

SOP for Quantitation of the Hepatitis C Virus (HCV) Genome by RT-PCR

1. Scope

- 1.1 This procedure describes a method for quantitation of the HCV genome in HCV-infected cells by RT-PCR.
- 1.2 For the preparation of HCV-infected cells, see _____.
- 1.3 For the preparation of competitor DNA, see _____.
- 1.4 For details about a thermal cycler (PTC-200; MJ Research), see _____, and for a densitometer (AE-6920M-03; ATTO), see _____.

2. Principles

- 2.1 The HCV genome in HCV-infected cells is quantitated by RT-PCR using, as template, total RNA extracted from the cells, with competitor DNA, as an internal standard, that is amplified in the same reaction tube. The amplification is targeted to the cDNA sequence of the 5'-noncoding region in which nucleotides are relatively strongly conserved in the HCV genome, and it is followed by nested PCR. The products of PCR are fractionated by agarose gel electrophoresis and bands are visualized under UV after reaction with ethidium bromide. The amount of HCV genome is calculated from the ratio of the density of the band of interest to that of the internal standard.

3. Reagents

- 3.1 ISOGEN™ (#311-02501; NIPPON GENE), dNTP Mixture (#27-2035-02; Pharmacia Biotech), diethyl pyrocarbonate-treated (DEPC-treated) water (#364-15; Nakalai Tesque), 5x First Strand Buffer [M-MLV RT 5x Buffer; 250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM MgCl₂, supplied with 0.1 M DTT; #18057-018; GIBCO BRL], AMV reverse transcriptase (RTase; #120248; Seikagaku Corporation), AmpliTaq DNA polymerase with buffer

(#N-808-0160; Perkin-Elmer), agarose (#A6013; Sigma), 50x TAE Buffer (#24710-030; GIBCO BRL).

4. Preparation of PCR primers

4.1 Primers for amplification of the HCV genome: 1st sense primer #5, 5'-AGTATGAGTGTCGTGCAGCCT-3'; 1st antisense primer #8, 5'-GCACTCGCAAGCACCTATCA-3'; 2nd sense primer #6, 5'-AGCCATAGTGGTCTGCGGAAC-3'; and 2nd antisense primer #7, 5'-TACCACAAGGCCTTTCGCGAC-3'. Dissolve the primers in DEPC-treated water at a final concentration of 10 μ M in each case.

5. Procedure

5.1 Wash HCV-infected human liver cells three times with 1 mL of PBS. Add 500 μ L of ISOGENTM and lyse the cells by pipetting. Transfer the lysed sample in ISOGENTM to a 1.5-mL centrifuge tube for the preparation of total RNA. The lysed sample be stored in a freezer at - 80°C (remaining: stable for at least one month) prior to the extraction of RNA.

5.2 Add 200 μ L of chloroform to the lysed sample in ISOGENTM, mix on a vortex mixer for 15 sec and allow the mixture to stand for 3 min at room temperature. Centrifuge at 20,000 x g (r, 9 cm; 14,000 rpm), for 15 min at 4°C. Transfer 300 μ L of the supernatant (aqueous phase) to a centrifuge tube that contains glycogen (2 μ L/tube; prepare during centrifugation). Add 300 μ L of isopropanol to the tube and mix on a vortex mixer for 10 sec. Then freeze at - 80°C for at least 30 min (or overnight). Centrifuge at 20,000 x g for 15 min at 4°C; remove and discard the supernatant (no need to remove completely yet). Add 700 μ L of 70% ethanol and centrifuge at 20,000 x g for 5 min at 4°C. Without mixing or disturbing the pellet, remove and discard the supernatant and centrifuge for 1 min again to bring ethanol on the inner surface of the tube to the bottom. Remove the supernatant completely using a micropipette and discard (do not allow the precipitated total RNA to dry out completely).

5.3 Reverse transcription: Add RT-1 mixture, as indicated below to the total RNA

(30 μL /tube), and mix well to dissolve; keep the mixture on ice. Then incubate the mixture for 5 min over boiling water and chill in iced water (at least 5 min). Centrifuge the tube briefly at 20,000 x g at 4°C to bring the mixture on the inner surface of the tube to the bottom. Pipette a few times to mix and then remove an aliquot of 12 μL to a tube for RT-PCR. Add 8 μL of RT-2 mixture (see below) and mix by pipetting. Using a thermal cycler (PTC-200; MJ Research), incubate at 43°C for 30 min and then at 99°C for 5 min. Store at 4°C. You should prepare RT-1 mixture and RT-2 mixture without AMV-RTase during the centrifugation that follows the addition of chloroform for RNA extraction in step 5.2. Add AMV-RTase during the chilling of the mixture of RNA plus RT-1 in iced water.

RT-1 mixture (per tube of RNA sample)

1st antisense primer #8 (10 μM)	10 μL
DEPC-treated water	20 μL

	30 μL

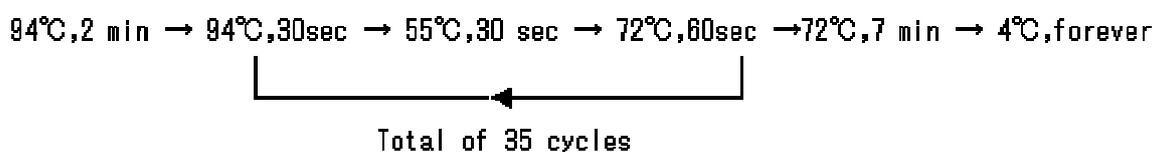
RT-2 mixture (per tube of RNA sample)

5x First Strand Buffer	4 μL
0.1 M DTT	2 μL
dNTP mixture (10 mM each dNTP)	1 μL
AMV-RTase (30 U/ μL)	1 μL

	8 μL

5.4 Amplification of cDNA (first PCR): Place 5 μL of the solution of cDNA in each of three tubes for RT-PCR in the presence of competitor DNA (10^3 , 10^4 and 10^5 copies/tube respectively) as the internal standard. Add 45 μL of first PCR mixture (see below) and mix by pipetting. Amplify the cDNA by PCR in a programmed thermal cycler.

Program for the thermal cycler for PCR



First PCR mixture (per sample of cDNA)	
10x PCR buffer	5.0 μL
1st sense primer #5 (10 μM)	0.5 μL
1st antisense primer #8 (10 μM)	0.5 μL
dNTP mixture (10 mM each dNTP)	1.0 μL
DEPC-treated water	27.75 μL
AmpliTaq DNA polymerase (5 U/ μL)	0.25 μL
Internal standard cDNA (competitor)	10.0 μL

	45.0 μL

- 5.5 Transfer 5 μL of the product of the first PCR to a new tube for the second PCR. Add 45 μL of second PCR mixture (see below) and mix by pipetting. Perform the second PCR using the same program as for the first PCR.

Second PCR mixture (per sample of cDNA)	
10x PCR buffer	5.0 μL
1st sense primer #6 (10 μM)	2.5 μL
1st antisense primer #7 (10 μM)	2.5 μL
dNTP mixture (10 mM each dNTP)	1.0 μL
DEPC-treated water	33.75 μL
AmpliTaq DNA polymerase (5 U/ μL)	0.25 μL

	45.0 μL

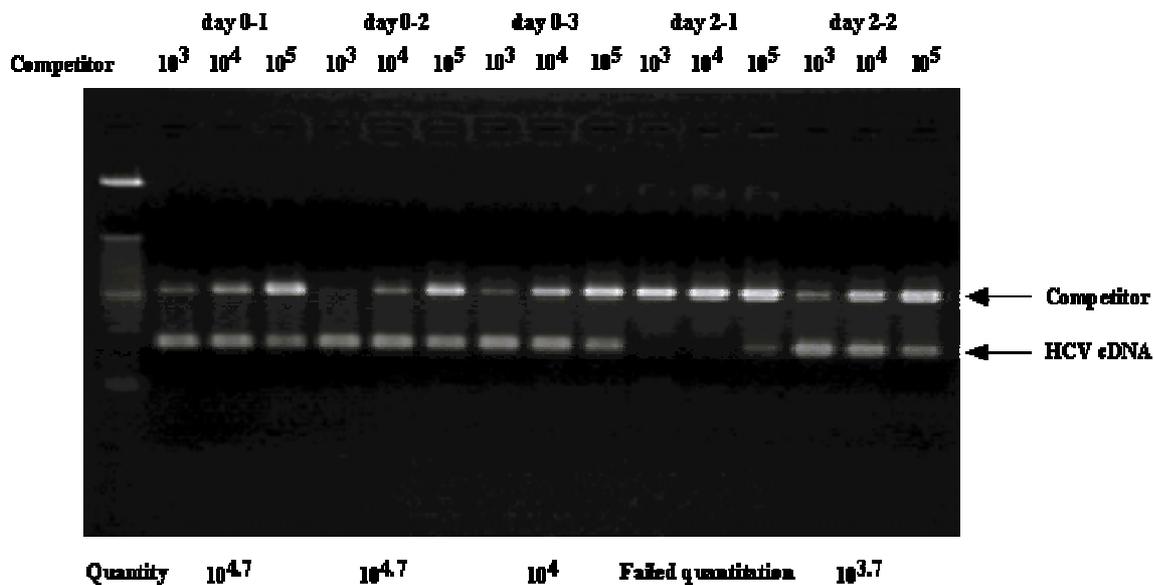
- 5.6 The amplified product of PCR is examined by agarose gel electrophoresis. Suspend agarose (3% w/v) in 1x TAE buffer (diluted with Milli-Q water). Heat the suspension to dissolve agarose completely, then allow to cool but not to gel. Pour 40 mL of the solution into a large plate of a Mupid-2 gel-maker set (#EM-2; Advance), and leave to cool and harden. To 10 μL of the solution of the product of the second PCR, add 2 μL of 6x gel-loading buffer (0.25% bromophenol blue/0.25% xylene cyanol/30% glycerol). Load 10 μL of the mixture per lane and allow electrophoresis to proceed in 1x TAE buffer for 30 min at 100 volts.
- 5.7 Soak the gel in a solution of ethidium bromide in Milli-Q water (2 $\mu\text{g}/\text{mL}$) for 10 min at room temperature. Then wash the gel with tap water for 5 min. Visualize cDNA under UV (312 nm) irradiation, and capture the image with a

densitograph (AE-6920M-03; ATTO). Analyze images with "Zone Densitometry" software and determine the density of the bands of HCV cDNA and competitor DNA. Save the data as a text file using computer software "Microsoft Excel 5.0".

6. Calculation

6.1 Calculate the number of copies of the HCV genome using the data provided by the "Zone Densitometry" program. For example, if the ratio of the intensity of the band of HCV cDNA to that of the band of competitor of which you used 10^5 copies (HCV genome/competitor) is 1 ± 0.1 , the initial number of copies of HCV RNA was 10^5 , that is to say the same as the number of copies of competitor. To obtain the number of HCV genomes in your initial culture, multiply this number by 10.

6.2 Example of agarose gel after electrophoresis.



LaneNo.	HCY genome	Competitor	HCY/Competitor		
Comp.10 ³	1	8780	4070	2.2	
Comp.10 ⁴	2	9690	8450	1.1	Day0-1
Comp.10 ⁵	3	6680	15810	0.42	10 ^{5.7} copies/well
	4	10730	790	13	
	5	10800	5940	1.8	Day0-2
	6	8300	14820	0.56	10 ^{5.7} copies/well
	7	11030	3330	3.3	
	8	10380	11330	0.92	Day0-3
	9	7890	16270	0.48	10 ⁵ copies/well
	10	840	15360	0.054	
	11	820	16950	0.048	Day2-1
	12	3880	17670	0.23	failed quantitation
	13	11480	4730	2.4	
	14	10150	12240	0.83	Day 2-2
	15	7280	14360	0.51	10 ^{4.7} copies/well