of unknown samples is interpolated based on the generated standard curve. HCV High, Medium, Low controls should form linear standard curve with $\mathbf{R^2} > 0.95$. If $\mathbf{R^2}$ is not met, run is invalid and should be repeated.
- The negative control must generate no **Ct** value in the green channel, but must have a **Ct** value of <30 in the red channel.
- For all standards and samples, there must be a **Ct** value generated in the red channel in order for the result to be valid. The internal control must have a **Ct** value <30.

Table 6. Results for Controls

Control	Stage for Control	FAM/ Green	Cy5/Red	Interpre-tation
Negative Control	Isolation	NEG	POS	Valid result
HCV High/Medium/ Low Controls	Isolation and Amplification	POS	POS	Valid result

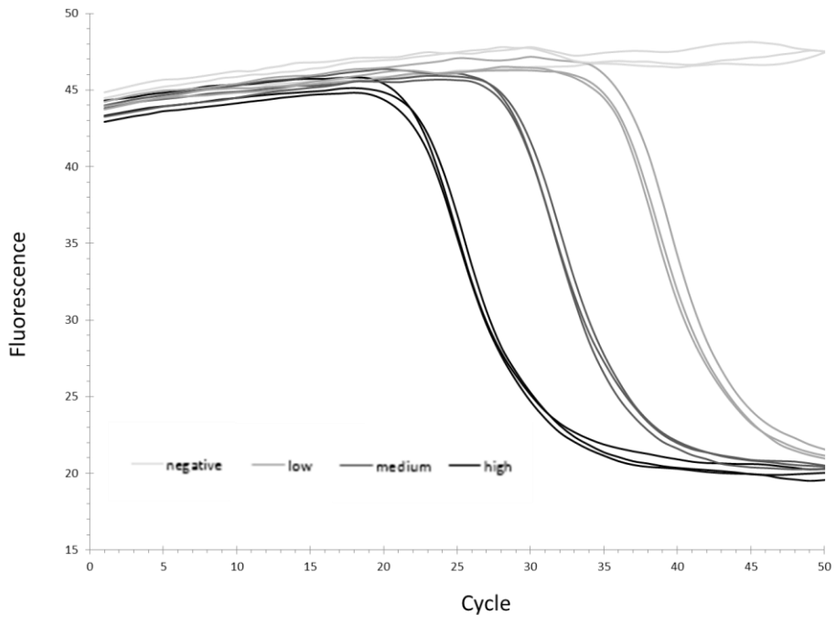
PERFORMANCE CHARACTERISTICS

Analytical sensitivity will vary based on extraction method and platform used.

In a New York State Department of Health approved validation, the AMPIPROBE® HCV Assay Kit was found to have the following characteristics:

- LOD serum = 5.46 IU/mL via 95% Probit analysis
- LOD plasma = 7.91 IU/mL via 95% Probit analysis
- LOQ serum = 10 IU/mL via 95% hit rate analysis
- LOQ plasma = 10 IU/mL via 95% hit rate analysis
- Linear range = 5 - 2.5e7 IU/mL

REPRESENTATIVE PLOT



Representative plot of typical data in the FAM channel, using high, medium, and low quantitation controls, and the negative control.

FREQUENTLY ASKED QUESTIONS

Can this assay be run on an ABI 7500?

Yes, most thermal cyclers can be used, provided the software is able to detect a negative (loss of) signal.

What is the composition of the HCV Low, Medium, and High Controls? Do they contain live virus?

The HCV Low, Medium, and High Controls are HCV RNA template and do not contain live virus. However, all reagents should be handled with care and treated as potentially infectious or hazardous.

What is the composition of the Negative Control?

The Negative Control is HCV-negative human serum. All reagents should be handled with care and treated as potentially infectious or hazardous.

Can the HCV Low, Medium, and High Controls be purchased separately?

Yes, the titered HCV Low, Medium, and High Controls can be purchased separately as the AMPIPROBE® HCV Control Set, ENZ-GEN201.

Can the reaction volume be reduced to conserve reagents?

No, the reaction volume is a key factor in the sensitivity of the assay. If the reaction volume is reduced, the sensitivity will be decreased.

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