

PrimerDesign™ Ltd

Quantification of Hepatitis C Virus (HCV) genomes

Core Protein Region

For general laboratory and research use only

Advanced Kit





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Introduction to Hepatitis C Virus

The hepatitis C virus (HCV) is a small, enveloped, single-stranded, positive sense RNA virus in the family Flaviviridae. HCV mainly replicates within hepatocytes in the liver, although there are clear evidence for replication in lymphocytes or monocytes. Circulating HCV particles bind to receptors on the surfaces of hepatocytes and subsequently enter the cells. Two putative HCV receptors are CD81 and human scavenger receptor class BI (SR-BI). However, these receptors are found throughout the body. The identification of hepatocyte-specific cofactors that determine observed HCV liver tropism are currently under investigation.

Once inside the hepatocyte, HCV utilizes the intracellular machinery necessary to accomplish its own replication.[1] Specifically, the HCV genome is translated to produce a single protein of around 3011 amino acids. This "polyprotein" is then proteolytically processed by viral and cellular proteases to produce three structural (virion-associated) and seven nonstructural (NS) proteins. Alternatively, a frameshift may occur in the Core region to produce an Alternate Reading Frame Protein (ARFP). HCV encodes two proteases, the NS2 cysteine aurotease and the NS3-4A serine protease. The NS proteins then recruit the viral genome into an RNA replication complex, which is associated with rearranged cytoplasmic membranes. RNA replication takes place via the viral RNA-dependent RNA polymerase of NS5B, which produces a negative-strand RNA intermediate. The negative strand RNA then serves as a template for the production of new positive-strand viral genomes. Nascent genomes can then be translated, further replicated, or packaged within new virus particles. New virus particles presumably bud into the secretory pathway and are released at the cell surface.

Based on genetic differences between HCV isolates, the hepatitis C virus species is classified into six genotypes with several subtypes within each genotype. Subtypes are further broken down into quasispecies based on their genetic diversity. The preponderance and distribution of HCV genotypes varies globally. For example, in North America, genotype 1a predominates followed by 1b, 2a, 2b, and 3a. In Europe, genotype 1b is predominant followed by 2a, 2b, 2c, and 3a. Genotypes 4 and 5 are found almost exclusively in Africa. Genotype is clinically important in determining potential response to interferon-based therapy and the required duration of such therapy. Genotypes 1 and 4 are less responsive to interferon-based treatment than are the other genotypes (2, 3, 5 and 6).[2]

References

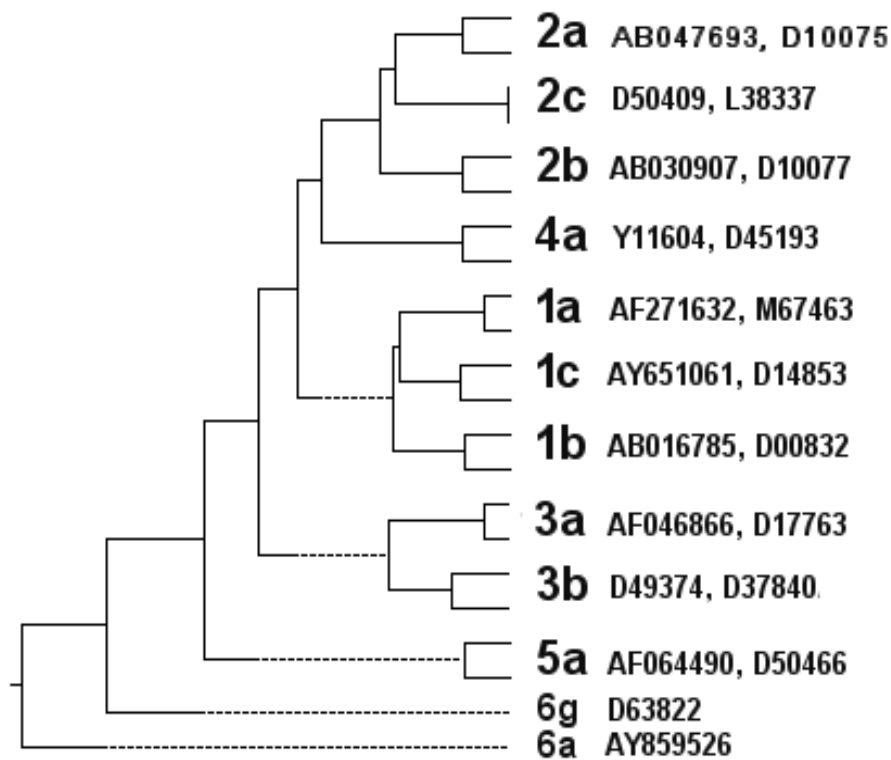
- 1: Lindenbach B, Rice C (2005). "Unravelling hepatitis C virus replication from genome to function.". *Nature* 436 (7053): 933-8. PMID 16107832.
- 2: Simmonds P, Bukh J, Combet C, Deléage G, Enomoto N, Feinstone S, Halfon P, Inchauspé G, Kuiken C, Maertens G, Mizokami M, Murphy D, Okamoto H, Pawlotsky J, Penin F, Sablon E, Shin-I T, Stuyver L, Thiel H, Viazov S, Weiner A, Widell A (2005). "Consensus proposals for a unified system of nomenclature of hepatitis C virus genotypes.". *Hepatology* 42 (4): 962-73. PMID 16149085.



Specificity

The *PrimerDesign™ Quantification Kit for Hepatitis C virus (HCV) Genomes* is designed for the *in vitro* quantification of all HCV subtypes. The primers have 100% homology with all reference sequences included in the phylogenetic tree below and therefore have a very broadest detection profile. However, due to the inherent instability of RNA viral genomes, it is not possible to guarantee detection of all clinical isolates.

Table I: Accession numbers for detected HCV isolates





Kit Contents

- Pathogen specific primer/probe mix (150 reactions **BROWN**)
- Pathogen positive control template (for Standard curve **RED**)
- Internal extraction control RNA (150 reactions **BLUE**)
- Internal extraction control primer/probe mix (150 reactions **BROWN**)
- Endogenous ACTB primer/probe mix (150 reactions **BROWN**)
- Internal extraction control/Pathogen/ACTB RT primer mix (150 reactions **GREEN**)
- RNase/DNase free water

Reagents and equipment to be supplied by the user

- Real-Time PCR Instrument
- Mastermix or Mastermix components
This kit is designed to work well with all commercially available Mastermixes. However, we recommend the use of PrimerDesign 2x Precision™ Mastermix.
- Pipettors and Tips
- Vortex and centrifuge
- Thin walled 1.5 ml PCR reaction tubes

Kit storage and stability

This kit is stable at room temperature. Once the lyophilised components have been re-suspended the kit should be stored at -20°C. Unnecessary repeated freeze/thawing should be avoided. Under these conditions reagents are stable for six months from date of purchase.



Suitable sample material

All kinds of sample material suited for PCR amplification can be used. Please ensure the samples are suitable in terms of purity, concentration, and RNA/DNA integrity (An internal PCR control is supplied to test for non specific PCR inhibitors). Always run at least one negative control with the samples. To prepare a negative-control, replace the template RNA sample with RNase/DNase free water.

Dynamic range of test

Under optimal PCR conditions PrimerDesign pathogen detection kits have very high priming efficiencies of >95% and can detect between 1×10^8 and 1×10^2 copies of target template.



Principles of the test

Real-time PCR

A pathogen specific primer and probe mix is provided and the target sequence can be detected through the **FAM** channel.

The primer and probe mix provided exploits the so-called TaqMan[®] principle. During PCR amplification, forward and reverse primers hybridize to the pathogen DNA/cDNA. A fluorogenic probe, is included in the same reaction mixture which consists of an oligonucleotide labeled with a 5'-reporter dye and a downstream, 3'-quencher. During PCR amplification, the probe is cleaved and the reporter dye and quencher are separated. The resulting increase in fluorescence can be detected on a range of real-time PCR platforms.

Positive control

For copy number determination, and as a positive control for the PCR set up, the kit contains positive control template. This can be used to generate a standard curve of pathogen copy number / CT value. Alternatively the positive control can be used at a single dilution for a qualitative analysis of the samples. Each time the kit is used, at least one positive control reaction must be included on the run. A positive result indicates that the primers and probes for quantification of the target pathogen gene are working properly in your particular experimental scenario. If a negative result is obtained the test results are invalid and must be repeated. Care should be taken to ensure that the positive control does not contaminate any other kit component which would lead to false positive results. This can be achieved by handling this component in a Post PCR environment.

Negative control

To confirm absence of contamination, a negative control reaction should be included every time the kit is used. For this reaction, the RNase/DNase free water should be used instead of template. A negative result indicates that the reagents have not become contaminated while setting up the run. If a positive result is obtained the results should be ignored and the test samples repeated. Possible sources or contamination should first be explored and removed.



Internal RNA extraction control

When performing RNA extraction, it is often advantageous to have an exogenous source of RNA template that is spiked into the lysis buffer. This control RNA is then co-purified with the sample RNA and can be detected as a positive control for the extraction process. Successful co-purification and real-time PCR for the control RNA also indicates that PCR inhibitors are not present at a high concentration.

A separate RT primer mix and a real-time PCR primer/probe mix are supplied with this kit to detect the exogenous RNA using real-time PCR. The PCR primers are present at PCR limiting concentrations which allows multiplexing with the target sequence primers. Amplification of the control cDNA does not interfere with detection of the pathogen target cDNA even when present at low copy number. The Internal control is detected through the **VIC** channel and gives a CT value of 26+/-3.

Endogenous ACTB control

To confirm extraction of a valid biological template a primer and probe mix is included to detect the Actin Beta (ACTB) gene. Detection of ACTB is through the FAM channel and it is **NOT** therefore possible to perform a multiplex for ACTB and the pathogen primers. A poor ACTB signal may indicate that the sample did not contain sufficient human material.

Carry-over prevention using UNG (optional)

Carry over contamination between PCR reactions can be prevented by including uracil-N-glycosylase (UNG) in the reaction mix. Some commercial mastermix preparations contain UNG or alternatively it can be added as a separate component. UNG can only prevent carry over from PCR reactions that include deoxyuridine triphosphate (dUTP) in the original PCR reaction. PrimerDesign recommend the application of 0.2 U UNG per assay with a 15 minute incubation step at 37°C prior to amplification. The heat-labile UNG is then inactivated during the Taq polymerase activation step (95°C for 10 minutes).



Bench side Protocol

To minimize the risk of contamination with foreign DNA, we recommend that all pipetting be performed in a PCR clean environment. Ideally this would be a designated PCR cabinet. Filter tips are recommended for all pipetting steps.

1. Pulse-spin each tube in a centrifuge before opening.

This will ensure lyophilised primer and probe mix is in the base of the tube and is not spilt upon opening the tube.

2. Reconstitute the kit components according to the table below

To ensure complete resuspension, vortex each tube thoroughly, allow to stand for 5 minutes and vortex again before use.

Component	Volume
Pre-PCR box	
Pathogen Primer/Probe mix (BROWN)	165 µl
Internal extraction control RNA (BLUE)	600 µl
Internal extraction control primer/probe mix (BROWN)	165 µl
Internal extraction control/Pathogen/ACTB RT primer mix (GREEN)	165 µl
Endogenous ACTB primer/probe mix (BROWN)	165 µl
Post PCR bottle	
Positive Control Template (RED) *	500 µl

* This component contains high copy number template and is a VERY significant contamination risk. It must be opened and handled in a separate laboratory environment, away from the other components.

RNA extraction

The internal extraction control RNA can be added either to the RNA lysis/extraction buffer or to the RNA sample once it has been resuspended in lysis buffer.

DO NOT add the internal extraction control RNA directly to the biological sample as this will lead to degradation and a loss in signal strength.

1. Add 4µl of the Internal extraction control RNA (**BLUE**) to each sample in RNA lysis/extraction buffer

2. Complete RNA extraction according to the manufactures protocols



Reverse Transcription

The protocols below are for PrimerDesign *Precision*TM Reverse Transcription kit. If you are using an alternative RT kit then follow the manufacturer's instruction.

1. For each RNA sample prepare a reaction mix according to the table below:

Component	1 reaction
RNA template	X μ l
Internal extraction control/Pathogen/ACTB RT primer mix (GREEN)	1.0 μ l
dNTP mix 10mM of each (ORANGE)	1.0 μ l
RNAse/DNAse free water (WHITE)	X μ l
Final volume	10 μl

2. Heat samples to 65°C for 5 minutes.

This can be achieved using a thermostatically controlled “hot block” or heated water bath. Do not heat the RNA to a higher temperature as this can lead to RNA degradation.

3. Immediately cool the tubes in an ice water bath.

It is important that the samples are transferred directly from 65°C to the ice. Do not allow the samples to cool prior to cooling on ice.

4. For each RNA sample prepare a reaction mix according to the table below:

Component	1 reaction
MMLV 5Xbuffer (CLEAR)	4.0 μ l
RNA/DNAse free water (WHITE)	5.2 μ l
MMLV enzyme (CLEAR)	0.8 μ l
Final volume	10 μl

5. Add 10 μ l of this mix to each of the samples on ice.

6. Apply a lid to each sample, mix tubes by briefly vortexing and incubate at 42°C for 60 minutes.

This can be achieved using a thermostatically controlled “hot-block” or heated water bath

7. Store cDNA samples at -20°C until use.



Real-time PCR detection

1. Prepare a reaction mix according to the tables below:

Include sufficient reactions for the standard curve wells (8 samples in duplicate) and also the negative control.

Pathogen detection mix

Component	l reaction
2X Precision™ Mastermix	10 µl
Pathogen primer/probe mix (BROWN)	1.0 µl
Internal extraction control primer/probe mix (BROWN)	1.0 µl
RNase/DNase Free water	3.0 µl
Final volume	15 µl

Endogenous ACTB detection mix (Optional)

Component	l reaction
2X Precision™ Mastermix	10 µl
Endogenous ACTB primer/probe mix (BROWN)	1.0 µl
RNase/DNase Free water	4.0 µl
Final volume	15 µl

2. Pipette 15µl of this mix into each well according to your real-time PCR experimental plate set up.

3. Prepare sample cDNA templates for each of your samples (suggested concentration 5ng/µl) in RNase/DNase free water.

If the concentration of RNA that was used to make the cDNA is not known, then dilute your RT reaction mix 1:20 (10µl of sample DNA and 90µl of water)

4. Pipette 5µl of diluted cDNA template into each well, according to your experimental plate set up.

For negative control wells use 5µl of RNase/DNase free water. The final volume in each well is 20µl



5. Preparation of standard curve dilution series

- 1) Pipette 900µl of RNase/DNase free water into 7 tubes and label 2-8
- 2) Pipette 100µl of Positive Control Template (**RED**) into tube 2
- 3) Vortex thoroughly
- 4) Change pipette tip and pipette 100µl from tube 2 into tube 3
- 5) Vortex thoroughly

Repeat steps 4 and 5 to complete the dilution series

Standard Curve	Copy Number
Tube 1 Positive control (RED)	2×10^7 per µl
Tube 2	2×10^6 per µl
Tube 3	2×10^5 per µl
Tube 4	2×10^4 per µl
Tube 5	2×10^3 per µl
Tube 6	2×10^2 per µl
Tube 7	20 per µl
Tube 8	2 per µl

6. Pipette 5µl of standard template into each well, according to your experimental plate set up.

The final volume in each well is 20µl



Amplification Protocol

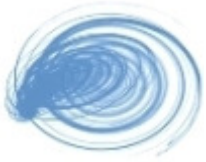
Amplification conditions using PrimerDesign 2X Precision™ MasterMix.

	Step	Time	Temp
	UNG treatment (if required) **	15 mins	37°C
	Enzyme Activation (if required)***	10 mins	95°C
50 cycles	Denaturation	10s	95°C
	DATA COLLECTION*	60s	60°C

* Fluorogenic data for the control DNA should be collect during this step through the FAM and VIC channels

** Required if your mastermix includes UNG to prevent PCR carryover contamination

*** Not all Mastermixes require this enzyme activation step. Follow the manufactures instructions for your mastermix.



Interpretation of Results

Pathogen	Internal control	Negative control	Positive control	Interpretation
+ve	+ve	-ve	+ve	+ve
+ve	-ve	-ve	+ve	+ve
-ve	+ve	-ve	+ve	-ve
-ve	-ve	-ve	-ve	Exp Fail
+ve	+ve	+ve	+ve	Exp Fail

Internal PCR control

When used according to the above protocols, assuming a 100% extraction efficiency, that 1:4 of extracted RNA is used in the RT reaction and that 1:20 of the RT reaction is used in an individual well, then a CT value of 26 is expected. However this can vary significantly depending on the extraction efficiency, the quantity of RNA added to the RT and PCR reaction and the individual machine settings. CT values of 26 ± 3 are within the normal range. When amplifying a pathogen sample with high genome copy number, the internal extraction control may not produce an amplification plot. This does not invalidate the test and should be interpreted as a positive experimental result.

Endogenous ACTB control

The signal obtained from the ACTB primer and probe set will vary according to the amount of biological material present in a given sample.



Notices and disclaimers

During the warranty period PrimerDesign pathogen detection kits allow precise and reproducible data recovery combined with excellent sensitivity. For data obtained by violation to the general GLP guidelines and the manufacturer's recommendations the right to claim under guarantee is expired.

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