

Instruction Manual for HCV Genotype Primer Kit

HCV Genotype Determination Kit for Research Purpose

Thoroughly read this instruction manual before use of this kit

Background

Study of nucleotide sequence of hepatitis C virus (HCV) has brought to light many of its variants, and reports on its grouping in 5 major genotypes as determined by respective genomic sequences in a fairly well conserved portion as well as geographic locality of each subtype have subsequently been made^{1,2}.

Genotyping of HCV is now considered to be clinically helpful in pinning and tracking down the source and route of its infection and will be instrumental in further elucidation of the virus.

Recent report also suggests difference in resistance against therapy and course of the disease of each viral subtype predicting the therapeutic significance of its genotyping³.

This kit is developed for genotyping HCV in 5 groups, types I (1a), II (1b), III (2a), IV (2b), and V (3a), determined by Okamoto, et al according to genomic sequences in its core region⁴.

Feature

This kit determines HCV genotypes by simply electrophoresing HCV core region genes of type-specific sequence length amplified by Reverse-Transcription Polymerase Reaction (RT-PCR) and features accurate and simple determination of HCV genotypes.

Kit Configuration

This kit contains the following components.

Label Color	Label Description	Component	Quantity	Volume
Red	RT Primer Solution	Primer for reverse transcription	5 vials	15 µL / vial
Green	1st Primer Solution	Primer for 1st amplification	5 vials	60 µL / vial
Yellow	2nd Primer Solution	Primer for 2nd amplification	5 vials	60 µL / vial
White	10x dNTPs	dNTPs	5 vials	100 µL / vial
Blue	Sterilized Water	Sterilized water	5 vials	1 mL / vial
Purple	Marker	Type-specific marker	5 vials	110 µL / vial
Brown	Gel Loading Buffer	Buffer for applying samples	5 vials	100 µL / vial

Application

Determination of HCV genotypes according to its RNA nucleotide sequence.

Principle of Determination

This kit is for determination of HCV-RNA genotypes by comparison in sizes of the products amplified by RT-PCR using HCV genotype-specific primers, and then subjecting the product to electrophoresis (Fig. 1).

Portion (272 bp) of the core region is first amplified by RT-PCR with primers common to all 5 genotypes. The product is then amplified in second PCR with 5 genotype-specific primers and is electrophoresed for determination of HCV-RNA genotype by its size.

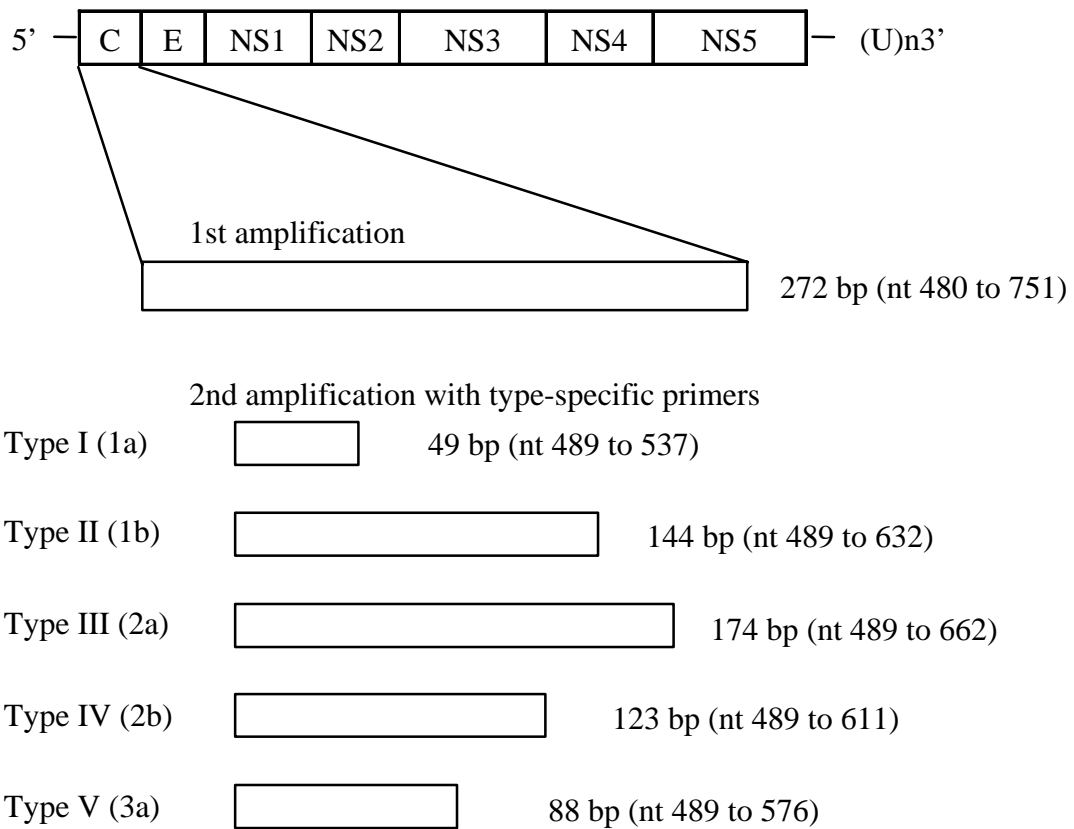


Fig. 1 Principle of HCV Genotype Determination

Reference) Okamoto, et al: Journal of General Virology, **74**: 2385-2390, 1993.

Operational Procedures

- Note: (1) For preparation of the solution before PCR amplification, it is essential to use a variable-volume single channel pipetter or pipettors. Pipetter tips must be provided with filter and must be replaced for every reagent and sample.
- (2) To prevent inactivation of enzyme or non-specific reaction, the reaction solution must be prepared in crushed ice.
- (3) Do not handle the PCR products with a variable-volume single channel pipetter or pipettors exclusive for preparation of the solution before PCR amplification.
- (4) Contamination of the solution before PCR amplification with PCR products may result in pseudo positive reaction and false determination. It is recommended that area, tools, and instruments for handling of PCR products should be separated before and after PCR amplification.

1. Materials required for the assay but not supplied with the kit

1-1 Reagents, tools and equipments required but not supplied with this kit (For RT-PCR amplification.)

- Variable-volume single channel pipetter
- Micro pipetter tips (with filter and sterilized in an autoclave)
- Plastic tubes for PCR (0.5 mL, sterilized in an autoclave, thin walled tube)
- Vortex mixer
- Microtube centrifuge
- Thermal cycler
- Temperature stable DNA polymerase (AmpliTaq[®])
- Reverse transcriptase (SuperScript[®] II by BRL)
- RNase inhibitor (Promega)
- Mineral oil (DNase/RNase free light white oil)

1-2 Reagents, tools and equipments required but not supplied with this kit (For electrophoresis)

- Electrophoresis unit
- Electrophoresis gel marker
- Variable-volume single channel pipetter
- Trans UV illuminator
- Instant camera (with orange-red color filter)
- Film for instant camera (ISO 3000)
- UV protective equipment
- NuSieve[®] GTG agarose
- SeaKam[®] GTG agarose
- Tris hydroxymethyl aminomethane (Tris base)
- Glacial acetic acid
- EDTA
- Ethidium bromide (Carcinogenic chemical. Handle with absolute care)

2. Preparation of reagents

- 50× TAE buffer: (To be stored at room temperature.)
Composition: Add sterilized water to mixture of Tris base 242 g, glacial acetic acid 57.1 mL and 0.5M EDTA (pH 8.0) 100 mL to make 1000 mL solution. (Dilute 50 folds with deionized water to make working solution.)
- Ethidium bromide staining solution. (To be stored at room temperature.)
Dissolve in distilled water to make 1 µg/mL concentration.
- 4% agarose gel (NuSieve[®] : SeaKam[®] = 3 : 1)
Assemble a gel frame according to the instruction manual provided to the electrophoresis unit.
Add 4% agarose gel (NuSieve[®] : SeaKam[®] = 3 : 1) to 1× TAE buffer and thoroughly dissolve in an autoclave (121°C, 3 ~ 5 min.). Thoroughly mix the solution at room temperature. When temperature of the agarose gel solution goes down to about 60°C, carefully pour it in the gel frame forming no bubble on the gel surface. Carefully set a comb and allow the solution thoroughly to cure at room temperature. Apply sparse 1× TAE buffer to the comb and carefully extract it from the gel. This gel can be stored in the 1× TAE buffer for several days.

3. Operation protocol

3-1 Synthesis of cDNA

- (1) Heat the RNA solution to 70 °C for 2 min to destruct the tertiary structure. (This is optional and not essentially required.)
- (2) Prepare the following RT reaction solution in a micro tube for PCR (0.5 mL, thin walled sterilized tube).

Composition of the RT reaction solution (for 1 test)

RNA solution	5.00 µL	(Isolated from 100 µL of serum)
5× RT buffer	2.00 µL	(Provided to SuperScript [®] II)
20 mM dNTP mix	0.25 µL	
0.1 M DTT	1.00 µL	(Provided to Super Script [®] II)
RT primer solution	1.00 µL	(A component of this kit)
RNase inhibitor	0.25 µL	(RNasein [®] by Promega, 40 u/µL)
Reverse transcriptase	0.50 µL	(SuperScript [®] II by BRL, 200 u/µL)
	10.00 µL	

- (3) Add to the above tube 25µL of mineral oil (DNase/RNase free light white oil).

- (4) Through mix the reaction solution and allow reaction according to the following cycle.

Reverse transcription reaction	42 °C, 60 min
	↓
Inactivation of enzyme	95 °C, 15 min
	↓
Storage	4 °C

3-2 1st PCR Reaction

- (1) Prepare the following 1st PCR reaction solution.

Composition of the 1st PCR reaction solution (for 1 test)

1st PCR primer solution	5.00 µL	(A component of this kit)
10× dNTPs	4.00 µL	(A component of this kit)
AmpliTaq [®]	0.25 µL	(by Roche Molecular Systems)
10×PCR buffer	3.00 µL	(A component of AmpliTaq [®] containing MgCl ₂)
Sterilized water	27.75 µL	(A component of this kit)
	40.00 µL	

- (2) Dispense the above 1st PCR reaction solution in a tube (0.5 mL, sterilized thin walled tube) and cover the top of the solution with 25 µL of mineral oil (DNase/RNase free light white oil).
- (3) Add to the tube 10 µL of synthesized cDNA in the above step.
- (4) After mixing on a vortex mixer, spin by a microtube centrifuge at 1000 ~ 3000 rpm for 10 ~ 30 sec to clear the tube inner wall of the mixed solution.
- (5) Set the program controller of a PCR thermal cycler according to the following conditions to amplify DNA.

DNA denaturation	94°C, 30 sec	} 35 cycles
Annealing	55°C, 30 sec	
DNA amplification	72°C, 60 sec	
	↓	
After completion of cycles	72°C, 7 min	
	↓	
Storage	4°C	

Note: Depending upon the instrument, above conditions may have to be changed. Adjust the conditions as required.

3-3 2nd PCR Reaction

- (1) Prepare the following 2nd PCR reaction solution.

Composition of the 2nd PCR reaction solution (for 1 test)

2nd PCR primer solution	5.00 μ L	(A component of this kit)
10 \times dNTPs	5.00 μ L	(A component of this kit)
AmpliTaq [®]	0.25 μ L	(by Roche Molecular Systems)
10 \times PCR buffer	5.00 μ L	(A component of AmpliTaq [®] containing MgCl ₂)
Sterilized water	33.75 μ L	(A component of this kit)
	49.00 μ L	

- (2) Dispense the above 2nd PCR reaction solution in a tube (0.5 mL, sterilized thin walled tube) and cover the top of the solution with 25 μ L of mineral oil (DNase/RNase free light white oil).
- (3) Add to the tube 1 μ L of the 1st PCR product.
- (4) After mixing on a vortex mixer, spin by a microtube centrifuge at 1000 ~ 3000 rpm for 10 ~ 30 sec to clear the tube inner wall of the mixed solution.
- (5) Set the program controller of a PCR thermal cycler according to the following conditions to amplify DNA.

DNA denaturation	94°C, 30 sec	} 25 cycles
Annealing	60°C, 30 sec	
DNA amplification	72°C, 30 sec	
After completion of cycles	72°C, 7 min	
Storage	4°C	

3-4 Electrophoresis

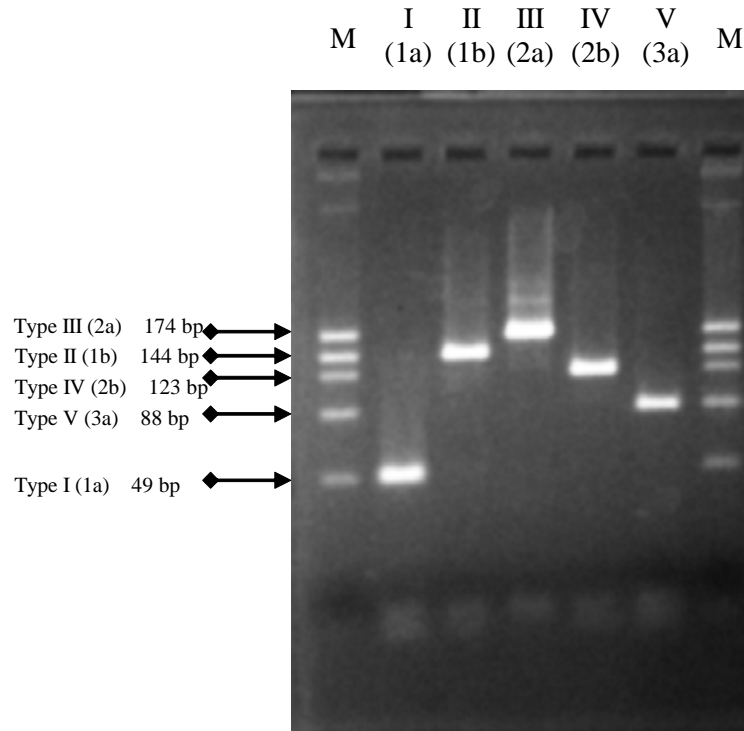
- (1) Dispense gel and the buffer (1 \times TAE) in the electrophoresis bath.
- (2) Add 2 μ L of the gel loading buffer to 10 μ L of the 2nd PCR product and mix them. Load this mixture to the well. Dispense 10 μ L of the type-specific marker in the well.
- (3) Apply 100V for 40 min and electrophorese.
- (4) Immerse the gel in ethidium bromide and rock for approximately 15 min.
- (5) Take a picture of the electrophoresed pattern using a trans UV illuminator.

Determination

Determination by HCV-RNA genotype-specific amplification

HCV-RNA genotypes are determined by the electrophoresis patterns.

As shown in the picture below, the amplified bands of type I (1a), II (1b), III (2a), IV (2b), and V (3a) appear at the positions of 49 bp, 144 bp, 174 bp and 123 bp, and 88 bp, respectively. Determine genotypes from the positions of the band. When concentration of HCV-RNA is high, the amplified band of the 1st PCR product may appear at 272 bp.



Pattern of HCV-RNA genotypes

M : Type-specific marker

I (1a): Type I (1a)

II (1b): Type II (1b)

III (2a): Type III (2a)

IV (2b): Type IV (2b)

V (3a): Type V (3a)

Cautions for specimen handling

(1) Samples

Fresh serum samples should be used for this test.

(2) Contamination

Samples contaminated with the PCR product may cause incorrect results. Carefully handle them.

Cautions for handling and operation

(1) Return the kit and samples to room temperature before use and thoroughly and carefully mix each component so as not to form bubbles.

(2) Do not mix components of different lot numbers.

- (3) Store the kit under conditions as instructed and use up before expire date.
- (4) Avoid contamination of kit components with microorganisms.
- (5) Store the kit avoiding direct, strong light.
- (6) Handle samples carefully to avoid infection with viruses.
- (7) Do not use the kit and kit components for other purposes than HCV-RNA genotyping.
- (8) Before discarding samples, reagents, components and tools (tubes, tips, etc.), disinfect them by either of the followings.
 - Immerse in 1% formalin for 1 hr or longer.
 - Immerse in 2% glutaraldehyde for 1 hr or longer.
 - Immerse in 0.1% sodium azide for 1 hr or longer.
 - Autoclave at 121°C for 1 hr or longer.

Package, storage conditions, shelf life and product code

Name of Kit	Package	Storage	Shelf life	Product Code
HCV Genotype Primer Kit	50 tests	-20°C, in the dark	Shown on the package	8C01

References

- 1) Okamoto H, et al: Typing hepatitis C virus by polymerase chain reaction with type-specific primers: application to clinical surveys and tracing infectious sources. *Journal of General Virology* **73**: 673-679, 1992.
- 2) Okamoto H, et al, Full-length sequence of a hepatitis C virus genome having poor homology to reported isolates: comparative study of four distinct genotypes. *Virology* **188**: 331-341, 1992.
- 3) Kanai K, et al, HCV genotypes in chronic hepatitis C and response to interferon. *Lancet* **339**: 1543, 1992.
- 4) Okamoto H, et al, Characterization of the genomic sequence of type V (or 3a) hepatitis C virus isolates and PCR primers for specific detection. *Journal of General Virology* **74**: 2385-2390, 1993.

Others

NuSieve[®], SeaKam[®], and SuperScript[®] are trade marks of FMC, and AmpliTaq[®] is trade mark of Roche Molecular Systems, respectively.

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