RTA® HCV Genotyping qPCR Kit

Handbook

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Identification of genotypes of Hepatitis C virus
For *research use only*.
For professional use only

REF 09024024 – 24 tests

Kit Contents

	CapColor	Reagents	24 Tests
1	BROWN	HCV Gntyp Reaction Mix A	335 µl
2	BROWN	HCV Gntyp Reaction Mix B	250 µl
3	BROWN	HCV Gntyp Reaction Mix C	250 µl
4	BROWN	HCV Gntyp Reaction Mix D	250 µl
5	YELLOW	HCV Gntyp Enzyme Mix A	18 µl
6	YELLOW	HCV Gntyp Enzyme Mix BC	27 µl
7	BLUE	HCV Gntyp Internal Control	75 µl
8	RED	HCV Gntyp Positive Control	100 µl
9	WHITE	HCV Gntyp Negative Control	100 µl

Storage

All reagents of RTA HCV Genotyping qPCR Kit should be stored at -20°C. Storage at higher temperatures should be avoided (e.g. +4°C). Under these conditions, kit contents should be stable through the expiration date printed on the label. The reagents should not be freeze-thawed more than 2 times; otherwise the shelf of the kit will reduce. During the working steps all reagents should be kept on ice.

Intended Use

RTA HCV Genotyping qPCR Kit is an in vitro nucleic acid amplification assay for identification of the genotypes of Hepatitis C virus (HCV) RNA in human serum or plasma (EDTA) using RTA Viral RNA Isolation Kit or RTA Viral Nucleic Acid Isolation Kit and BIO-RAD CFX96-IVD Real-Time PCR Detection System. RTA HCV Genotyping qPCR Kit detects HCV genotypes 1a, 1b, 2, 3, 4, 5 and 6. RTA HCV Genotyping qPCR Kit is not intended for screening of blood and blood products for the presence of HCV RNA or confirmation of the diagnosis of infection with HCV.

Product Use Limitations

- All reagents of the kit is for research use only.
- RTA HCV Genotyping qPCR Kit is not intended for screening of blood and blood products for the presence of HCV RNA or confirmation of the diagnosis of infection with HCV.
- This kit has been validated for use with human serum or human plasma collected in EDTA anticoagulant. Test with other sample types may result in inaccurate results.
- This kit has been validated for use with RTA Viral RNA Isolation Kit or RTA Viral Nucleic Acid Isolation Kit. Using other isolation kits may adversely affect the performance characteristics of the kit.
- This kit has been validated for use with BIO-RAD CFX96-IVD Real-Time PCR Detection System. Using other instruments may adversely affect the performance characteristics of the kit.
- This kit has been optimized for use with specific PCR plastic consumables listed under Additional Materials Required section of the Handbook. Using other PCR plastic consumables may adversely affect the performance characteristics of the kit.
- Trustworthy results depends on proper sample collection, transport, storage and processing methods.
- It is intended for professional use by properly trained personnel.
- The instructions in user manual should be followed strictly for optimum PCR results.
- The expired kits should not be used. Kit components from different lots should not be mixed.

Product Description

RTA HCV Genotyping qPCR kit is based on real-time reverse transcription-PCR technology. The method is performed directly on RNA extracted from the patient specimens. The identification of HCV-RNA genotypes/subtypes 1a, 1b, 2, 3, 4, 5 and 6 is done in 4 different reactions in which specific Internal Control (IC) is simultaneously detected. In tube A, one primer-probe set amplifies the non-structural 5b (NS5b) region of genotype 1a, and the other set amplifies 5′ untranslated region (5′ -UTR) of genotype 2. In tube B, one primer-probe set amplifies the non-structural 5b (NS5b) region of genotype 1b, and the other set amplifies the non-structural 5b (NS5b) region of genotype 5. In tube C, one primer-probe set amplifies the non-structural 3 (NS3) region of genotype 3, and the other set amplifies the non-structural 5b (NS5b) region of genotype 4. In tube D, one primer-probe set amplifies the non-structural 5b (NS5b) region of genotype 6.

General Information

The HCV RNA genome sequences are highly heterogeneous. At present, HCV is classified into six genotypes (designated as 1-6) differing in their nucleotide sequence by 30%-35%. Within HCV genotype, several subtypes (designated as a, b, c, etc.) can be defined that differ in their nucleotide sequence by 15%-30%(1).
Globally, HCV genotypes 1, 2, and 3 are common and genotypes 4, 5, and 6 are localized to limited regions (2,3). Genotype 1a is the most common in Northern Europe and North America, and 1b is the most common in Far East Asia and Europe. Genotype 2 is less common than genotype 1. Genotype 3 is common in Southeast Asia and genotype 4 is common in the Middle East, Egypt, and Central Africa. Genotype 5 is commonly found in South Africa and genotype 6 is common in Hong Kong, Macau, and Vietnam. Common HCV genotypes in South Korea are genotype 1b (45- 59%) and 2a (26-51%); types 1a, 2b, 3, 4, and 6 are rare in South Korea (4,5). HCV genotype 1b is predominant in Turkey (6).

References
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Warnings and Precautions

- All clinical specimens and all resulting waste materials should be treated as potentially infectious; the samples should be prepared in Bio-safety Level 2 area.
- Before and after work all surfaces should be disinfected with a freshly prepared solution of 10% bleach or antiviral agents.
- Dispose of unused reagents, waste and specimens in accordance with country or local regulations.
- Do not pipette by mouth.
- Do not eat, drink or smoke in laboratory work areas.
- Wear protective disposable gloves, laboratory coats and eye-wear when handling clinical specimens and kit reagents. Wash hands thoroughly after handling specimens and test reagents.
- Avoid contact of reagents with the skin, eyes or mucous membranes. If contact does occur, immediately wash with large amounts of water.
- The procedures should preferably be performed in four separated areas (i.e. for RNA extraction, PCR setup, sample addition, amplification) to aid in preventing contamination.. All supplies for a particular procedure should be stored in the area where that procedure is performed and should not be moved between areas Gloves should be removed and disposed of before leaving one area to proceed to the next. Lab coats should be specific to an area and never worn outside of that area. The work should flow in one direction, beginning in the extraction area, moving to the PCR setup area in which PCR Master Mix is prepared, then moving to the third area in which samples, negative control and quantification standards are added, finally moving to amplification area in which real-time PCR equipment is run.
- Use all pipetting devices and instruments with care and follow the manufacturer's instructions for calibration and quality control; to prevent sample contamination, use new, sterile aerosol barrier or positive displacement Rnase/DNase-free pipette tips and sterile pipettes.
- Handle all materials containing specimens or controls according to Good Laboratory Practices in order to prevent cross-contamination of specimens or controls.
- Store the kit away from any source of contaminating RNA or RNA, especially amplified nucleic acid.
- Do not mix reagents with different lot numbers or substitute reagents from other manufacturers.
- Do not use a kit after its expiration date.

Performance Characteristics

Analytical Sensitivity

Analytical sensitivity was analyzed by use of a dilution series of RTA HCV Genotype Reference samples (1a, 1b, 2, 3, 4, 5, 6) whose viral loads were calibrated against the HCV WHO standard, and genotype/subtypes were identified by sequencing. A dilution series of a RTA HCV Genotype Reference samples was prepared to give the final concentrations of 3,000, 1,000, 300, 100, 30 and 10 IU/ml. Each dilution was tested in 24 replicates. Lower limit was calculated by probit analysis done by PASW Statistics 18 program. For each genotype/subtype, Limit of Detection (LoD) values and 95 % confidence ranges are summarized in Table 1.

Table 1: HCV Genotyping qPCR Kit - Limit of Detection (LoD) values and 95 % confidence ranges

HCV genotype/subtype	Limit of Detection (IU/ml)	95 % confidence lower limit	95 % confidence upper limit
Genotype 1a	46	35	90
Genotype 1b	41	28	191
Genotype 2	106	85	148
Genotype 3	155	120	241
Genotype 4	88	69	131
Genotype 5	34	26	60
Genotype 6	178	127	358

Precision

In this study, precision of the kit was evaluated for intra-assay, inter-assay, inter-batch, different extraction kits (RTA Viral RNA Isolation Kit (Cat No: 09010) vs. RTA Viral Nucleic Acid Isolation Kit (Cat No: 09029)) , and different specimen types (plasma EDTA vs. serum). For each genotype/subtype and different assay, 24 replicates of 10^3 IU/ml RTA HCV Genotype Reference samples (1a, 1b, 2, 3, 4, 5, 6) were used. Descriptive statistics were analyzed by PASW Statistics 18 program. Overall precision assays associated with Ct values were summarized in Table 2.

Table 2: Overall descriptive statistics of HCV genotypes/subtypes precision data.

Descriptive Statistics					
	N	Mean	Std.Deviation	Variance	Coefficient of variation (%)
Genotype 1a	120	30,0337	,14527	,021	0,48
Genotype 1b	120	29,2161	,13896	,019	0,48
Genotype 2	120	31,0055	,12607	,016	0,41
Genotype 3	120	27,9594	,08651	,007	0,31
Genotype 4	120	30,3558	,24190	,059	0,80
Genotype 5	120	28,5387	,12425	,015	0,44
Genotype 6	120	28,9874	,61403	,377	2,12

Diagnostic Specificity

HCV RNA negative clinical specimens were analyzed to determine the diagnostic specificity of RTA HCV Genotyping qPCR Kit. 49 Hepatitis C virus RNA negative clinical **serum** specimens and 82 Hepatitis C virus RNA negative clinical **EDTA plasma** specimens were used. None of the 131 HCV negative clinical specimens gave positive test result for HCV Genotyping. Diagnostic specificity of RTA HCV Genotyping qPCR Kit is 100 %. All of the Internal Controls of tests gave positive result.

Cross Reactivity

To examine the specificity of an assay, cross-reactivity studies should be performed for potential cross-reactive markers. In this study, the specificity of the assay was evaluated by testing. 9 reference organism and 15 clinical specimens which were positive.
RTA HCV Genotyping qPCR Kit do not show any cross-reactivity with other potential cross-reactive markers given in the table 3 below:

Table 3: Potential cross-reactive markers tested in the study.

Organism	Source	Test Result
Hepatitis B virus (HBV)	NIBSC (Cat. No: 10/264)	Negative
Human Immunodeficiency Virus 1 (HIV-1)	NIBSC (Cat. No: 10/152)	Negative
Human Immunodeficiency Virus 2 (HIV-2)	NIBSC (Cat. No: 08/150)	Negative
Human Cytomegalovirus (HCMV)	NIBSC (Cat. No: 09/162)	Negative
Epstein-Barr Virus (EBV)	NIBSC (Cat. No: 09/260)	Negative
Human Herpes Simplex virus type 1 (HSV-1)	NIBSC (Cat. No: 08/224)	Negative
Human Herpes Simplex virus type 2 (HSV-2)	NIBSC (Cat. No: 08/226)	Negative
Parvovirus B19	NIBSC (Cat. No: 99/686)	Negative
Varicella Zoster Virus(Type B)	NIBSC (Cat. No: 08/310)	Negative
West Nile virus	5 Clinical specimens	Negative
Hepatitis B virus (HBV)	2 Clinical specimens	Negative
Human Immunodeficiency Virus 1 (HIV-1)	4 Clinical specimens	Negative
Hepatitis G virus	4 Clinical specimens	Negative

Diagnostic Sensitivity Study

Total 120 clinical and reference samples were tested. 21 samples were subtype 1a, 26 samples were subtype 1b, 21 samples were genotype 2, 22 samples were genotype 3, 20 samples were genotype 4, 6 samples were genotype 5, 2 samples were genotype 6, and 2 samples were mixed HCV genotype infection. According to the results, the data gathered by RTA HCV Genotyping

qPCR Kit is highly compatible with the results of NS5B nucleotide sequencing. 119 of 120 clinical samples with single HCV genotype infection were 100 % compatible with sequencing.

Additional Materials Required

- RTA Viral RNA Isolation Kit (Cat No: 09010; RTA Laboratories, Turkey) or RTA Viral Nucleic Acid Isolation Kit (Cat No: 09029; RTA Laboratories, Turkey),
- BIO-RAD CFX96-IVD Real-Time PCR Detection System,
- Disposable powder-free gloves
- Micropipettes (0.5 µl – 1000 µl),
- Sterile micropipette tips with filters,
- Microcentrifuge tubes,
- Vortex mixer,
- Desktop microcentrifuge for 2.0 ml tubes and for PCR strip tubes,
- PCR Workstation,

Real-Time PCR reaction tubes or plates:

- Hard-Shell Thin-Wall 96-Well Skirted PCR Plates with Bar Codes (BIO-RAD, Cat#: HSP-9955)
- Hard-Shell Thin-Wall 96-Well Skirted PCR Plates (BIO-RAD, Cat#: HSP-9655)
- Microseal 'B' Adhesive Seals, optically clear (BIO-RAD, Cat#: MSB-1001),
- Individual PCR Tubes, Low Tube Strips, 8-tubes strip, 0.2 ml Low Profile, White (BIO-RAD, Cat#: TLS0851)

Sample Preparation

This kit has been validated for use with human serum or human plasma collected in EDTA anticoagulant. Aseptic techniques must be employed during collection to prevent the introduction of micro-organisms into the patient's anatomical space, and to prevent the sample from being contaminated during the process of collection. All samples should be regarded as potentially infectious and standard precautions guidelines should be followed by all healthcare workers during sample collection and handling. Samples must be collected into appropriate containers before despatch to the laboratory. Be careful to check for cracks in the containers and to ensure that the lids of containers are properly tightened to prevent leakage of samples during handling and transportation. This can pose infection hazards to transport and laboratory staff. Ensure that the outer surfaces of the containers are not contaminated by the patients' samples.
Store whole blood at room temperature for no longer than 4 hours. Centrifuge blood within 4 hours of collection. Transfer serum or plasma to a screw cap cryovial tube.
Transportation of whole blood, serum or plasma must conform to country or local regulations for the transport of etiologic agents. Serum or plasma samples may be stored at 2-8°C for up to 3 days or frozen at -70°C or colder for long-term storage. Avoid multiple freeze/thaw cycles of specimens.

Protocol

Viral RNA Isolation RTA Viral RNA Isolation Kit (Cat No: 09010; RTA Laboratories, Turkey) or RTA Viral Nucleic Acid Isolation Kit (Cat No: 09029; RTA Laboratories, Turkey) should be used for viral RNA extraction from clinical samples. Please follow the manufacturer's instructions as stated in the kit manual.

Internal Control During RNA isolation, addition of the supplied internal control (IC) is necessary. IC allows the user to monitor RNA extraction step as well as to determine any PCR inhibition. For each sample, add 2.5 µl IC together with Solution RL of the isolation kit for a 50 µl elution. Depending on your final elution volume, the volume of IC to be added can be calculated (0.05 µl IC/1 µl Elution Buffer). There might be no amplification of internal control in the tests where high positive HCV samples are amplified because there is a competition between internal control template and HCV RNA template for using PCR components. The Ct value of internal control of a *negative sample* should be equal to 33 ± 4, otherwise, it denotes a problem during purification. Internal control is co-amplified in all of 3 reaction tubes

Positive Control Positive Control includes in vitro transcribed RNA containing an insert. The insert contains primer binding sequences for oligonucleotides HCV 1a forward and HCV 1a reverse, and HCV 1a probe. Positive Control is amplified by Reaction Tube A. The Ct value of Positive Control should be equal to 30 ± 3, otherwise, it denotes a problem during amplification.

PCR Protocol

1. Thaw all components, except HCV Gntyp Enzyme Mix A and BC, at room temperature. Thaw HCV Gntyp Reaction Mixes at 37°C for 5 min if there is a precipitate. Put HCV Gntyp Enzyme Mixes on ice. Mix each component thoroughly, then centrifuge briefly before use. Transfer all the reagents onto ice or cooling block.

2. The final volumes of Master Mixes are calculated by multiplying single reaction volumes of Reaction Mix and Enzyme Mix by the total sample size. The number of negative control and the positive control should be added to Master Mix A when calculating total sample size. Against possible pipetting errors, addition of an extra sample to the total sample size is recommended.

3. To prepare Master Mix A, add 9.5 µl of HCV Gntyp Reaction Mix A (brown tube) and 0.5 µl of HCV Gntyp Enzyme Mix A (yellow cap) for each sample to the Master Mix tube A. To prepare Master Mix B, add 9.5 µl of HCV Gntyp Reaction Mix B (brown tube) and 0.5 µl of HCV Gntyp Enzyme Mix BC (yellow cap) for each sample to the Master Mix tube B. To prepare Master Mix C, add 9.5 µl of HCV Gntyp Reaction Mix C (brown tube) and 0.5 µl of HCV Gntyp Enzyme Mix BC (yellow cap) for each sample to the Master Mix tube C. To prepare Master Mix D, add 9.5 µl of HCV Gntyp Reaction Mix D (brown tube) and 0.5 µl of HCV Gntyp Enzyme Mix A (yellow cap) for each sample to the Master Mix tube A (Figure 1).

4. The final volumes of Master Mixes are calculated by multiplying single reaction volumes of Reaction Mix and Enzyme Mix by the total sample size. The number of positive control and negative control should be taken into account when calculating total sample size for Master Mix A. Against possible pipetting errors, addition of an extra sample to the total sample size is recommended for each Master Mix (Table 4)

5. After preparation of Master Mixes, vortex the tubes gently and spin down briefly in a microcentrifuge. Add 10 µl of each Master Mix into Real-Time PCR reaction tubes/plate for each sample. Add 10 µl RNA of each sample into each of 4 Master Mixes, and add negative control and positive control into the Master Mix A (Table 5). Spin down briefly.

Figure 1: Preparation schemas of Master Mixes.

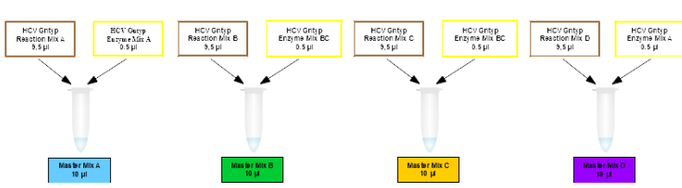

Table 4: Preparation table of Master Mixes for 24 samples.

	Master Mix A		Master Mix B		Master Mix C		Master Mix D	
	1 Reaction (µl)	24 +3 Reactions (µl)*	1 Reaction (µl)	24 +1 Reactions (µl)**	1 Reaction (µl)	24 +1 Reactions (µl)**	1 Reaction (µl)	24 +1 Reactions (µl)**
Volume of Reaction Mix	9,5	256,5	9,5	237,5	9,5	237,5	9,5	237,5
Volume of Enzyme Mix	0,5	13,5	0,5	12,5	0,5	12,5	0,5	12,5
Total volume	10	270	10	250	10	250	10	250

*: One reaction for Positive Control, one reaction for Negative Control, and one extra reaction against possible pipetting errors.

**: One extra reaction against possible pipetting errors.

Table 5: Distrubition schema of Master Mixes, samples and controls.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Sample 1	Sample 9	Sample 1	Sample 9	Sample 1	Sample 9	Sample 1	Sample 9				
B	Sample 2	Sample 10	Sample 2	Sample 10	Sample 2	Sample 10	Sample 2	Sample 10				
C	Sample 3	Sample 11	Sample 3	Sample 11	Sample 3	Sample 11	Sample 3	Sample 11				
D	Sample 4	Sample 12	Sample 4	Sample 12	Sample 4	Sample 12	Sample 4	Sample 12				
E	Sample 5	Pos.Con.	Sample 5		Sample 5		Sample 5					
F	Sample 6	Neg.Con.	Sample 6		Sample 6		Sample 6					
G	Sample 7		Sample 7		Sample 7		Sample 7					
H	Sample 8		Sample 8		Sample 8		Sample 8					
	Master Mix A		Master Mix B		Master Mix C		Master Mix D					

6. Perform the following protocol for BIO-RAD CFX96-IVD: 45°C for 30 min; 95°C for 1 min, 1 cycle; 95°C for 15 sec, 60°C for 45 sec, 68°C for 30 sec, 45 cycles (Table 6). Choose 20 µl as sample volume.

7. Fluorescence is measured at 68°C. FAM, HEX and Texas Red channels should be chosen. Plate type should be chosen as BR White from the Settings menu in the Plate Editor.

8. Refer to the Operator's Manual of the related instruments to program and analyze the results.

Table 6: Programming of Thermal Cycler.

Program Name	Cycles	Program for BIO-RAD CFX96-IVD
cDNA Synthesis	1	45ºC, 30 min
Hot Start	1	95ºC, 1 min
Amplification*	45	95ºC, 15 sec
		60ºC, 45 sec
		68ºC, 30 sec

*** Fluorescence is measured at 68°C; FAM, HEX and Texas channels should be chosen**

Data Analysis

During analysis on BIO-RAD CFX96-IVD software, adjust threshold fluorescence values manually by entering 500 for FAM and HEX, and 250 for Texas Red.
To be able to evaluate the experiment, the Ct value of Positive Control in the HEX channel should be equal to 30±3, and Negative Control in all channels should be negative. Otherwise, the experiment should be repeated.
The result is invalid if the test sample result is invalid for one or two or all three Master Mixes (A, B, C). This sample should be tested again.

The interpretation on the results can be done as follows:

Master Mix A

Signal detected in FAM channel	Signal detected in HEX channel	Signal detected in Texas Red channel	Conclusion
Yes	No	Yes/No	Genotype 2
No	Yes	Yes/No	Subtype 1a
Yes	Yes	Yes/No	2/1a mix genotypes.
No	No	Yes (Ct=33 ± 4)	The result is valid. No genotype/subtype is identified
No	No	No or Ct is not equal to 33 ± 4	The result is invalid. No diagnostic interpretation can be done.

Master Mix B

Signal detected in FAM channel	Signal detected in HEX channel	Signal detected in Texas Red channel	Conclusion
Yes	No	Yes/No	Genotype 5
No	Yes	Yes/No	Subtype 1b
Yes	Yes	Yes/No	5/1b mix genotypes.
No	No	Yes (Ct=33 ± 4)	The result is valid. No genotype/subtype is identified
No	No	No or Ct is not equal to 33 ± 4	The result is invalid. No diagnostic interpretation can be done.

Master Mix C

Signal detected in FAM channel	Signal detected in HEX channel	Signal detected in Texas Red channel	Conclusion
Yes	No	Yes/No	Genotype 3
No	Yes	Yes/No	Subtype 4
Yes	Yes	Yes/No	3/4 mix genotypes.
No	No	Yes (Ct=33 ± 4)	The result is valid. No genotype/subtype is identified
No	No	No or Ct is not equal to 33 ± 4	The result is invalid. No diagnostic interpretation can be done.

Master Mix D

Signal detected in FAM channel	Signal detected in HEX channel	Signal detected in Texas Red channel	Conclusion
Yes	No	Yes/No	Genotype 6
No	No	Yes (Ct=33 ± 4)	The result is valid. No genotype/subtype is identified
No	No	No or Ct is not equal to 33 ± 4	The result is invalid. No diagnostic interpretation can be done.

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